Multidimensional Computational Modeling of Potent BACE1 (β-Secretase) Inhibitors Towards Alzheimer's Disease Treatment

Samuel Chima Ugbaja

2021

A thesis submitted to the School of Laboratory Medicine & Medical Sciences, University of Kwa-Zulu Natal, Howard, in fulfillment of the degree of

Doctor of Philosophy

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This is to certify that the contents of this thesis are the original research work of Samuel Chima Ugbaja. As the candidate's supervisors, we hereby approve this thesis for submission.

Supervisor:	**Co-Supervisor:**
Name: Dr. Hezekiel M. Kumalo	**Name: Dr. Monsurat M. Lawal**
Signed:	**Signed:**

PREFACE

This thesis is written in chapters that are briefly highlighted herein to include:

CHAPTER ONE

This chapter is a brief introduction of the study background, with a general description of neurons' communication network in the brain. It also highlights BACE1 structural properties, the multi-target therapeutic approach for the treatment of Alzheimer's disease, the methods employed in computational drug design as well as the intended aims and objectives of the study.

CHAPTER TWO

Submitted and accepted for publishing as presented following the required format of the journal in which it was submitted for publication

This chapter is the general literature review on BACE1 and Alzheimer's disease. It extensively deals with the prevalence and pathology of the disease with a focus on the current drug pharmacological and design processes involved. It covers the inhibitors' properties as well as the mechanism involved in BACE1 inhibition. It highlights the general *in silico* methodologies of drug discovery while focusing on the methods involved in BACE1 inhibitor identification. Finally, it unravels the achievements on the application of theoretical and computational BACE1 inhibitor design.

CHAPTER THREE

Published work as presented following the required format of the journal in which it was accepted for publication, hence it is the final version of the accepted manuscript.

This chapter is a second review article on BACE1 exosites-binding antibody and allosteric inhibitor development as therapies. It also covers BACE1 biological roles, the associated disease mechanisms, and the enzyme's conditions for amyloid precursor protein (APP) sites splitting with a little overview on BACE1 gene properties and substrates.

CHAPTER FOUR

This chapter deals with the introduction to the applied computational chemistry techniques which encompasses quantum mechanical methods, molecular mechanical methods, and a hybrid of both methods. It briefly explains some of the contemporary computational methods employed in molecular modelings

such as the gaussian application of density functional theory (DFT) and time-dependent density functional theory (TDDFT). It also gives a brief description of conventional and accelerated molecular dynamics as well as ONIOM methods as succinctly applied in the study that constitutes this thesis. Molecular docking and molecular dynamic simulations using Swissdock a web-server software is also discussed.

CHAPTER FIVE

Published work as presented following the required format of the journal in which it was accepted for publication, hence it is the final version of the accepted manuscript.

This chapter focuses on applying the quantum mechanics of DFT and hybrid QM/MM ONIOM methods to investigate the chemical properties of AM-6494 (a novel drug) which showed high selectivity for BACE1 relative to CNP-520 (another BACE1 inhibitor). It further explores the use of molecular electrostatic potential (MESP) in the analysis of the effect of atomic charge distribution and natural bond orbital (NBO) on the studied BACE1 inhibitors.

CHAPTER SIX

Published work as presented following the required format of the journal in which it was accepted for publication, hence it is the final version of the accepted manuscript.

This chapter focuses on the further elucidation of the structural and binding dynamisms of AM-6494 in comparison to umibecestat (CNP-520). It deals with the application of the computational instruments of conventional molecular dynamic simulations (cMD) and accelerated molecular dynamic simulations (aMD) in the study of the chosen inhibitors. It covers the conformational monitoring of the flap covering of the active site of the studied inhibitors when bound to BACE1. Comparison of the binding free energy of the studied inhibitors as well as principal component analysis (PCA) was also covered in this chapter.

CHAPTER SEVEN

This is the concluding chapter; it also contains some recommendations.

ABSTRACT

Alzheimer's disease (AD), as a progressive multifactorial neurodegenerative abnormality of the brain, is often connected with loss or death of neurons as its primary pathogenesis. Another kind of dementia is associated with memory loss and unstable and irrational behaviors, especially among the elderly above 60 years. In South Africa, there are over four million people above the age of 60 years, with an approximation of one hundred and eighty-seven thousand living with dementia. The two distinguishing features (hallmarks) of AD are neurofibrillary tangles and β-amyloid plaques. The β-amyloid plaques result when amyloid precursor protein (APP) is cleaved by β-amyloid precursor protein cleaving enzyme1 (BACE1), otherwise known as β-secretase. Since 1999 the first BACE1 was discovered, it has become a major interest in attempting to develop drugs for the inhibition or reduction of the β-amyloid aggregates in the brain. Reducing or inhibiting the accumulation of β-amyloid has long been the target in the design of drugs for AD treatment.

Having a good knowledge of the characteristic properties (BACE1) would assist in the design of potent selective BACE1 inhibitors with fewer or no side effects. Hitherto, only five drugs have been approved by the Food and Drug Administration (FDA) for the remediation of Alzheimer's disease, and none of the approved drugs targets BACE1. In about twenty years of its discovery, several past and ongoing studies have focused on BACE1 therapeutic roles as a target in managing AD. Several attempts have previously been made in designing some small drug molecules capable of good BACE1 inhibition. Some of the initially discovered BACE1 inhibitors include verubecestat, lanabecestat, atabecestat, and umibecestat (CNP-520). Although these inhibitors significantly lowered β-amyloid plaques in persons having neurological Alzheimer's at its clinical trials (phase 3), they were suddenly terminated for some health concerns. The termination contributed to the reasons why there are insufficient BACE-targeted drugs for AD treatment. Lately, a novel potent, orally effective, and highly selective **AM-6494 BACE1** inhibitor was discovered. This novel BACE1 inhibitor exhibited no fur coloration and common skin alteration, as observed with some initial BACE1 inhibitors. AM-6494 with an IC50 value of 0.4 nM *in vivo* is presently selected and at the preclinical phase trials. Before this study, the inhibition properties of this novel BACE1 inhibitor at the atomistic and molecular level of BACE1 inhibition remained very unclear.

The first manuscript (chapter two) is a literature review on Alzheimer's disease and β-secretase inhibition: An update focusing on computer-aided inhibitor design. We provide an introductory background of the subject with a brief discussion on Alzheimer's pathology. The review features computational methods involved in designing BACE1 inhibitors including the discontinued drugs. Using the topical keywords BACE1, inhibitor design, and computational/theoretical study in the Web of Science and Scopus database,

we retrieved over 49 relevant articles. The search years are from 2010 and 2020, with analysis conducted from May 2020 to March 2021.

Our second manuscript (chapter three) reviewed BACE1 exosite-binding antibody and allosteric inhibition as an alternative therapeutic development. We studied BACE1 biological functions, the pathogenesis of the associated diseases, and the enzymatic properties of the APP site cleavage. We suggested an extensive application of advanced computational simulations in the investigation of anti-BACE1 body and allosteric exosites. It is believed that this investigation will further help in reducing the associated challenges with designing BACE1 inhibitors while exploring the opportunities in the design of allosteric antibodies. The review also revealed that some molecules exhibited dual binding sites at the active site and allosteric site. As a result, we recommend an extensive investigation of the binding free energy beyond molecular docking (such as advanced molecular dynamic simulations) as this promises to reveal the actual binding site for the compounds under investigation.

Chapter four contains the detailed computational science techniques which cover the application of the vitally essential methods of molecular mechanics (MM), quantum mechanics (QM), hybrid of QM/MM, basis sets, and other computational instruments employed in this study.

In the third manuscript (chapter five), we carried out computational simulations of AM-6494 and CNP-520. CNP520 was one of the earliest BACE1 drugs that were terminated, chosen in this study for comparative reasons. This simulation was to elucidate and understand the binding affinities of these two inhibitors at the atomistic level. We explored the quantum mechanics (QM) density functional theory (DFT) and hybrid QM/MM of Our Own N-layered Integrated molecular Orbital and Molecular Mechanics (ONIOM) in these simulations. These computational approaches helped in predicting the electronic properties of AM-6494 and CNP-520, including their binding energies when in complex with BACE1. Considering the debates on which protonated forms of Asp 32 and Asp 288 gives a more favorable binding energy, we analysed the two forms which involved the protonation and un-protonation of Asp 32 and Asp 228. The ONIOM protonated model calculation gave binding free energy of -33.463 kcal/mol (CNP-520) and 62.849 kcal/mol (AM-6494) while the binding free energy of -59.758 kcal/mol was observed for the unprotonated AM-6494 model. These results show the protonated model as a more favourable binding free energy when compared with the un-protonation AM-6494 model. Further thermochemistry processes coupled with molecular interaction plots indicate that AM-6494 has better inhibition properties than CNP-520. However, it was observed that the protonation and the un-protonation of Asp 32 and Asp 228 models could adequately illustrate the interatomic binding of the ligands-BACE1 complex.

To further explicate the binding mechanism, conformational and structural dynamism of AM-6494 relative to CNP-520 in complex with BACE1, we carried out advanced computational simulations in the fourth manuscript (chapter six). The extensive application of accelerated molecular dynamics simulations, as well as principal component analysis, were involved. From the results, AM-6494 further exhibited higher binding affinity with van der Waals as the predominant contributing energy relative to CNP-520. Furthermore, conformational analysis of the β-hairpin (flap) within the BACE1 active site exhibited efficient closed flap conformations in complex with AM-6494 relative to CNP-520, which mostly alternated between closed and semi-open conformational dynamics. These observations further elucidate that AM-6494 shows higher inhibitory potential towards BACE1. The catalytic dyad (Asp32/228), Tyr14, Leu30, Tyr71, and Gly230 constitute essential residues in both AM-6494 potencies CNP-520 at the BACE1 binding interface. The results from these extensive computational simulations and analysis undoubtedly elucidate AM-6494 higher inhibition potentials that will further help develop new molecules with improved potency and selectivity for BACE1. Besides, grasping the comprehensive molecular mechanisms of the selected inhibitors would also help in fundamental pharmacophore investigation when designing BACE1 inhibitors.

Finally, the implementation of computational techniques in the designing of BACE1 inhibitors has been quite interesting. Nevertheless, the designing of potent BACE1 inhibitors through the computational application of the QM method such as the density functional theory (DFT), MM, and a hybrid QM/MM method should be extensively explored. We highly recommend that experimentalists should always collaborate with computational chemists to save time and other resources.

Iqoqa

Isifo se-Alzheimer (AD), njengoba siqhubeka siyinhlanganisela yezimbangela ze- neurodegenerative engajwayelekile ebuchosheni, isikhathi esiningi kuxhumana nokulahleka noma ukufa kwama-neurons njengongqaphambili we-pathogenesis. Kungolunye uhlobo lwedementia oluhambisana nokulahlekelwa ukukhumbula kanyenokuxenga kanye nokuphanjanelwa ingqondo, ikakhulukazi kubantu abadala esebeneminyaka engaphezulu kuka-60. ENingizimu Afrikha, kunabantu abangaphezulu kwezigidi ezine abangephezulu kweminyaka ewu-60, ngokuhlawumbisela nje abayinkulungwane namashumi ayisishayangolombili nesikhombisa baphila nedemetia. Zimbili izimpawu ezihlukanisekayo ze-AD ziba-ama-neurofibrillary tangles kanye ne-B-amyloid plaques. I-B-amyloid plaques ingumphumela ngesikhathi i-amyloid eyiprotheni egijimayo iqhwakele oketshezini i-enzyme1 (BACEI), ngale kwalokho yaziwa njenge B-secretase. Kusukela ngo 1999 i-BAC1 yatholakala, isiphenduke ungqaphambili emizamweni yokwakha isidakamizwa sokwehlisa i-B-amyloid

ngokwezinga lengqondo. Ngokunciphisa ukwanda kwe-B-amyloid isiphenduke okuqondiwe mayelana

nokuqopha isidakamizwa ukuze kwelashwe i-AD.

Ukuba nolwazi oluhle oluthinta isici sezakhi ze-BACE1 kuzosiza ekubazeni amandla akhethiwe i-BACE1 ukuvimbela imiphumela engaqondiwe. Kuze kube manje mihlanu imithi esiphasisiwe ngabezokuphatha ukudla kanye nezidakamizwa (FDA) ukwelapha isifo se-Alzheimer kanye nokuthi azikho kulezi eziphasisiwe izidakamizwa ebhekana ngqo ne-BACE1. Emva kokuba selitholakele lapho nje eminyakeni engu 20, sekunezinye esikhathini esedlule kanye nezifundo ezisaqhubeka zigxile ngokubheka kakhulu iqhaza lokwelapha i-BAC1 njengokuqondiswe ekungameleni u-AD. Imizamo eminingana yenziwa esikhathini esedlule ukuqopha uketshezi lwezidakamizwa olukwazi ukuvimba kahle i-BACE1. i-B-amyloid plaques kumuntu one-neurological ye-Alzheimer's kumzamo (isigaba 3), kwabuye kwanqanyulwa ngenxa yokukhathazeka ngokwezempilo. Ukunqanyulwa kwanikela kuzizathu zokusilele kwezidakamizwa okuqondene nokulashwa kwe-AD. Kamuva, i-novel enamandla, ngisho ngawo umlomo kanye neyakhethwa ngezinga eliphezulu i-AM-6494 BACE1 evikelayo yatholakala. Le noveli i-BACE1 evimbayo yabukisa hhayi ukushintsha kombala woboya kanye nokushintsha kwesikhumba okujwayelekile, njengoba kubukwa nezivimbo zokuqala ze-BACE1. I-AM-6494 ne-IC50 enobumqoka buka 0.4nM kuyo i-vivo ekhethwa ngokwamanje kanye nesigaba sembulambethe yemizamo. Ngaphambi kwalesi sifundo, izakhi zesivimbela zale noveli i-BACE1zivimba ngokwe-atomistic kanye neqophelo le-molecular ye-B ACE1evimbayo kusale nje kungacacile. Umqulu wokuqala (isahluko sesibili) ukubuyekezwa kwesifo se-Alzheimer's kanye no-B-secretase ovimbayo: ezikhumbuzayo ezigxile ngokusizwa yikhompuyutha eyisivimbo ngokwakhiwa. Sethula isendlalelo sesifundo kanye nengxoxo kafushane nezimbangela nemiphumela ye-Alzheimer. Ukubukezwa kwezimpawu zendlela zobukhompuyutha kufaka ekuqopheni isivimbo se-BACE1 nokuqhutshekiswa kwesidakamizwa. Ngokusebenzisa ofeleba begama BACE1, kusho ukwakha isivimbo, kanye nesifundo senjulalwazi kulwembu lobuchwepheshe kanye ne-Scopus sesizindalwazi. Sathola amaphepha acwaningiwe anokuhlobana angaphezulu kuka 49. Unyaka wokuthungatha usukela ku2010 kuya ku2020, nohlaziyo lwenziwa kusukela kuNhlaba 2020 kuya kuNdasa 2021.

Umqulu wethu wesibili (isahluko sesithathu) sabuyekeza i-BACE ehlanganisa i-exosite antibody kanye ne-allosteric yokuthuthukisa ukwelashwa. Sakufunda ukusebenza kwesayensi yokuphila ye-BACE1, i-pathogenesis ehambisana nezifo kanye nezakhi zama-enzymatic esizinda sokuhlukana se-APP. Saphakamisa ukufakwa okunzulu nokucokeme kokulinganisa ngobuchwepheshe bekhompuyutha ekuphenyeni ama-anti-BACE1 omzimba kanye ne-allosteric ye-exosites. Kuyakholeka ukuthi uphenyo luzoqhubeka nokusiza ekwehliseni izinselelo ezihambisana nokwakha isithiyo se-BACE1 ngesikhathi kuhlolwa amathuba okwakheka kwe-allosteric yama-antibodies. Ubuyekezo luphinde lwaveza uketshezi olubukisa isizinda sokuhlanganisa kabili kusizinda esikhuthele kanye nesizinda se-allosteric. Umphumela,

kube ukwenza isincomo mayelana nocwaningo olunzulu oluzohlanganisa umfutho okhululekile odlulele

ku-molecular docking (njengesicokeme se-molecular yokuhlukahlukana kokulinganisa) njengoba lokhu kuthembisa ukuveza isiza esibopha ngempela ama-compounds angaphansi

Isahluko sesine siqukethe imininingwane ngamaqhinga e-computational sayensi efaka isicelo esibalulekile sezindlela ezibalulekile ze-molecular mechanics (MM), i-quantum mechanics (QM), i-hybrid ye-QM/MM, ngesisekelo samasethi kanye namanye amathuluzi ekhompuyutha akhethwa kulesi sifundo.

Kumqulu wesithathu (isahluko sesihlanu), siqhube isilinganiso se-computational ye-AM-6494 kanye CNP-520. I-CNP-520 kwakungenye yezidakamizwa zokuqala zeBACE1 ezashatshalaliswa, zakhethwa kulesi sifundo ngezizathu zokuqhathanisa. Ukulinganisa kwakuchaza kanye nokuqonda ukusondelana ngokuhlanganiswa kwezithiyo ezimbili kusigaba se-atomistic. Kwahlolwa i-quatum mechanics (QM) yesisindo yokusebenza kwenjulalwazi (DFT) kanye ne-hybrid QM/MM yokwethu okuno-N oluwugqinsi lwe-molecular Orbital kanye ne-Molecular Mechanics (ONIOM) kulolu linganiso.

Lezi zindlelakwenza ze-computational zasiza ekuqageleni kwezakhiwo zama-electronic e-AM-6494 kanye CNP-520, kungena namandla okuhlanganisa ngesikhathi kuba lukhuni ne-BACE1. Ngokucabanga izinkulumo mpikiswano mayelana nokuma kwe-protonated ye-Asp32 kanye Asp288 kunika ukuvumelana namandla okuhlanganisa, nokuhlaziya izimo ezimbili ezifaka i-protonation kanye ne-unprotonation ye-Asp32 kanye Asp228. I-ONIOM ye-protonated yomfanekiso wokubala wanikeza amandla akhululekile okuhlanganisa -33,463kcal/mol (NP-520) kanye 62.849 kcal /mol kwavela i-unprotonate ye-AM6494. Imiphumela itshengisa ukuthi i-protonated iyisifanekiso njengoba kuyisona esivumela ukuhlanganiswa ngokukhululeka ngesikhathi lapho bekuqhathanisa ne-unprotonation yomfanekiso u-AM-649. Kuqhutshelwa phambili nemisebenzi ye-thermochemistry kuhlangana nokudlelana ne-molecular plots

kutshengisa ukuthi i-AM-649 inezakhiwo ezinhle zokuvimba kune CNP-520. Yize kunjalo kwabonakala ukuthi i-protonation kanye ne-unprotonation ye-Asp32 kanye neyomfanekiso owu- Asp228 bekungatshengisa ngokwenele ukuhlanganisa ngokwe-interatomic yama-ligands EBACE1 ebilukhuni.

be-BACE1 ngokwedlulele isilinganiso se-computational. Ukwenza ngokujulile kuphangiswa isilinganiso se-molecular ngokuhlukana, kwakakwa nohlaziyo olusemqoka lwezingxenyana. Imiphumela ye-AM-6494 yaqhubeka yatshengisa ukusondelana kokuhlanganiswayo no-van der Waals njengohamba phambili ekunikeleni amandla ahlobene ne-CNP-520. Ukuvuma kohlaziyo lwe-B-hairpin ngaphakathi ku-BACE1 kutshengiswa esizeni esiphilayo esivala ngendlela umnyakazo wokuvuma kobunkimbinkimbi be-AM-6494 ehlobene neCNP-520, ngokuvamile eshitshashintshayo phakathi kwevalekile kanye nezishaya sakuvuleka kokuvuma okunhlobonhlobo.

Lokhu kuhlolwa kuqhubeke kwachazwa ngokuthi i-AM-6494 itshengisa ukuvimba okukhulu nokunethemba mayelana ne-BACE1. Isikhuthazizinguquko se-dyad (Asp32/228), Tyr14, Leu 30, Tyr 71,

kanye ne-Gly230 kwakha izinsalela ezibalulekile nxazombili kuAM-6494ne-potencies yeCNP-520 kuBACE1 nesixhumanisi esihlanganisayo. Imiphumela ivela kulama-computational anzulu ayisilinganiso kanye nohlaziyo olucacisa ngokungangabazi i-AM-6494 enesivimbelo esiphakeme esingakwazi ukuqhubeka nokusiza intuthuko yama-molecules amasha anamandla athuthukile kanye nakhethelwe i-BACE1. Ngaphandle kwalokhu, ukucosha izinkambiso ezibanzi ze-moleculor mayelana nezivimbo ezikhethiwe kuzosiza mayelana nophenyo olubalulekile lwe- pharmacophore ngesikhathi kuqoshwa izivimbo se-BACE1. Ekugcineni, ukwenziwa kwe-computational ngokwamacebo ekubazeni izivimbo ze-BACE1 kube into ehlaba umxhwele. Nokho ukubaza izivimbo ezinamandla ze-BACE1 ngokusebenzisa i-

computational yendlela ye-QM njengenjulalwazi yesisindo esisebenzayo (DFT), MM, kanye nendlela ye-hybrid QM/MM kufanele iphenywe kanzulu. Sincoma kakhulu ukuthi ongoti abenza izibonisi kufanele njalo bahlangane nama-computational chemists ukonga isikhathi kanye

nezinye izinsiza.

DECLARATION I – PLAGIARISM

I, Samuel Chima Ugbaja, declare that

1. Research reported here in this thesis is originally mine, except where otherwise indicated.

2. This thesis has not been submitted for any degree or examination at any other university or institution of learning.

3. No other person's data, images, graphs, annotations, or information is contained in this thesis unless specifically acknowledged as being sourced from other persons.

4. This thesis contains no other person's writing unless specifically acknowledged as being sourced from other researchers. Moreover, in cases where other written sources were quoted, then:

a. their words have been re-constructed while the general information attributed to them was appropriately referenced.

b. in places where their exact words were used, their writings were italicized and quoted while also appropriately referenced.

5. No text, graphics, or tables directly copied from the internet are pasted in this thesis unless specifically acknowledged and sources appropriately indicated and referenced.

Also presented herein is a detailed list of contributions to publications, including those that form part of this thesis as stated appropriately.

Signed: S.C Ugbaja

DECLARATION II-LIST OF PUBLICATIONS

1. Samuel C. Ugbaja, Zainab K. Sanusi, Patrick Appiah-Kubi, Monsurat M. Lawal, Hezekiel M. Kumalo, Computational modelling of potent β-secretase (BACE1) inhibitors towards Alzheimer's disease treatment. *Biophysical Chemistry*, **2021**, 106536

   Contributions:

   Samuel C. Ugbaja: Conceptualization, data curation, formal analysis, investigation, project administration, visualization, original draft writing, review, and editing.

   Zainab K. Sanusi: Assisted in data curation, formal analysis, review, and editing.

   Patrick Appiah-Kubi: Assisted in conceptualization, review, and editing.

   Monsurat M. Lawal: Assisted in data curation, formal analysis, supervision, writing, review, and editing.

   Hezekiel M. Kumalo: Supervision.

2. Samuel C. Ugbaja, Patrick Appiah-Kubi, Monsurat M. Lawal, Nelisiwe S. Gumede, Hezekiel M. Kumalo, Unravelling the molecular basis of AM-6494 high potency at BACE1 in Alzheimer's disease: an integrated dynamic interaction investigation. *Journal of Biomolecular Structure and Dynamics*, **2021**, 1-13.

   Samuel C. Ugbaja: Conceptualization, data curation, formal analysis, investigation, project administration, visualization, original draft writing, review, and editing.

   Patrick Appiah-Kubi: Assisted in conceptualization, data curation, original draft writing, formal analysis, review, and editing.

   Monsurat M. Lawal: Ass/isted in supervision, review, and editing.

   Nelisiwe S. Gumede: Assisted with review and editing.

   Hezekiel M. Kumalo: Supervision.

3. Samuel C. Ugbaja, Monsurat M. Lawal, Hezekiel M. Kumalo, An overview of β-amyloid cleaving enzyme 1 (BACE1) biological functions – elucidating its exosite-binding antibody and allosteric inhibitor, Current Medicinal Chemistry, 2021, 34102967.

Contributions:

Samuel C. Ugbaja: Original draft writing, review, and editing.

Monsurat M. Lawal: Supervision, review, and editing.

Hezekiel M. Kumalo: Supervision.

4. Samuel C. Ugbaja, Isiaka A. Lawal, Monsurat M. Lawal, Hezekiel M. Kumalo, Alzheimer's disease and β-secretase inhibition: An update with a focus on computer-aided inhibitor design.

Contributions:

Samuel C. Ugbaja: Original draft writing, review, and editing.

Isiaka A. Lawal: Review and editing

Monsurat M. Lawal: Supervision, review, and editing.

Hezekiel M. Kumalo: Supervision.

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Jesus asked “Were there not ten lepers healed, but only one returned to say ‘Thank You? I am the one who has returned to say “Thank You Jesus” my Lord, Savior, helper, and intercessor. Thank you, Abba Father, for your enabling mercy and grace, sustenance and provision.

My unalloyed loyalty goes to Dr. H.M. Kumalo (supervisor), Dr. Monsurat M. Lawal (co-supervisor), Rtd.Air Vice Marshal Kingsley Lar (Godfather), Revs. Chido&Joy Onuoha (guardians), Bayode Kolade (friend and business colleague), Dr. Patrick Apiah-Kubi (best friend in SA), Binta Max Gbinije (spiritual mother), Dr. Terence& Jothi Govender (my next of kin in SA), Engr. Mrs. Bahijjahtu Abubakar (Boss4life), Professor Emeka Eneh (papam), Bishop Walter Mbamara (spiritual father), Sam Chika (twin brother), Oludotun Babayemi (best man & my colleague), Barr.Chidi & Joy Metu (counselors), Dr. Tumelo &Vincent Njiru, Dr. Tomi Ajayi, Vincent Obakachi, and Murtala Ejalonibu, Chukwudi Okpala, Festus Uchenna Okoro and Uchenna Udorji (Cousin in Joburg), Dr. Akebe Luther King Abia.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
Å	Angstrom
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADMET	Adsorption distribution, metabolism, excretion, and toxicity
aMD	Accelerated molecular dynamics
APP	Amyloid precursor protein
Asp-PR	Aspartate protease
Aβ	β-amyloid
B3LYP	Becke,3-parameter, Lee Yang Parr
BACE1	β-amyloid precursor protein cleaving enzyme 1
BBB	Blood-brain barrier
BuChE	Butyrylcholinesterase
CADD	Computer-aided drug design
ChE	Cholinesterase
cMD	Conventional molecular dynamics
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Classification tree
CTF	Carboxy-terminal fragment
DCCM	Dynamic cross-correlation matrix
DFT	Density functional theory
esu	Electrostatic unit of charge
FBDD	Fragment-based drug discovery
FB-QSAR	Fragment-based quantitative structure-activity relationship
FB-QSSR	Fragment-based quantitative structure selectivity relationship

FDA	Food and drug administration
FEP	Free energy perturbation
FRET	Fluorescence resonance energy transfer
GAFF	General AMBER force field
GPD	Gross domestic product
HB	Hydrogen bond
HPT	Hyperphosphorylated-tau
IC_{50}	Half inhibitory concentration
IDPs	Intrinsically disordered proteins
LIE	Linear interaction energy
MAA	Moving average analysis
MD	Molecular dynamics
MESP	Molecular electrostatic potential
ML	Machine learning
MM	Molecular mechanics
MM/GBSA	Molecular mechanics/generalized Born surface area
MMPBSA	Molecular mechanics Poisson-Boltzmann surface area
MO-QSPR	Multi-objective quantitative structure properties relationship
NAC	Natural atomic charges
NBO	Natural bond orbital
NFTs	Neurofibrillary tangles
nM	Nanomolar
NMDA	*N*-methyl- D-aspartate
NMR	Nuclear magnetic resonance
NPT	Number of moles, pressure, and temperature
ONIOM	Our Own N-layered Integrated molecular Orbital and Molecular Mechanics
PCA	Principal component analyses
PD	Pharmacodynamic
PDB	Protein data bank

PET	Positron emission tomography
P-gp	P-glycoprotein
PHFs	Paired helical filaments
PK	Pharmacokinetic
QM	Quantum mechanics
QM/MM	Quantum mechanics/molecular mechanics
QSAR	Quantitative structure-activity relationship
QTAIM	Quantum theory atom in molecules
RCSB	Research Collaboratory for structural bioinformatics
RF	Random forest
Rg	Radius of gyration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
sAPPα	Soluble α-amyloid precursor protein
sAPPβ	Soluble β-amyloid precursor protein
SMD	Solvation model based on solute electron density
vdW	van der Waals
μM	Micro molar

LIST OF AMINO ACIDS

One Letter code	Three Letter code	Amino acid name
A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartate
C	Cys	Cysteine
E	Glu	Glutamate
Q	Gln	Glutamine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

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Figure 10. Representative binding interaction modes of CNP-520 with BACE1 extracted from the (A) initial, (B) intermediate, and (C) final stages of the simulation.

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CHAPTER ONE

INTRODUCTION

BACKGROUND

One of the main causes of dementia is Alzheimer's disease (AD), detected more than a hundred years ago by a Deutsch Neurologist Dr. Alois Alzheimer's has been described as a disease that is caused by abnormalities in the neurons and the brain (1, 2). It has two significant distinctive characteristics, which include β-amyloid accumulation and tau entangles (3-5). The aggregation of β-amyloids occurs when amyloid precursor protein (APP) is concurrently split by β and γ secretases (6). This cleavage by β-secretase produces more toxic species at the APP N-terminus (7).

In 2020, AD facts and figures estimated that about six million Americans were living with dementia-related AD, and this number was projected to increase to about fourteen million by the year 2050 (8). However, there has been scarce information due to insufficient research in dementia-related AD in South Africa and sub-Saharan Africa at large (9). A recent World Alzheimer's declaration stated that the number of persons above sixty in South Africa was 4.4 million, of which 187 thousand were living with dementia (9, 10). This number was estimated to rise to about 250 thousand with a corresponding increase in persons above sixty years to seven million (9). In Nigeria, the most prominent black nation in Africa, it was estimated that over sixty-three thousand people (above 60 years) were living with dementia in 1995, and this figure rose to above three hundred thousand in 2015. This increment represents about 400%, which is very worrisome, as the number of people within 60 years and above increases too (11). The annual economic burden of about 600 billion US$ (American dollar) is utilized in caring for approximately thirty-five million persons with dementia-related AD worldwide. This figure accounts for one percent of the Gross Domestic Product (GDP) globally (12).

The β-secretase, otherwise called β-amyloid precursor protein cleaving enzyme-1 (BACE1), is majorly responsible for splitting peptide bonds; hence, its classification as aspartate protease (13, 14). Previously, scientists paid much attention to the design of drug molecules targeted at γ-secretase inhibition (1, 15). This attention resulted in the discovery of semagacestat, the first γ-secretase inhibitor at phase three clinical trial but was discontinued (April 2011) due to skin cancerous (accelerated skin cancer) risk and lack of potency (1, 16). Furthermore, other deleterious side effects were prevalent during further clinical trials involving γ-secretase, such as off-target effects associated with notch receptors. Finally, due to these health and pharmacological risks (including the impairment of memory and elevation in the APP γ-secretase substrates), γ-secretase was discontinued as an AD therapeutic target (1, 16, 17). Sequel to the success of HIV protease (other aspartyl proteases) inhibitors, it became easier to develop BACE1 inhibitors with

highly selective and reduced side effects (18). This success has also led to an appreciable development in discovering BACE1 inhibitors targeted at Alzheimer's disease therapy (19).

Over the years, BACE1 has been considered a primary treatment target in the management of AD (20). Having a good insight into its drug-inhibiting properties is imperative in designing an effective and potent BACE1 inhibitor that exhibits higher selectivity for the purpose (20). It is believed that BACE1 plays a significant role in the development, maintenance, and repair of the neurons as well as myelin sheath formation in the brain. Therefore, drug therapy should be targeted at regulating BACE1 levels instead of completely eradicating them (21-25). BACE1 has a similar structure with other aspartyl proteases families; hence an in-depth understanding of its interactions with inhibitors at the atomistic level is a prerequisite in achieving an efficient BACE1-ligand complex (26). When the inhibitor (ligand) is adequately accommodated at the active site, the BACE1 catalytic dyad (aspartate 32 and 228) gets actively involved in hydrogen bond formation and thus prevents the protease mechanism of coordinating a water molecule and non-cleaving of APP (27). In addition, the ligand (inhibitor) establishes a better contact with the specificity pocket and the BACE1 flap giving an imperfect yet stable BACE1-ligand complex. Several laboratories, biological and computational studies on BACE1-ligand inhibition properties have been carried out, and many others are currently ongoing (1, 28). There is no doubt that the terminated BACE inhibitors (Verubecestat, Umibecestat, Atabecestat, and Lanabecestat) significantly lowered β-amyloid plaques in persons with neurological Alzheimer's, they were, however, terminated for some adverse health concerns (29). Their termination contributed to the shortage of BACE1-targeted AD therapeutic drugs (30-32). In 2019, only a few β-amyloid targeted studies in phase3 trials compared with the numbers in 2017 and 2018 (33, 34). Sequel to the ongoing, this research pays close attention to BACE1 properties and their interactions when bound with ligand molecules.

BACE1 Structural Properties and description

BACE1 (figure.2) consists of an active site situated between the C and N terminals having the aspartates 32 and 228 (catalytic dyad) centrally located within its binding region (35, 36). The flap (hairpin loop) includes residues 67–75 and situated at the N terminus, and it regulates the entrance of substrates into the active site through its dynamic conformations. The binding site also contains other pockets (subsites: S1, S2, S3, S4, S1', S2', S3', S4'). The hydrophobic residues are found within S3 and S1 (Leu30, Phe108, Ile110, Ile118, Trp115), while the hydrophilic (solvent-exposed) residues are within S4 and S2 (Lys9, Ser10, Thr72, Gln73, Thr231, Thr232, Arg235, Arg307, Lys321). The S4' and S3' (Pro70, Thr72, Glu125, Arg128, Agr195, Trp197) are other hydrophilic pockets, while S2' (Ser35, Val69, Tyr71, Ile126, Tyr198) situated closer to S4' is both hydrophobic and hydrophilic (amphipathic). Finally, the catalytic dyad is centrally located within the S1' (Ile226 and Val332) pocket (28, 36).

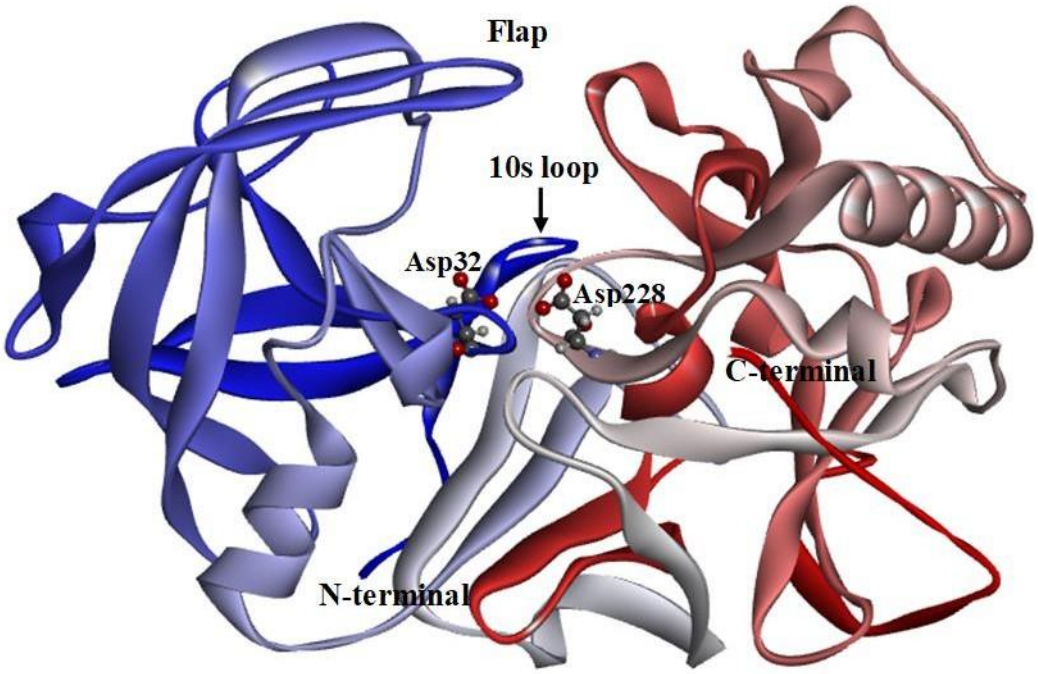
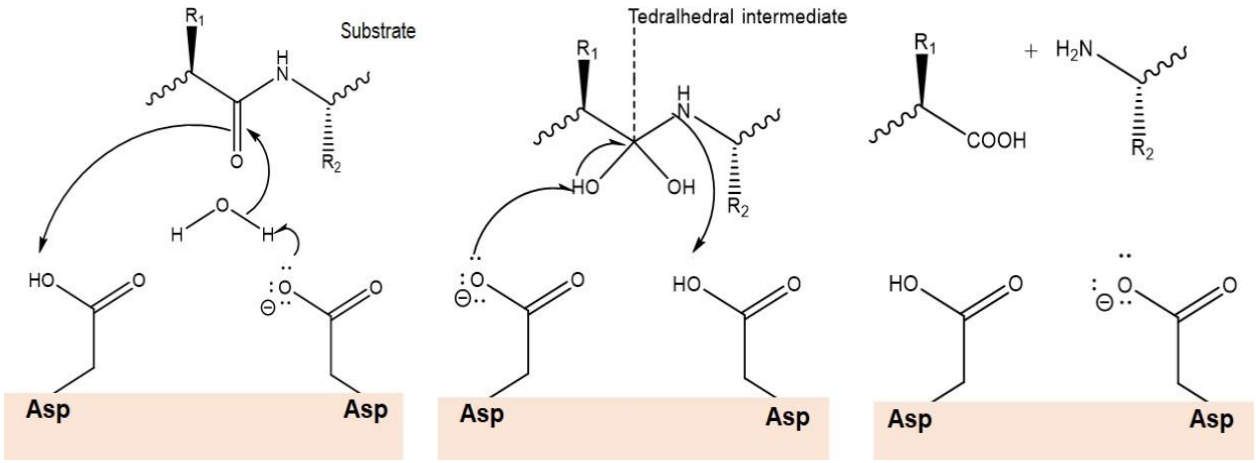


Figure 1. 3D structure of BACE1 showing the N and C terminals, the catalytic dyad (Asp32 and 228), the flap (residues 67–75), and the 10S loop (residues 5–16).

Mechanism of BACE1 peptide bonds breaking by the catalytic dyad

Based on extensive investigations by several researchers on aspartic proteases, it has been reported that one of the two characteristic water molecules located closer to the catalytic dyad (Asp 32 and Asp 228 for BACE1) is responsible for the breaking of the peptide bond (27, 37). The other water molecule plays a vital role in hydrogen bond formation and stabilizing the enzyme structure (38, 39). The mechanism of the breaking of the peptide bond in BACE1 as well as other protease families is like an acid-base catalytic reaction (27). A proton is released to Asp 32 by a water molecule located at the active site, thereby producing a potential nucleophilic species. Afterward, the peptide's carbonyl carbon undergoes a nucleophilic attack with the removal of one electron which is then transferred to Asp 228, making it positively charged and leaving Asp 32 negatively charged. The generated tetrahedral intermediate is further split in a reaction like serine catalysis and acid-base catalytic mechanism (27, 40, 41).



Scheme 3: Proposed acid-base mechanism of the protease activity

Multi-target therapeutic approach for AD treatment

Alzheimer's disease pathogenesis is multifactorial and requires a multi-targeted treatment approach; herein reported are three treatment routes currently being studied at various clinical trial stages. The first route involves identifying patients with accelerated risk and helping them with primary preventive control measures. The second route involves diagnosing persons at the preclinical phase (usually starts 10-20 years) while exploring modern techniques on genetic makeup and neuroimaging and CSF (cerebrospinal fluid) examinations. Finally, discovering disease-modifying molecules which lower the accumulation of β-amyloid plaques and inhibiting NFTs aggregation (42, 43). The latter constitutes the focus of this research. Due to the unpleasant failure rates of most of the BACE1 inhibitors at the clinical trials, scientists decided to shift in the direction of multitarget molecules towards designing BACE1 inhibitors. These multitarget-directed ligands (MTDLs) methods involved designing small molecules modulating BACE1 and other related targets (44, 45). Researchers suggested that lead optimization and drug discovery through MTDLs methods appear frustrating and very complex (46). Sequel to the ongoing multitargeting of BACE1 inhibitors seemed not to be the only way of out the challenge of failures in designing BACE1 inhibitor compounds that would scale through the phase 3 clinical trials. Therefore, attention is shifted to single target compounds with specificity and selectivity for the BACE1 active site. . The detailed literature reviews on this study are found in chapter two as an accepted manuscript for publication in a journal and chapter three, already published in a journal.

Having reviewed the previous experimental and some computational approaches in attempting to design successful BACE1 inhibitors, this present study delved into the computational investigation of the BACE1-ligand complex at the atomic and interatomic, and molecular levels. First, we considered a recent (2019) research by Pettus et al. (47) in identifying **AM-6494** as a potent BACE1 inhibitor and its subsequent

proposal for preclinical development. This novel drug (**AM-6494**) was an analog of verubecestat, but it showed better selectivity for BACE1, unlike the discontinued verubecestat, which showed hair-color changes traced to BACE2 off-target inhibition (47, 48). Sequel to this discovery, our group embarked on further computational studies on the novel drug (49, 50). we, therefore, extensively investigated **AM-6494** at the molecular level through conventional and accelerated molecular dynamics (cMD and aMD) simulations, end-point binding free energy analysis, and flap conformational analysis (50). The result provided a helpful understanding of the interatomic mechanical interactions of the BACE1–inhibitors' complex. The outcome can also help design additional BACE1 inhibitors with specific selectivity (50). we also employed density functional theory (DFT) and Our own N-layered Integrated Molecular Orbital and Molecular Mechanics (ONIOM) in predicting the binding free energy and electronic properties of BACE1–inhibitor complexes (49). The two theoretical models (49, 50) successfully elucidated the potency of **AM-6494** over the discontinued **CNP-520** (**Table 1**) BACE1 inhibitors. The details of these computational tools are duly explained in chapters four, five, and six of this report. We also observed preliminary research with a focus on targeting allosteric regions or exosites, we therefore embarked on allosteric site or exosite in the investigation on potent BACE1 inhibitors.

Finally, we shifted our attention from the conventional binding site of BACE1 to the exosite (allosteric site). We extensively reviewed existing experimental and computational exosite antibody and allosteric site inhibition for BACE1 (51-53). The details are found in chapter three of this report. Below is a brief description of the computational drug design methods used in this study. The detailed discussion, including other computational methods, is fully discussed in chapter four of this report.

Experimental studies are not adequate to elucidate the interatomic, structural, vibrational, and optical properties of inhibitor molecules targeted at BACE1 inhibition. Herein, we investigated and established the importance of constantly comparing experimental results with computationally derived results. Therefore, we hereby present a diagram illustrating some available computational chemistry methods (Figure 8) available for BACE1 inhibitor design (54-56). The detailed discussions are found in chapters two, three, and four of this study

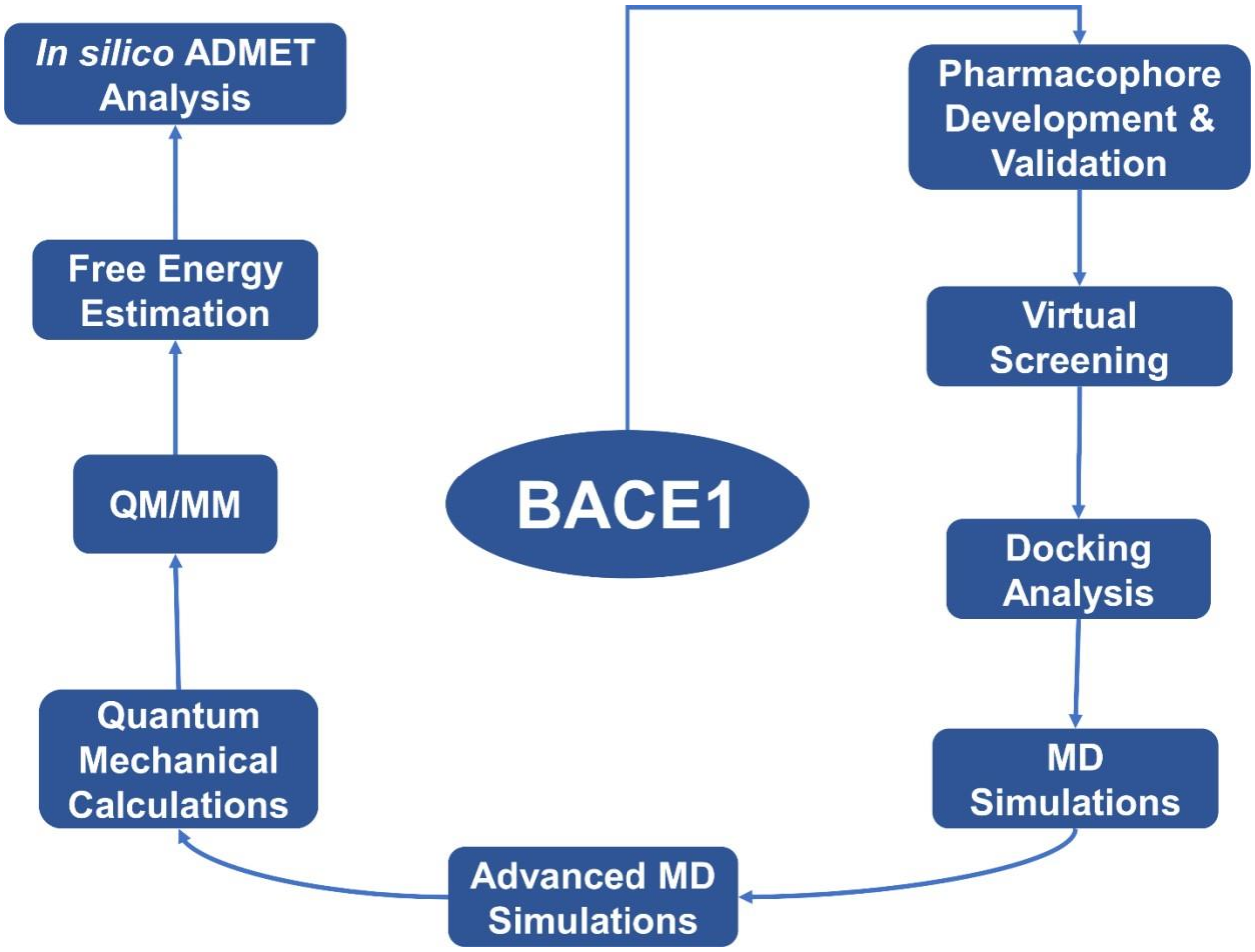


Figure 2. Schematic representation of some available computational methods for BACE1 inhibitor design.

Aims and objectives

The overarching aims of this work are:

i. To employ multidimensional computational instruments in exploring, investigating, and understanding the potent BACE1 inhibition properties at the interatomic and molecular level at large.

ii. To calculate and determine the binding free energies of the selected small molecules in complex with BACE1.

iii. To elucidate (at atomic and molecular levels) the efficiencies and effectiveness of some potent experimentally discovered BACE1 inhibitors by application of computational apparatus of quantum mechanics (QM), molecular mechanics (MM), and combined levels of theory of QM/MM.

iv. To also compare at the atomic level the inhibition potentials of the novel AM6494 with one of the terminated BACE1 drugs.

v. To investigate the existing debates on which protonated forms of Asp 32 and Asp 288 give a more favorable binding energy.

vi. To further substantiate the existence of some molecules with dual, allosteric, and exosite binding possibilities to the BACE1 enzyme

The objectives are:

i. To further investigate these potent drug molecules by employing conventional MD and accelerated MD simulations.

ii. To extensively explore and study ligand-BACE1 complex binding mechanisms.

iii. To leverage the interatomic and electron density calculations provided by Gaussian software and other computational software in determining the natural atomic charges, molecular electrostatic potentials, and binding free energy of the drug molecules under investigation.

iv. Furthermore, to review the provided insight into the computational approaches employed in drug development while mentioning how these methods have been used in designing potent BACE1 inhibitors over the last decade.

v. Finally, to provide detailed and extensive reviews on BACE1 and AD and BACE1 biological roles, genetic properties, and the exosites-binding antibodies and allosteric properties.

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INTERLINKING PAGE ONE

Chapter one is a brief introduction of the study background, with a general description of neurons' communication network in the brain. It also highlights BACE1 structural properties, the multi-target therapeutic approach for the treatment of Alzheimer's disease, the methods employed in computational drug design as well as the intended aims and objectives of the study. In chapter two, we present a literature review having an overview on Alzheimer's disease and β-Secretase inhibition with an update and focus on computer-aided inhibitor design. In this chapter two, the structural properties of BACE1 mentioned in chapter one are discussed in detail. The chapter also extensively deals with the prevalence and pathology of AD with a focus on the current drug pharmacological and design processes involved. It covers the inhibitors' properties as well as the mechanism involved in BACE1 inhibition. It highlights the general *in silico* methodologies of drug discovery while focusing on the methods involved in BACE1 inhibitor identification. Moreso, the review contains some of the computational techniques that are briefly discussed in chapter four of this study. The computational BACE1 inhibitor designs reviewed in this chapter two are mainly focused on the conventional active site of the enzyme. The inability of most of the BACE1 drugs to proceed beyond the clinical trials aroused curiosity to probe further. We embarked on another review which shifted attention from the conventional known active site to explore the possibility of other binding sites as discussed in chapter three.

CHAPTER TWO

MANUSCRIPT ONE

ALZHEIMER'S DISEASE AND β-SECRETASE INHIBITION: AN UPDATE WITH A FOCUS ON COMPUTER-AIDED INHIBITOR DESIGN

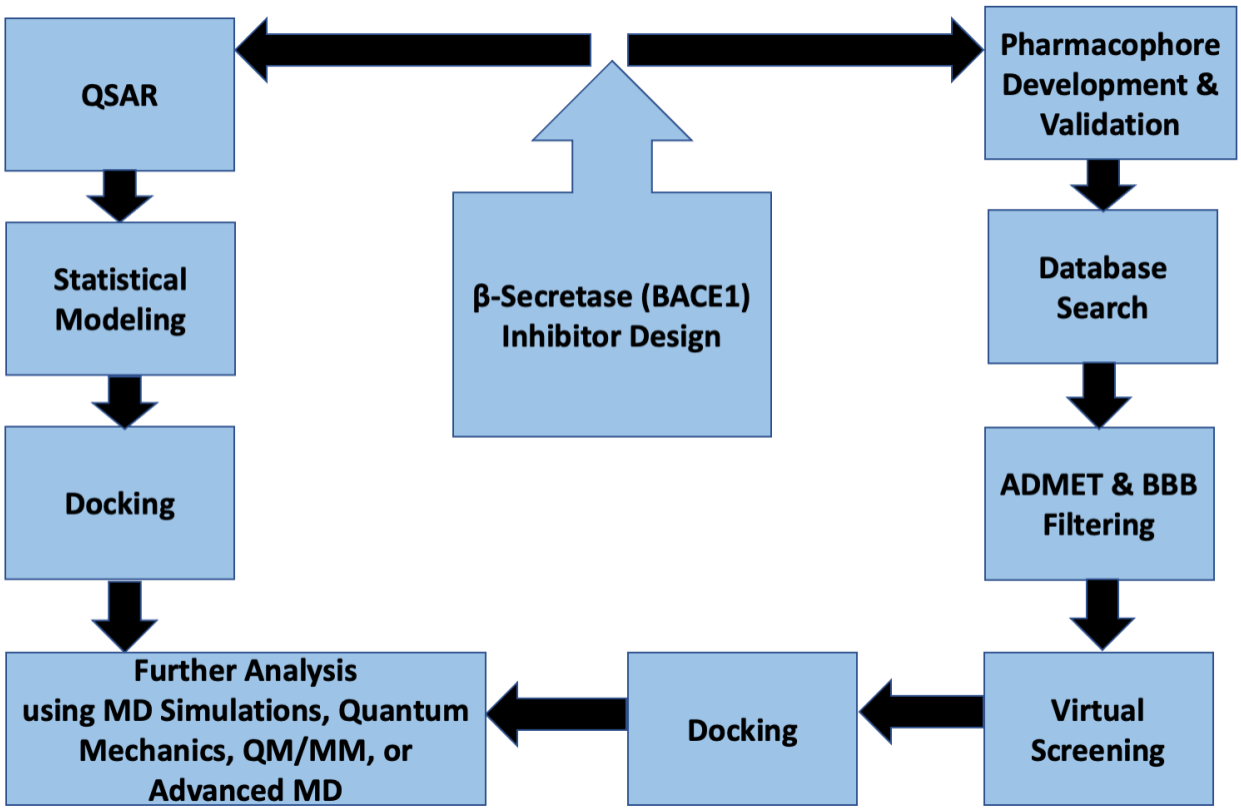
Samuel C. Ugbaja,[1] Isiaka A. Lawal,[2] Hezekiel M. Kumalo,[1] and Monsurat M. Lawal.[1]*

[1]Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu- Natal, Durban 4001, SouthAfrica.

[2]Chemistry Department, Faculty of Applied and Computer Science, Vaal University of Technology, Vanderbijlpark Campus, Boulevard, 1900, Vanderbijlpark, South Africa.

*Corresponding author: Dr. M. M. Lawal; Email: lawalmonsurat635@gmail.com

Graphical abstract



Synopsis: Schematic representation of common computational approaches for BACE1 inhibitor design.

Abstract

Introduction: Alzheimer's disease (AD) is an intensifying neurodegenerative illness due to its irreversible nature. Identification of β-site amyloid precursor protein (APP) cleaving enzyme1 (BACE1) has been a significant medicinal focus towards AD treatment, and this has opened ground for several investigations. Despite the numerous works in this direction, no BACE1 inhibitor has made it to the final approval stage as an anti-AD drug.

Method: We provide an introductory background of the subject with a general overview of the pathogenesis of AD. The review features BACE1 inhibitor design and development with a focus on some clinical trials and discontinued drugs. Using the topical keywords BACE1, inhibitor design, and computational/theoretical study in the Web of Science and Scopus database, we retrieved over 49 relevant articles. The search years are from 2010 and 2020, with analysis conducted from May 2020 to March 2021.

Results and discussion: Researchers have employed computational methodologies to unravel potential BACE1 inhibitors with a significant outcome. The most used computer-aided approach in BACE1 inhibitor design and binding/interaction studies are pharmacophore development, quantitative structure-activity relationship (QSAR), virtual screening, docking, and molecular dynamics (MD) simulations. These methods, plus more advanced ones including quantum mechanics/molecular mechanics (QM/MM) and QM, have proven substantial in the computational framework for BACE1 inhibitor design. Computational chemists have embraced the incorporation of *in vitro* assay to provide insight into the inhibition performance of identified molecules with potential inhibition towards BACE1. Significant IC_{50} values up to 50 nM, better than clinical trial compounds, are available in the literature.

Conclusion: The continuous failure of potent BACE1 inhibitors at clinical trials is attracting many queries prompting researchers to investigate newer concepts necessary for effective inhibitor design. The considered properties for efficient BACE1 inhibitor design seem enormous and require thorough scrutiny. Lately, researchers noticed that besides appreciable binding affinity and blood-brain barrier (BBB) permeation, BACE1 inhibitor must show low or no affinity for permeability-glycoprotein. Computational modeling methods have profound applications in drug discovery strategy. With the volume of recent *in silico* studies on BACE1 inhibition, the prospect of identifying potent molecules that would reach the approved level is feasible. Investigators should try pushing many of the identified BACE1 compounds with significant anti-AD properties to preclinical and clinical trial stages. We also advise computational research on allosteric inhibitor design, exosite modeling, and multisite inhibition of BACE1. These alternatives might be a solution to BACE1 drug discovery in AD therapy.

Keywords: β-Secretase; Alzheimer's disease (AD); BACE1 inhibition; anti-AD drugs, Computer-aided inhibitor design; Docking

1. Introduction

1.1 Clinical description of Alzheimer's disease

There are billions of cells known as neurons within a healthy human brain, which build transmission networks and communication within themselves. The movement of electrical impulses from the axon to the neurons ensures message transmission among the cells. Electrical impulses produce chemical messengers called neurotransmitters. As the transmitters pass through the synapses and neurons, they result in dendrites linking the next neuron (**Figure 1**). This established transmission network and communication results in the human brain's proper functioning [1-4]. There is an interruption of this smooth transmission network and communication in a brain with Alzheimer's disease (AD) [5-8]. AD, which was discovered over a century ago by Dr. A. Alzheimer's, a renowned Deutsch neurologist, and psychiatrist is an intensifying neurodegenerative brain illness [9, 10]. Other early symptoms common to AD patients are anterograde amnesia, speech problems, lack of concentration and comportment, apathy, and inability to carry out daily activities [11, 12].

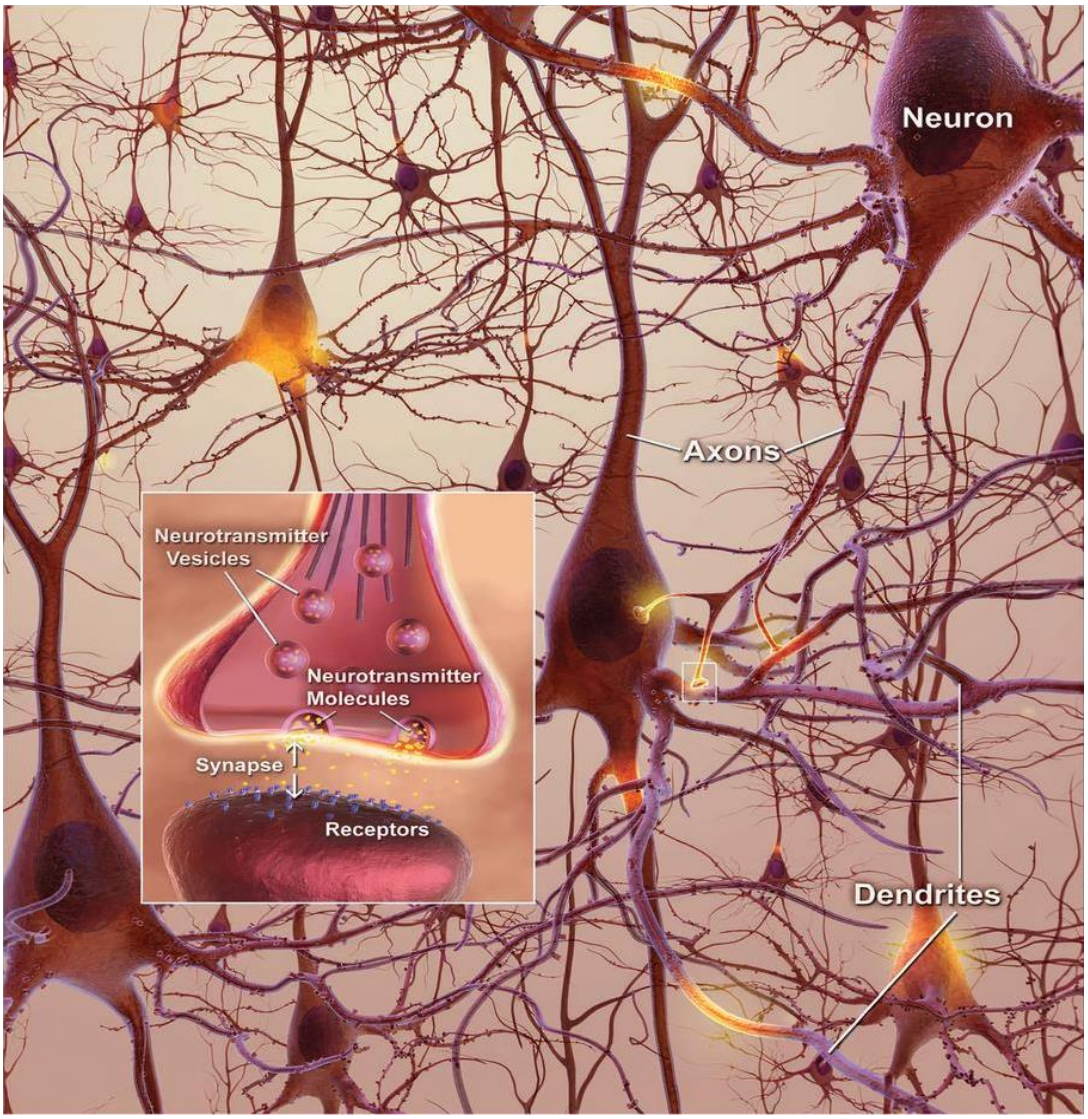


Figure 16: Schematic representation of the neurons' communication network in the brain. Image adapted from an open-source communication [13].

1.2 AD prevalence and pathology

In 2010, the world health organization (WHO) estimated that over 30 million persons have dementia worldwide, with an envisaged increase every two decades that might result in over 60 million by 2030 and 110 million by 2050 [14]. In another report in 2019 by a reputable organization (Alzheimer's disease facts and figures), 5 million Americans have dementia due to AD. This figure might rise beyond 13 million in 2050 due to high AD cases in Western Europe and America among older people above 65 years [15]. The expected cumulative expenditure in treating persons with dementia and AD in the US would rise above the 2017 figure ($259 billion) to more than $1.1 trillion in 2050 [16-22].

Authors proposed that Alzheimer's-diseased brain usually shows abnormal neuronal processes, astrogliosis, defective microglia, β-amyloid (Aβ) accumulation, and neurofibrillary tangles (NFTs) from hyperphosphorylated-tau

(HPT) [12]. Aβ and NFTs are typical biological features of AD [23-26]. α-secretase and γ-secretase perform the first two subsequent cleavages of the β-site amyloid precursor protein (APP), resulting in fragments that drive the usual electrical impulses. These impulses or charges are responsible for synaptic plasticity formation, which helps to improve emotional behavior, learning, memory, and longevity of neurons [27-29]. Further splitting of APP by β- secretase joins with the fragments from γ-secretase to form short bits known as β-amyloid. The aggregation of these short fragments gives rise to toxicity and malfunctioning of the neurons. These fragments aggregate further to form insoluble β-amyloid plaques, which leads to Alzheimer's disease [30-34].

Modification of tau protein forms neurofibrillary tangles formation. Within healthy human brain cells, tau stability is responsible for cellular materials and nutrients transport. This transportation occurs through microtubules to the different components of the neurons [35]. The falling apart of the tau results in different tau strands that form tangles. These tangles block nutrients and cellular materials transportation giving rise to some dead neurons in the AD brain. This process is responsible for abnormal behaviors like inability to think, memory loss, and weakness to live an independent life. All these behavioral changes are associated with AD patients [36, 37].

The production and subsequent aggregation of both Aβ and tau form the hallmark of AD [38]. Recently, there have been increased studies on tau and Aβ aggregation activities in Alzheimer's and other neurodegenerative diseases (**Figure 2**). The neurotoxicity correlation between Aβ and tau aggregation has also been investigated [39-41]. A study showed that Aβ partly exerts its toxicity via tau, and this process is enhanced by the Src kinase Fyn [42]. In another experimental study, Aβ and tau were reported to exhibit toxicity both separately and synergistically [39]. Therefore, further treatment options with a focus on either Aβ or tau aggregation are imperative for the improvement of current therapeutic strategies, such as immunotherapy, for AD treatment [39, 41].

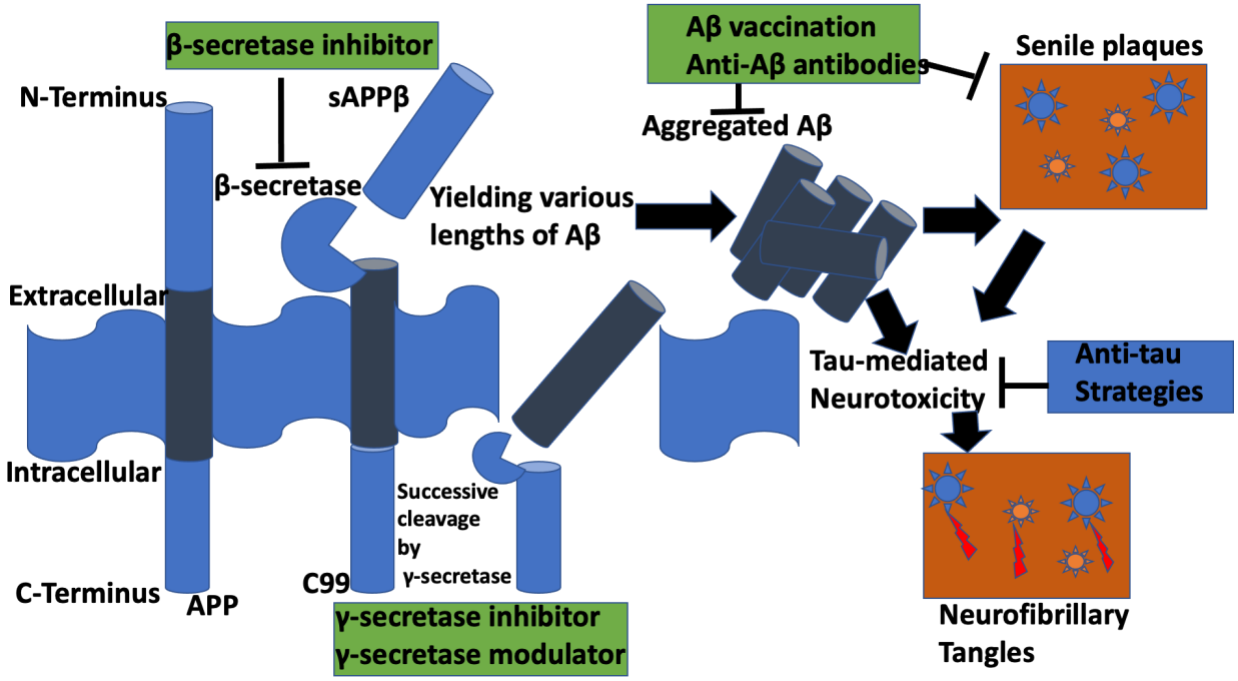


Figure 17: Amyloid hypothesis-based AD pathways showing the aggregated Aβ and tau-mediated neurotoxicity. Image redrew from [41].

1.3 General discussion on postulated hypotheses on AD development

Extracellular Aβ plaques and NFTs are predominantly the two typical biological features of AD. However, several other disease-causing pathways result in the aggregation of Aβ plaques and NFTs. These multifaceted pathways include deregulation of iron, fat biological processes, inflammation, and oxidative disruptions. Presently, the available AD management therapies include counterbalancing neuro transmitting disruption and interfering with the disease-causing pathway showing medical conditions associated with AD [43]. Higgins and Hardy postulated a hypothesis in 1992 called the amyloid cascade, which stated that disease-causing Aβ plaques and NFTs, neurodegeneration, loss of synapses, and eventual dementia in AD are responsible for cascade disrupting neurons and synapses caused by aggregation of Aβ [38]. β-amyloid brain aggregation is responsible for the imbalance of free radicals and antioxidants (oxidative stress) and an inflammatory response, which results in neurotoxicity and impairment of cognitive functions. The deadness and damaging of neurotransmitters, imbalance of free radicals and antioxidants, soreness, lack of calcium balance, pressure on the endoplasmic reticulum, disorder of mitochondria and synapses are all resultant effects of Aβ accumulation [43, 44]. However, surveys show that the amyloid-cascade hypothesis has failed to elucidate the pathologic process of AD – Aβ elimination could not prevent the disease- causing pathway. Therefore, researchers proposed the tau theory. Saturation of the tau phosphorylation sites enhances aggregation of paired helical filament (PHFs) that result in neurofibrillary tangles formation (**Figure 3**). In the 1990s, authors [43, 45, 46] reported fully saturated phosphorylated tau, a constituent of NFTs isolated from the AD brain. They identified tau as crucial to neurodegeneration [43, 45, 46].

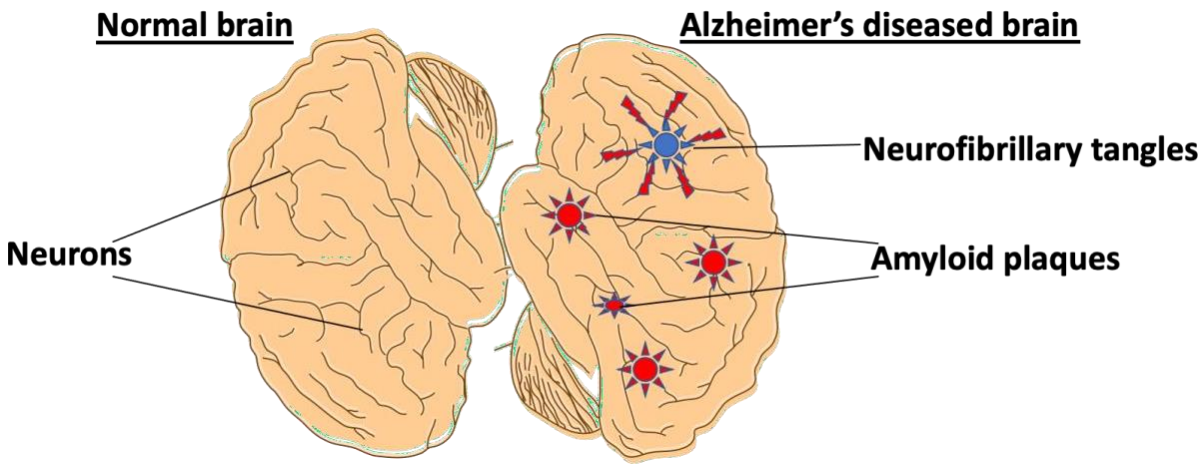


Figure 18: Diagram showing a healthy human brain and AD human brain, image redrew from the literature [47].

1.4 β-amyloid-based therapies and other ongoing clinical trial therapies

According to the cascade postulated theory, Alzheimer's disease-related signs and symptoms set in when there is an aggregation of β-amyloid. Of course, a healthy human brain contains the APP, which is split differently by α, β, and γ secretase enzymes. APP is first cleaved in the extracellular segment either by α or β secretase at the distal region.

The APP could be split by α-secretase within the Aβ domain to produce soluble sAPPα and a membrane-bound C83 (**Figure 4**). The C83 is further cleaved by γ-secretase, resulting in a P3 (unreactive peptide) fragment extracellularly. This cleavage is non-amyloidogenic because of the formation of the unreactive P3 [48-51]. Conversely, the APP could first be split by the β-secretase at the N terminal of the APP, producing soluble sAPPβ and a membrane-bound C99 [49]. The C99 is subsequently cleaved by γ-secretase to produce Aβ and carboxy-terminal fragment (CTF) intracellularly (**Figure 4**). The splitting by γ-secretase is amyloidogenic because it results in β-amyloid fragments aggregates in the middle domain with soluble sAPPβ N-terminal [49, 52]. Finally, the β-amyloid fragments produced are either cleared by a mechanism involving lysosome and protease or conjugate and induce disease-causing functions. The soluble sAPPβ is more harmful than the insoluble moiety [48, 50, 51].

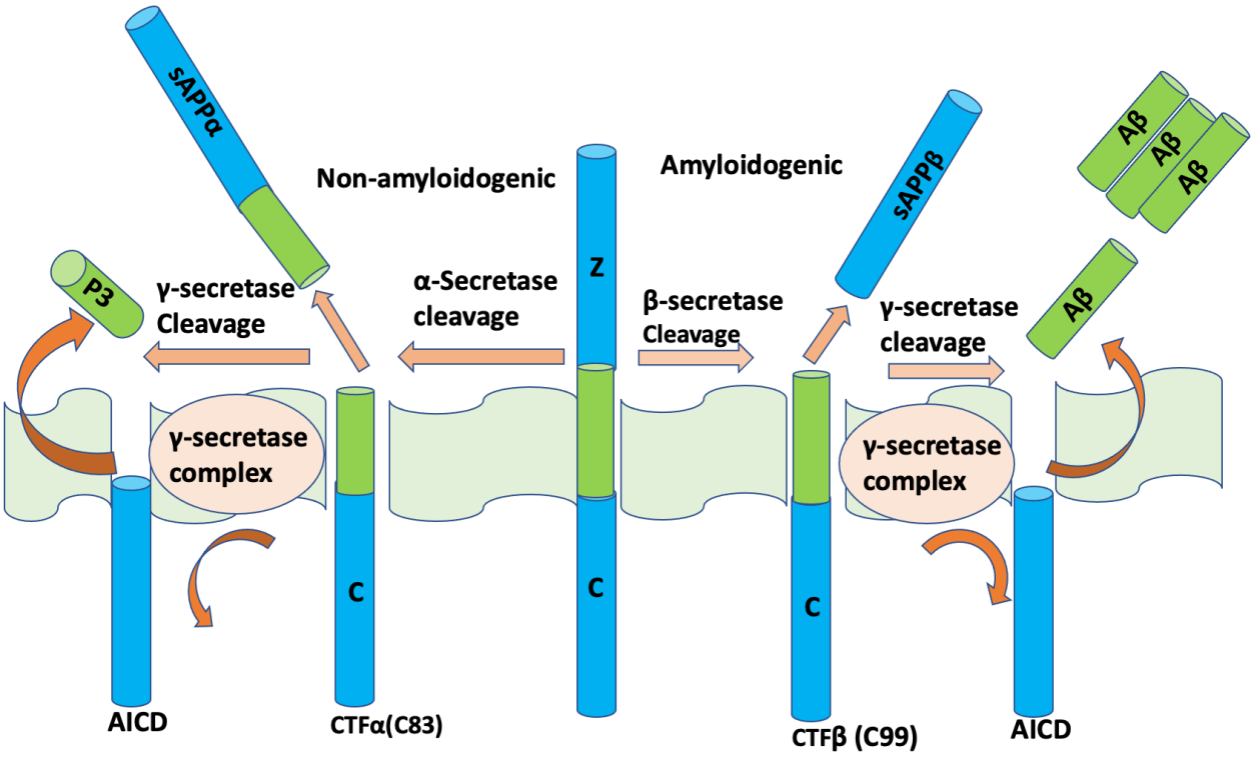


Figure 19: Diagram showing APP cleavage resulting in amyloidogenic and non-amyloidogenic cleavage pathways. Image redrew from Ref. [52].

Lately, researchers reported that out of the nine drugs that reached the phase 3 trials, eight of them were Aβ-based therapy *[53]*. They observed that none of the current clinical drug tests includes persons with an advanced stage of AD. This observation validates the theory that Aβ targeted intervention is not helpful for persons in the later phase of the disease. There are relatively fewer Aβ targeted studies at stage 3 clinical trials in 2019 relative to 2017 and 2018 studies. However, there has been tremendous advancement in the Aβ targeting; researchers are now considering experiments at the preclinical and early phases. AD therapeutic trial has also advanced to applying more useful clinical materials that directly measure some concepts. One could evaluate how a patient feels (surrogate biomarkers) through the use of cerebrospinal fluid (CSF), positron emission tomography (PET), and magnetic resonance imaging (MRI) for Aβ and tau [54-56].

In 2019, the only available immunotherapeutic intervention at the clinical stage was the combination of CNP-520 and CAD106 in treating patients with Apolipoprotein E4 [57]. CNP-520, also known as umibecestat, is a BACE1 inhibitor administered orally and targeted to inhibit the production and aggregation of Aβ. However, investigators discontinued CNP-520 usage because it showed a worsened cognitive function [58], but treatment with the CAD106 counterpart is still ongoing. Also terminated is another drug called Bapineuzumab, which uses certain parts of a person's immune system to fight AD. The drug showed no appreciable improvement after the subsequent two clinical trials in the patients [59]. Additional five other clinical trial drugs [60] (**Table 1**) were examined in 2019 using monoclonal antibodies, which were Aβ-based therapies.

Table 5: Status of β-amyloid-based clinical trial candidates for AD Management.

Drug name	Identification code for clinical trial	Target and therapeutic purpose	Mode of action	Stage 3 trial status
ANA VE X2-73	NCT03790709	Targeted at tau, β-amyloid, and neuroinflammation	Targeted at tau and β-amyloid	Recruiting
GV-971	NCT02293915	Amyloid-related	Aβ aggregation inhibitor	Completed
Elenbecestat E2609	NCT02956486 NCT03036280	Reduces amyloid production	BACE1 inhibitor	Discontinued
Solanezumab	NCT02008357	Removes amyloid and prevents aggregation	Monoclonal antibody	Active, not recruiting
Exchanging Plasma and immunoglobulin albumin	NCT01561053	Removes amyloid	Plasma-exchange	Completed
Gantenerumab and Solanezumab	NCT01760005	Removes amyloid/reduces amyloid production	Monoclonal antibody	Recruiting
Crenezumab	NCT02670083 NCT03114657 NCT03491150	Removes amyloid	A monoclonal antibody directed at oligomers	Completed
Gantenerumab	NCT02051608 NCT01224106 NCT03444870 NCT03443973	Removes amyloid	Monoclonal antibody	Some are active while some are not recruiting
ALZT-OP1a +ALZTOP1b	NCT02547818	Amyloid-related & targeted neuroinflammatory	Mast cell stabilizer, anti-inflammatory	Active, not recruiting

2. The BACE1 enzyme

BACE1 is an enzyme in humans encoded by the BACE1 gene and highly expressed in neurons, hence, its connection to neurodegenerative diseases, including Alzheimer's disease *[53, 61, 62].* BACE1 is classified as aspartate protease because it is highly involved in peptide bond splitting. Aspartate proteins are available in plants and animals as well as some other microscopic organisms. These proteinases play crucial roles in regulating blood pressure, health, and digestion in the human body [63]. OM99-2 and OM00-3 are among the many crystalline 3D structures of holoenzymes deposited in the protein data bank (PDB) [64]. These complexes are the first cocrystal structure that revealed the catalytic dyad within the BACE1 active site [65]. The catalytic dyad (Asp32/228) in BACE1 helps coordinate one water molecule, and it is involved in substrate cleavage. This catalytic mechanism is common to other aspartate proteases [66]. Within the catalytic sites are also found two other critical structures that reside at the active site of the BACE1, which are the β-hairpin loop and the 10S loop (**Figure 5**). The flap and the 10S play similar roles in holding firmly or binding ligands and substrates in the active sites [67].

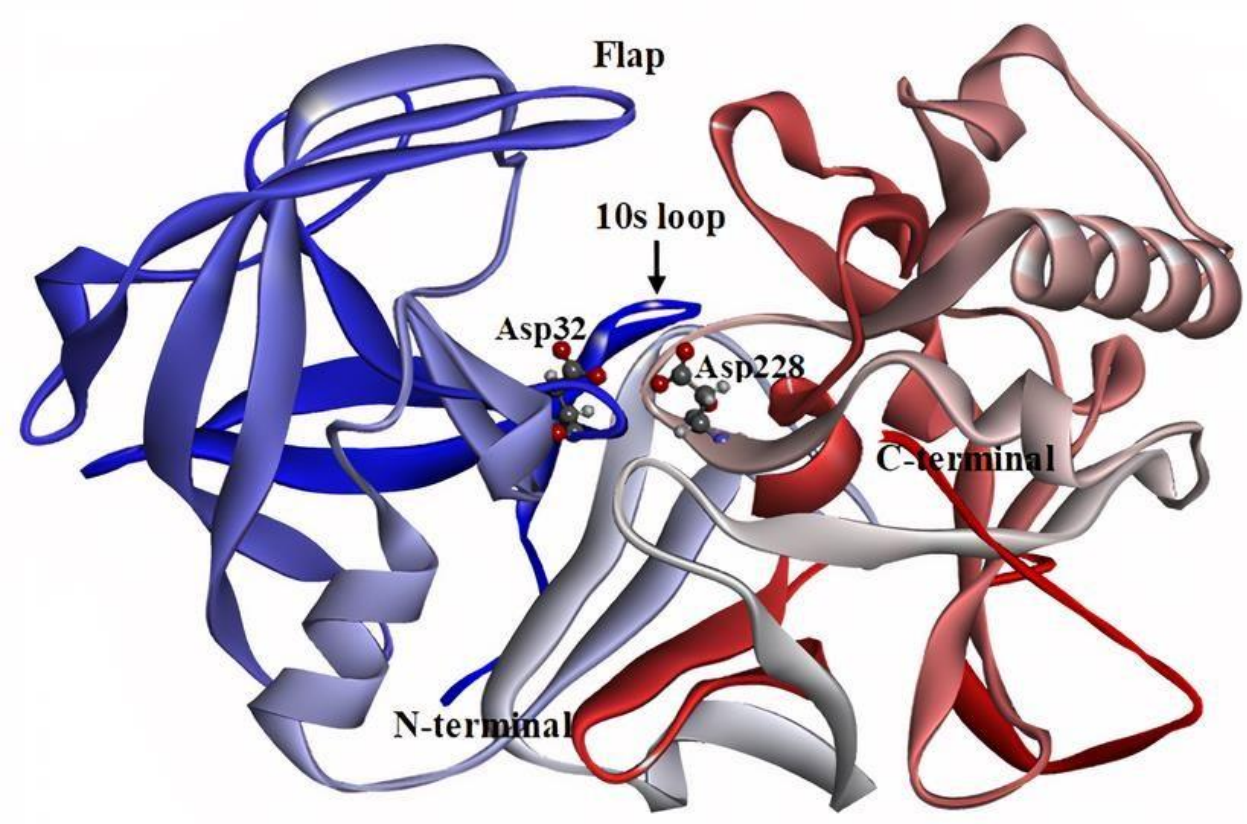


Figure 20: 3D structure of human β-secretase showing important domains within the enzyme. The two catalytic Asp32 and Asp228 dyad are indicated with ball and stick.

The BACE1 also consists of N and C terminals with the β-hair loop positioned at N-terminus with eleven constituent residues (Val67-Glu77). Notably, Tyr71 is a very common residue found at the β-hair loop, which changes its conformation when inhibitors are present. The 10S loop within the S3 sub-pocket is composed of Lys9-Tyr14. The 10S loop also contains Gly11, which forms hydrogen bonding with the substrate and facilitates stability. Rumsh and Andreeva carried out extensive research on aspartate proteases [68] and reported the functions of the two water molecules found at the active site. According to the literature, one of the water molecules located closer to the aspartates (Asp32/228) participates in substrate peptide bond splitting. The other water molecule is involved in continuous hydrogen bonding, enabling stability of the entire structure [68-71].

2.1 General BACE1 inhibition mechanism

The mechanism (**Scheme 1**) starts when one of the two water molecules found at the active site releases a proton to one (Asp32) of the catalytic dyads making the water a good nucleophile. This nucleophile subsequently attacks the carbonyl carbon of the substrate's scissile unit. An electron released through this process migrates to the other catalytic dyad (Asp228), producing a positive charge while Asp32 became negatively charged. This process results in a tetrahedral intermediate formation, which further splits in a mechanism that resembles serine protease catalysis and an acid and base mechanism [72]. However, unlike serine protease, no covalent bonds are formed between the

β-secretase side chains and substrates, and for this reason, the fragment can exit the system freely [63]. The most common drug strategy against BACE1 is its use as a competitive inhibitor. When the ligand is within the active site, the aspartic residues 32 and 228 participate in hydrogen bonding. Hence, the protease catalytic mechanism coordinating a water molecule will not occur, and the APP will not be cleaved. Meanwhile, the inhibitor interacts with the specific pocket and the enzyme flap. The combination of these factors makes for an imperfect but stable enzyme-substrate or enzyme-ligand complex.

Scheme 4: A proposed acid-base mechanism for BACE1 catalytic activity.

BACE1 targeted inhibitors are very useful in lowering the accumulation or aggregation of Aβ, but they come with significant side effects [73]. There are reports and evidence that β-secretase has a crucial function in the repair, development, and maintenance of the neurons in the body [74-78]. Therefore, the formation of plaques is due to an imbalance between β and α secretase, suggesting that a balanced amount of β-secretase is healthy while unhealthy when in excess. Drug therapy should have a goal to regulate β-secretase levels instead of eradicating them [79].

2.2 Overview of BACE1 inhibitors' properties

BACE1 has long been a prime target towards the reduction of Aβ aggregation in the brain since its discovery in 1999. BACE1 inhibition is one of the most important therapies targeted toward Alzheimer's disease management. Therefore, understanding its drug-inhibition properties is a prerequisite in designing a more efficient and suitable β- secretase inhibitor with high selectivity. Its structural similarity with other aspartyl proteases in the same family makes it very daunting to achieve selectivity in BACE1 inhibition without an off-target effect to one or any other proteases [80-82]. As a result of BACE1 expression at the blood-brain barrier (BBB), drugs/inhibitors targeted at BACE1 are supposed to overcome the BBB. Researchers have, however, argued that many BACE1 inhibitors under development exhibit poor BBB permeability and get absorbed back to the bloodstream via P-glycoprotein (P-gp) [83, 84]. More so, the relatively large size of the BACE1 active site (**Figure 5**), which consists of the catalytic, flap, and 10S loop, made it uneasy to develop larger drug molecules that can optimally and comfortably bind to the active site [85]. As a result of the above challenges, there have been reported cases of failed BACE1 inhibitors showing worsened cognitive impairment while testing patients with mild to moderate symptoms in the clinical trial phases.

Based on this disappointment, a holistic understanding of BACE1 inhibitor properties, as well as its adverse effects, is very imperative [86-90].

Generally, drug designs rely on absorption, distribution, metabolism, excretion, and toxicity (ADMET) measurement necessary to facilitate drug discovery, design, and development processes. Closer examination showed that the main reason for the high failure rate in drug development is the neglect of pharmaco-kinetical and ADMET drug functionalities [91]. Drugs have also been assessed based on their conformity to the famous Lipinski's rule of five [92]. However, the theory also makes room for potent molecules that may fall short of one or two of these properties and some compound classes that are biologically non-conformable to the rule [92].

More experimental and computational studies are required to understand BACE1 biological function and its inhibition properties. Authors have identified drug molecules that could effectively inhibit BACE1 using *in vivo* approach. Atabecestat, umibecestat, LY3202626, elenbecestat, lanabecestat, and verubecestat are examples of BACE1 inhibitors with appreciable preclinical outcomes. Among these BACE1 inhibitors, verubecestat is the first selected to reach stage 3 of the clinical trials. Unfortunately, in February 2018, its administration was discontinued and declared inefficient; subjects did not show improved condition [93]. It is no doubt that verubecestat immensely

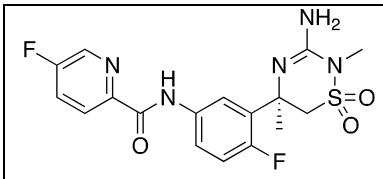
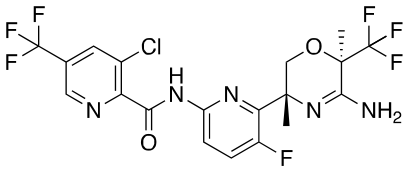
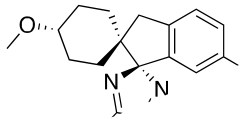
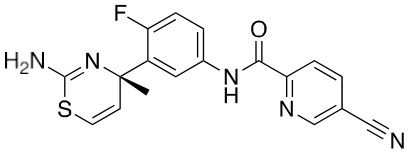
reduced Aβ levels in the brain. However, it has side effects such as lack of comportment, skin reactions, and dizziness

[47, 82, 94]. Another BACE1 inhibitor, atabecestat, was used on persons with no symptoms but likely with a high risk for AD. At first, atabecestat achieved an excellent depletion on the level of β-amyloid (about 90%) but later terminated in May 2018 due to severe adverse effects on the liver of over 500 persons tested with it [95].

Lanabecestat is another orally active potent BACE1 inhibitor which initially did not show any adverse health effects on all the patients from different parts of the world tested with it in the phase 1 study. There were preliminary observations of suppressed CSF Aβ peptides on persons whose cases were not severe. Sadly, they terminated the phase 3 clinical trial in 2018 due to a display of inefficacy for persons whose cases were at early/mild stages [96]. Wessels *et al*. [97] further investigated the safety and efficacy of lanabecestat in AD treatment. They observed adverse effects such as weight loss, change in hair color with worsened memory impairment in AD patients [97]. Considering the failure rates (**Table 2**) of most BACE1 inhibitors in preclinical and clinical trials for AD therapy [98, 99], it raises the curiosity for careful examination and research on the factors behind these failures. Therefore, it becomes necessary to find the missing link, elements, and properties that should be paramount when contemplating BACE1 inhibitor designs [47].

Table 6: Failed phase 3 trials BACE1 inhibitors for AD management

Name	Terminated year	Targeted AD patients	Reasons for failure
Verubecestat	2016	Mild-moderate/prodromal	Side effects such as skin reactions, lack of comportment, dizziness, and lack of efficacy

			
Umibecestat 	2019	Preclinical	Lack of efficacy
Lanabecestat 	2018	Early/mild	Weight loss, hair color change, and lack of efficacy
Atabecestat 	2018	Preclinical	Toxicity

2.3 Multitarget BACE1 approach towards drug discovery

Considering the failure rates of some BACE1 inhibitors at the clinical trials, researchers shifted attention to multitarget molecules towards the development of BACE1 inhibitors. These multitarget-directed ligands (MTDLs) approach involved developing compounds for the modulation of BACE1 and other related targets [62, 100]. This approach could improve the efficacy of the BACE1 inhibitors and enhance the clinical trial results. Studies showed that some compounds derived from coumarin displayed multitarget (dual) BACE1/AChE inhibition [62, 101, 102]. On further analysis, some compounds showed single-digit micromolar activities for BACE1 while displaying significantly low potency for AChE [62, 103]. Multitarget BACE1 inhibition appeared not to be a solution to the problems of the failed compounds at the clinical trials. Some researchers opined that lead optimization and drug discovery through an MTDLs approach are frustrating and complicated processes. Nonetheless, research works on multitarget inhibitor development are significantly available in the literature.

Ambure *et al*. [104] used a screening and machine learning-like approach to identify natural compounds as multitarget-directed ligands against AD. Verubecestat appeared along with two other drugs (**DB06925** and **DB08899**) in a data mining strategy and virtual screening by Ion *et al*. [105]. The drug repurposing model proved to be relevant in identifying therapeutic candidates for AD. In a report, the random forest (RF) machine learning (ML) model proved to be the most reliable predictor of Alzheimer's drugs and targets [106]. After this comparative ML study by Hu *et al*. [106], they developed an online server [http://47.106.158.30:8080/AD/] for predicting a molecule as a

multitarget drug for AD. A dataset of forty-two amino hydantoin derivatives screened using different ML algorithms showed BACE1 inhibitory activity [107]. The authors applied classification tree (CT), moving average analysis (MAA), and RF models. The suggested models provide potentials for lead molecule design as BACE1 inhibitors for AD management [107].

3. Computational methods for BACE1 inhibitor identification

Researchers have applied various computational approaches (**Figure 6**) in determining potent inhibitors targeting the pathological pathway of AD. As highlighted lately from our group [108], theoretical and computational methods have been reportedly useful in designing potent dual cholinesterase inhibitors. Herein, we briefly introduce and highlight some of these methods, followed by researchers' efforts in proposing novel BACE1 inhibitors using *in silico* techniques.

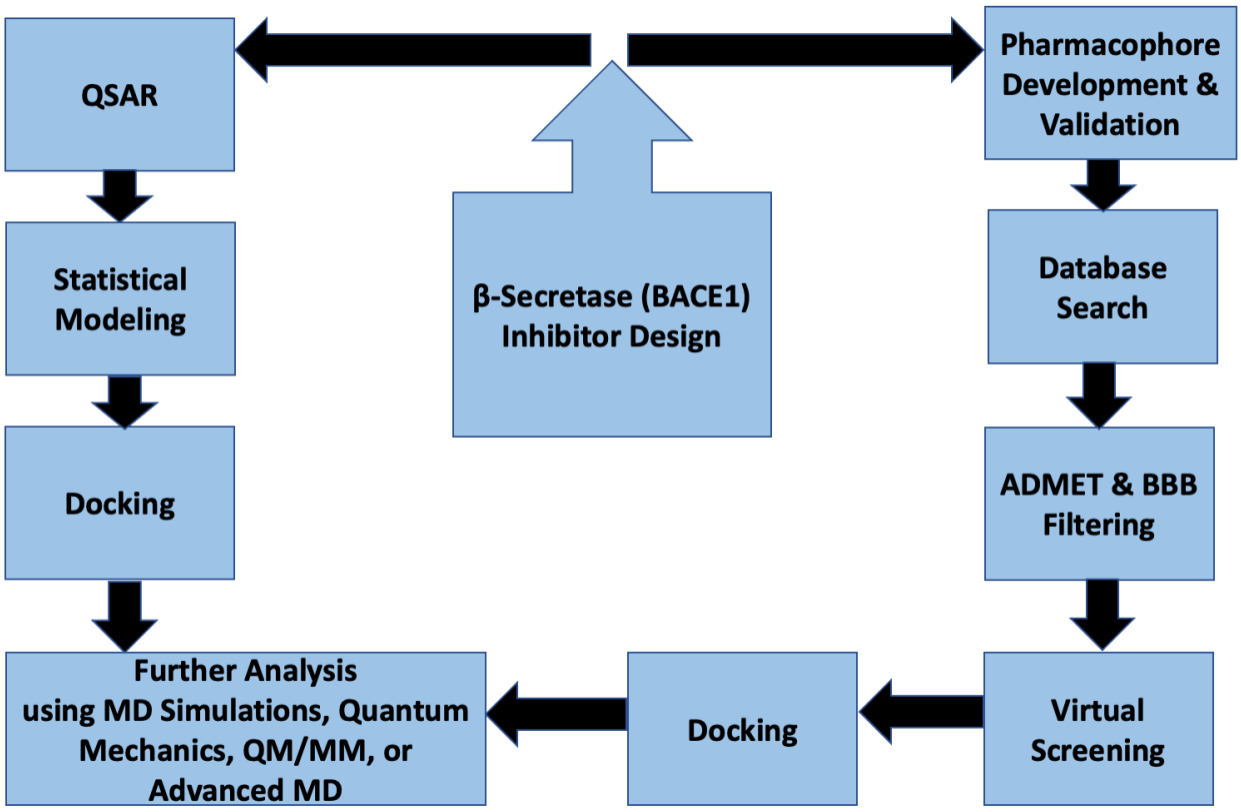


Figure 21: Schematic representation of common computational approaches for BACE1 inhibitor design.

The usefulness of *in silico* techniques in assisting *in vitro* filtering towards designing therapeutic drugs targeted at BACE1 inhibition for AD is commendable. Using the topical keywords "BACE1; inhibitor, and review or overview or update" in the Web of Science [www.clarivate.com/webofsciencegroup/solutions/web-of-science] and Scopus [www.scopus.com/] database published within 2010 and 2020, we retrieved 80 articles. Removing duplicates and less related pieces of works to the subject, a total of 35 review articles falls within our search protocols, indicating

that investigations in this direction are noteworthy. The year 2020 features about ten review articles [47, 109-117], four of which are most relevant to the present work.

Moussa-Pacha *et al*. [47] gave a comprehensive update on BACE1 inhibitors and focusing on the current status and future directions in managing AD. They highlighted the most promising BACE1 inhibitors, described and analyzed their pharmacodynamic and pharmacokinetic parameters. These authors [47] also documented some lead drug moieties targeting BACE1 and shed light on potential therapeutic options for targeting AD. Iraji *et al*. [112] discussed novel small molecules for AD treatment with a focus on BACE1. They [112] highlighted multitarget-directed ligands and computational modeling. Similarly, Mouchlis *et al*. [111] reviewed the computer-aided inhibitor design of BACE1, γ-secretase, and anti-tau for AD therapy. Gupta *et al*. [113] also reviewed studies on the design and development of anti-AD drugs and the amyloid and cholinergic theories. They [113] observed relentless efforts from researchers in identifying efficient non-peptidic BACE1 inhibitors targeting AD.

Indeed, a handful of review works are available on BACE1 inhibitor design. However, a streamlined update directly addressing BACE1 inhibitor design with computational models is scarce. Therefore, the present work documents the different investigations from researchers who have designed potent molecules inhibiting BACE1 using computer- aided drug design (CADD) methods. The overview features the applied computational methods, the identified molecule, published year, and references. In most cases, authors have complemented *in silico* research with *in vitro* or *in vivo* assay reporting IC_{50} or K_i of such potent compound. Using the topical keywords "BACE1; inhibitor design; and *in silico* or computational study or CADD" in the Web of Science and Scopus database, we retrieved 49 relevant articles. **Table 3** shows a summary of our survey within the years 2010 and 2020.

3.1 Quantitative structure-activity relationship

Drug design involves the application of different computer-aided methods to explore new drugs. The significant role of the quantitative structure-activity relationship (QSAR) as a statistical instrument for the correlation of biological activities and the molecular structures of compounds has been commendable. It finds application in the absence of 3D models of specific drug targets [118]. QSAR as a fragment-based drug design technique is applicable in the areas where the structures of proteins are not readily available [118]. It employs the framework of density functional theory, DFT-based chemical descriptors in the correlation of the structure of molecules and the biological activities [119, 120]. The application of high-throughput screening for filtering large compound libraries is no longer economically feasible as it involves capital intensive and time-consuming. Therefore, QSAR statistical modeling makes it easier by leveraging the active and inactive chemical data in selecting lead compounds [119, 121]. After sorting the lead compounds through the QSAR method, the investigator performs molecular docking with the BACE1 enzyme or other enzymes of choice.

Authors [122] have provided a model to justify fragment-based drug discovery (FBDD) using a novel computational procedure involving fragment-based quantitative structure-activity relationship (FB-QSAR) and a multi-objective approach. FB-QSAR allows weighing the fragment contributions to molecular selectivity thereby, enabling lead

identification of fragments with improved activity and selectivity. The integrated models are FB-QSAR, fragment- based quantitative structure selectivity relationship (FB-QSSR), and multi-objective quantitative structure properties relationship (MO-QSPR). These integrations could facilitate fragment development in identifying potential scaffolds in BACE1 inhibitor design, drug design and assist chemical synthesis [122]. Kuhn *et al*. [123] mentioned in a review that coupling and merging fragments of known inhibitors might be a promising approach to enhanced BACE1 inhibitor design. They [123] emphasized that a 3D combination of scaffolds from various crystal structures is a lucrative protocol to conjugate motifs from different molecular series.

Monceaux *et al*. [124] applied FB drug discovery and high throughput *in situ* filtering approach to identify compounds with potent inhibition against BACE1. Four of these compounds showed IC_{50} values ≤10 µM, with **A3Z10** showing an IC_{50} value of 2 µM (**Table 3**) in a cell-free fluorescence resonance energy transfer (FRET) assay. Also, researchers have discovered biphenylacetamide-based inhibitors of BACE1 using *de novo* fragment-based design, QSAR, virtual filtering, and experimental methods [125]. Similarly, authors [126] applied the FB approach to design a series of phthalimide and saccharin derivatives. The compounds conjugate through alicyclic fragments of piperazine, hexahydropyrimidine, 3-aminopyrrolidine, or 3-aminopiperidine. The study shows structures **26** (**Table 3**) and **52** as the most potent multitarget ligands of AChE and BACE1 with significant anti-Aβ aggregation potentials. Compound **26** showed the best potency towards human BACE1 with 33.61% inhibition at 50 µM [126].

Designing druggable molecules targeting BACE1 inhibition remains a challenge due to the complex nature of AD pathogenesis [10]. The application of CADD and 3D structural models has been significant in understanding and predicting the binding modes and energies of potent BACE1 drugs. MD simulations, docking, pharmacophore development/validation, end-point binding energy prediction, DFT calculation, and QSAR are the readily used CADD methods in identifying hit molecules targeting BACE1 (**Figure 6**). Integrating these methods has assisted researchers in designing and identifying more desirable inhibitors. Optimization or modification of existing active compounds can yield more active ones. Kiso and co-workers [127, 128] applied *in silico* conformational structure-based design to identify potent molecules against BACE1. The synthesized and tested molecules, including **KMI-1564** with **KMI- 429** as the parent compound, showed 70% inhibition at 2 µM [127]. **KMI-1027** and **KMI-1303** also showed significant IC_{50} values (**Table 3**) for BACE1 inhibition [128]. Wu et al. [129] also applied 3D-QSAR, molecular docking, and MD modeling methods to identify active BACE1 inhibitors from a dataset of 128 hydroxyethyl amine derivatives. The predicted IC_{50} ranges from 3.8 to 8.06 nM for the most active derivatives.

3.2 Pharmacophore development and validation

Pharmacophore development finds usage in hit identification and lead optimization, which could be ligand-based or structure-based. It is an approach in providing insight into the binding site of a protein when the structural data is not known. During structural database search, pharmacophore is very helpful in getting the potential lead. It is also applicable in monitoring optimal interactions of a targeted biological compound and its subsequent response [130- 132]. The products of hit identification and lead optimization could further proceed to virtual screening and

subsequent docking analysis. In one research [133] from our group, we used per-residue energy decomposition- based pharmacophore modeling to identify novel BACE1 inhibitors as anti-AD candidates. The sequential protocol adopted (**Figure 7**) in the study led to **ZINC30028065** and **ZINC29797869** identification as promising inhibitors of BACE1 [133]. This protocol could be akin to Chakraborty *et al*. [134] when screening a library of phytochemicals. The identified 24 highly potent BACE1 inhibitors with calculated IC_{50} below 50 nM could be potent therapeutics in AD [134].

A structure-based pharmacophore approach applied by authors [135] showed **TGN2** with potency against BACE1 inhibitor. This compound also displays substantial neuroprotection against Aβ-induced cytotoxicity at a concentration of 2.62 µM [135]. Lately, Gupta *et al*. [136] identified compound **B3** through a couple of computational and theoretical modeling protocols involving molecular docking, virtual filtering, web-based ADME screening, MD simulations, and free energy calculation. Researchers have applied multiple ligands pharmacophore models to screen natural compounds database for novel BACE1 inhibitors identification [137-139]. The investigation, which also employed molecular docking, free energy calculation, ADMET, and QSAR IC_{50} calculation led to **Narirutin** [138], **C000000956** [140], **NPC469686**, **NPC262328**, **NPC29763** and **NPC86744** [139] identification as potent BACE1
inhibitors. The identified natural compounds by Kumar *et al*. [137] showed calculated IC_{50} values within 0.7 to 894.4 nM for BACE1 inhibition towards AD therapy. Compounds **M-1** and **M-4** identified through pharmacophore mapping, docking, and ranking displayed IC_{50} values of 15.1 and 15.4 nM, respectively, in an investigation [141]. Besides, *in silico* ADME calculation showed that **M-1** and **M-4** have laudable absorption and distribution after oral administration [141].

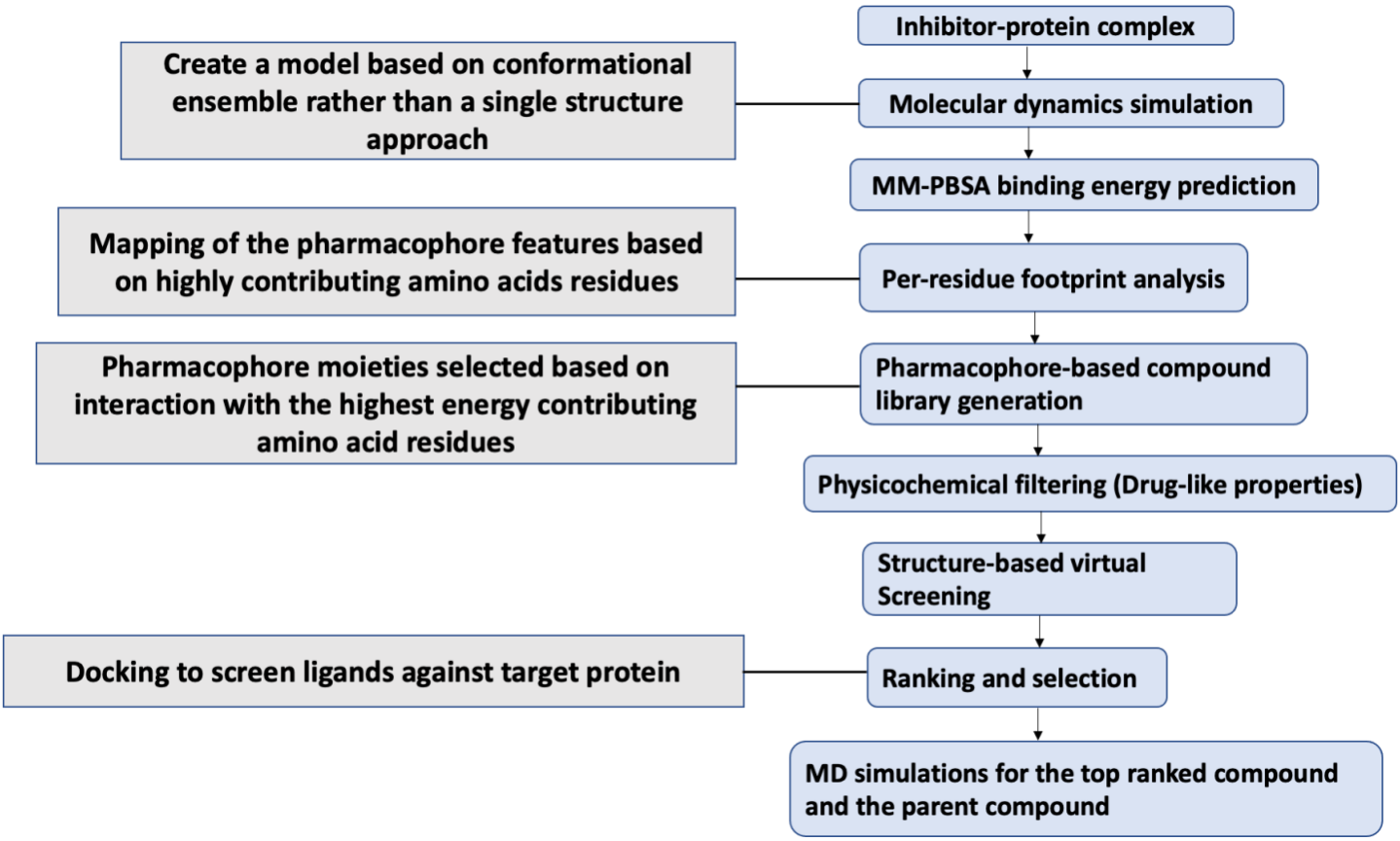


Figure 22: Flowchart of the *in silico* procedures for BACE1 inhibitor design using per-residue energy decomposition pharmacophore modeling [133].

3.3 ADMET and blood-brain barrier screening

The drug-likeness and medicinal properties of a potent drug molecule could be computationally simulated. For instance, SwissADME [142] is a robust web-based tool for predicting potent molecule suitability as a drug candidate. The software can estimate the drug-likeness and pharmacokinetic properties of small molecules [142]. Outcomes from *in silico* ADME prediction could serve as a guide for synthesis, preclinical and clinical studies. Scholars have summarized ADMET, QSAR, and physicochemical predictions and related tools [143-145] for interested users. Although the results from some *in silico* ADME predictions do not necessarily guarantee experimental expectations, it is, however, predictable based on our ongoing investigations that if the potent molecule has poor BBB permeation, the likelihood of its success is thin. The relative BBB impermeability of BACE1 inhibitors currently under development might be significant to an extent because experimental studies showed that the complete knockout of BACE1 causes the production of myelin coating around an axon in the brain [146]. Alternative delivery mechanisms to overcome the BBB for larger-sized potentially active BACE1 inhibitors are available in the literature [147-150].

Screening and docking protocols have found applications in identifying **Triptofordin B1** from the world's largest traditional Chinese medicine (TCM) database [185] as a potent BACE1 inhibitor. Also, **NCI0262634** was found by Al-

Nadaf *et al*. [151] in a combined screening and docking analysis. Khalid *et al*. [152] screened a library of biaryl scaffold-containing compounds to identify multitarget ligands for AD enzymes. The study showed **ZINC000002010548** (**Table 3**) affinity for BACE1 inhibition while some biaryl sulphonamides are potent multi- directed ligands. Gurjar *et al*. [153] designed some non-peptide inhibitors based on amino aromatic heterocyclic scaffolds using ADME filtering and molecular docking. The research features the smallest potent molecule (3-methyl- 1,2,4-thiadiazol-5-amine, **8**) inhibiting BACE1 in this survey. Compound **8** showed IC_{50} of 5.96 µM from the FRET assay to appear as the most potent inhibitor of BACE1 [153].

3.4 Virtual screening

Virtual screening involves assessing commercial deposition (libraries) of large quantities of molecules identify structures that are most likely to bind to a protein or enzyme. The protocol proceeds with further refinement of the best binding molecules to ascertain their selectivity for the intended target through other computational methods (**Figure 6**) or experimental analysis [154]. Virtual screening involves analyzing the targeted 3D structures of the molecules experimentally generated through either nuclear magnetic resonance or X-ray crystallography [154]. When the screening incorporates database assessment of known drug molecules for target inhibition, it is called drug repurposing. Lately, Coimbra *et al*. [155] successfully applied integrated theoretical and experimental approaches to identify novel BACE1 inhibitors from a library of compounds. The employed molecular model technique involved virtual screening combined with various fragment-based models. The developed pharmacophore model was applied to screen a database, and 34 compounds showed potency as BACE1 hits [155]. Molecular docking revealed 13 compounds with appreciable binding towards BACE1, and the most desirable molecule **11** (**Table 3**), displayed an IC_{50} of 15 µM. They concluded that the identified hit BACE1 inhibitors could serve as the kickoff for additional structure refining towards hit to lead identification steps [155].

3.5 Docking

Molecular docking involves virtually docking a small molecule into the receptor's binding site executed with a selected software. Docking predicts the inhibitor-enzyme complex conformation after scanning different structural orientations of the ligand in the enzyme binding site. The procedure involves subsequent scoring of the different poses and using it to determine the binding free energy of the complexes [156, 157]. Further analysis of the docked compounds for improved prediction requires quantum mechanics (QM), molecular mechanics (MM), or hybrid methods. Molecular docking is the most popular computational approach to quickly gain insight into the potential binding affinity and interaction between potent molecules and targets [108]. It is involved in nearly all *in silico* drug discovery protocols (**Figures 6 and 7**).

3.6 Molecular dynamics simulations

The integration of MD simulations in studying biological systems enables exploring the physical motion of atoms and molecules, which are uneasy by any other means [158, 159]. The output from atomistic simulation often provides a

detailed perspective into the dynamics evolution within the biological systems. Such data could provide insight into the conformational changes and molecule association within a complex structure [158]. Several MD simulation studies focusing on the dynamics of apo BACE1 and its inhibitor-bound forms are available in the literature. Lately, Saravanan *et al*. [160] researched to determine the structural properties, mode of binding, conformational stability, and charge density parameters of verubecestat at the active site of BACE1 using the MD approach, molecular docking, and quantum mechanics [160]. More recently, we applied both the classical and accelerated MD approaches to elucidate the binding mechanism and structural changes of the lately identified **AM-6494** relative to umibecestat (**CNP-520**) when bound with BACE1 [159]. The incorporation of advanced computational analytic approaches enabled us to evaluate some concepts with high accuracy. The results indicate that **AM-6494** exhibited a higher binding affinity to BACE1 than umibecestat. Conformational monitoring of the β-hairpin flap covering the active site revealed an effective flap closure when bound with **AM-6494** compared to **CNP-520**, which predominantly alternates between semi-open and closed conformations. The observed effective flap closure of AM-6494 depicts its improved binding power towards BACE1 [159].

3.7 Hybrid quantum mechanics/molecular mechanics (QM/MM) methods

To provide solutions to the challenges of analyzing larger molecules like enzymes while considering less resource consumption, QM/MM was initiated [161-163]. In 2012, authors reported on the underlying techniques behind this combined method [163]. The concept of this hybrid method is to divide large molecules such as protein into two or three segments based on the study aim. The modeling involves partitioning the most active species like ligands and the most active residues at the QM region, with the other part at the MM region [161, 164]. Considering system fragmentation into two or three parts, the QM and MM Hamiltonian give rise to subtractive and additive forms [163]. Our Own N-layered Integrated molecular Orbital and Molecular Mechanics (ONIOM) [164, 165] and umbrella sampling [166, 167] methods are examples of the subtractive and additive forms, respectively. The application of hybrid QM/MM methods has proven reasonable in binding [168-170] and activation energy [171-174] predictions for aspartate proteases.

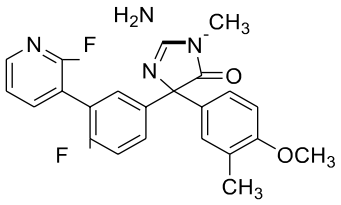
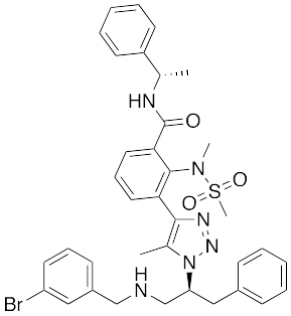
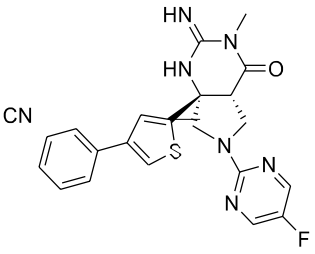
Frush *et al*. [175] used a QM/MM linear interaction energy (LIE) based binding free energy algorithm to predict the binding affinities of ligand-protein complexes. The method proved to be more precise than the widely used binding free energy approaches. The protocol enabled them [175] to screen 140 inhibitors against BACE1 and three other therapeutic targets. The authors suggested that the precision achieved with QM/MM LIE approach could be implemented in the small molecule *in silico* drug design software [175] for improved prediction. The ONIOM modeling [164] allows fragmenting an entire system, such as an enzyme, into different parts according to their biological importance. Lately, we designed a potentially active molecule using an experimentally identified **AM-6494** as a parent compound [170]. Pettus *et al*. [176] recently reported **AM-6494** (compound **20**) with substantial inhibition potency for BACE1. We applied DFT and ONIOM methods to modify the amide bond of **AM-6494** through electronic induction. This process results in **20—SCH$_3$** (**Table 3**) with improved binding affinity relative to the parent

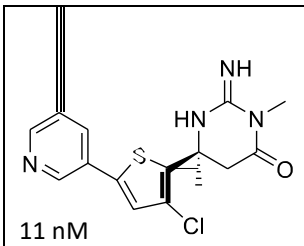
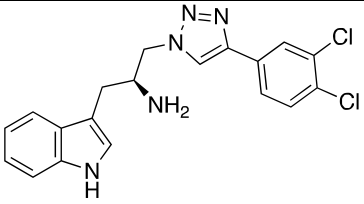
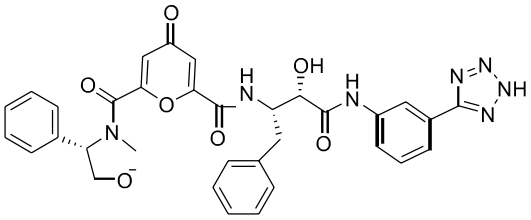
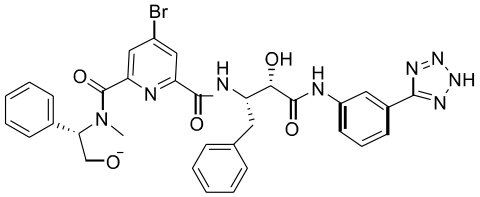
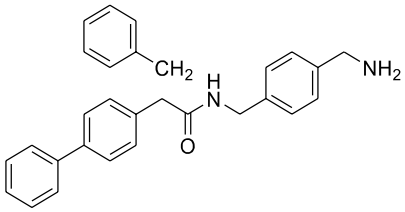
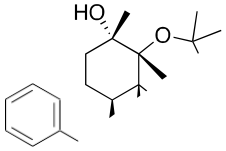
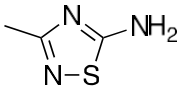
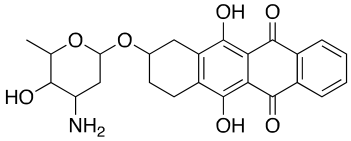
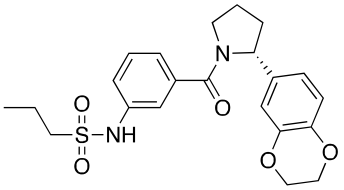
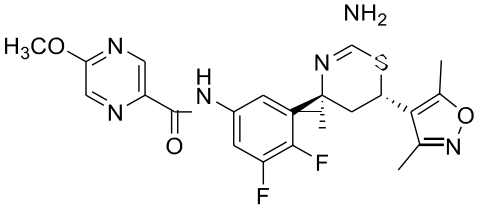
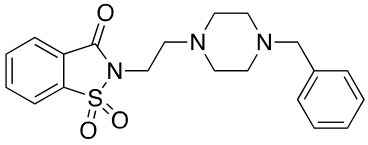
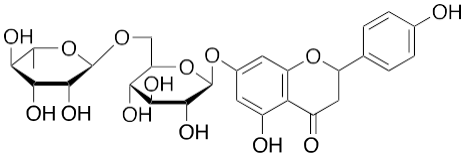
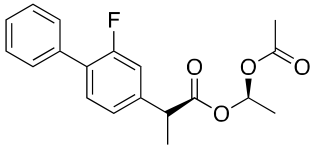
structure [170]. Related work is from Gutierrez *et al*. [177] on aminopyrimidine rational substitution, which resulted in compound **4b** identification with the best potency to inhibit BACE1. The integrated computational protocols are docking analysis, MD simulations, and quantum theory atom in molecules (QTAIM) calculations [177].

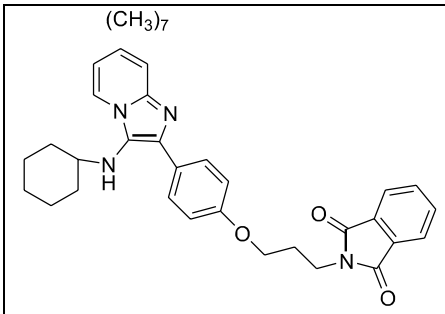
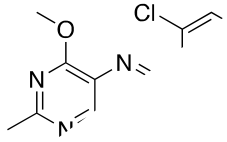
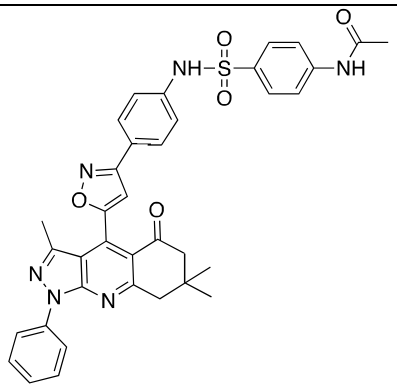
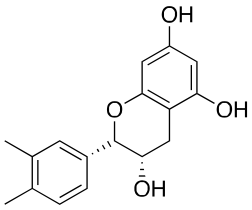
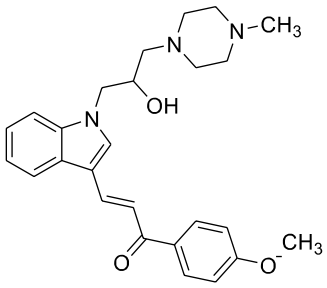
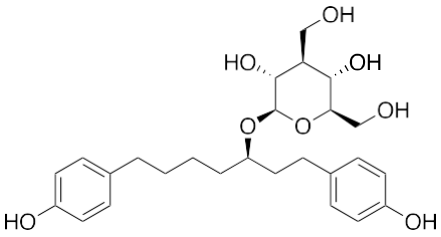
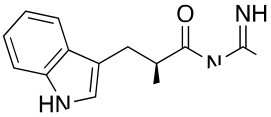
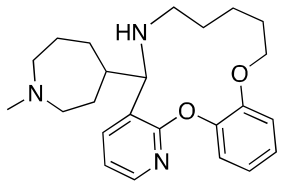
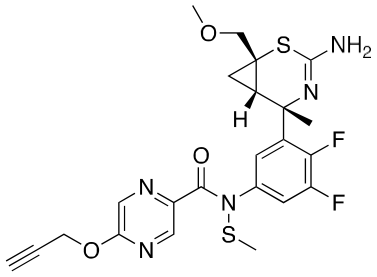
3.8 Advanced MD simulations

The world of science kept advancing, and so are methods in accurately predicting molecular properties using *in silico* approach [178]. One such improvement is using advanced MD simulation methods to elucidate reaction pathways, investigate conformational changes, and evaluate electric field fluctuations at the enzyme's active site. The umbrella sampling technique [166] is a signature to both advanced MD and QM/MM. In this method, the reaction coordinate is controlled and drawn close to a targeted number called bias potential, which allows the complete sampling of the momentum space [167]. Analyzing the fluctuations of an enzyme's electric field during catalytic activity/binding could provide a clearer picture of the specific residue involved with the highest electric field fluctuation to serve as a guide towards enzyme-based inhibitor design [179]. Researchers have applied a statistical approach, *in silico* planting (chain growth) method, molecular dynamics, and free energy perturbation (FEP) to identify potent BACE1 inhibitors [180]. Keranen *et al*. [181] rationally modified a series of acyl guanidine BACE1 inhibitors. They synthesized these substituents and predicted their energetics using FEP calculations. The obtained molecules showed high potencies for BACE1 inhibition with assays up to 1 nM. The work features the application of FEP as a computational model for molecular and drug design [181].

Table 7: Some computer-aided designed potent BACE1 inhibitors in the literature within 2010 and 2020. The table shows the compound name (according to the authors), 2D structure, IC_{50} or predicted pIC_{50} value, and the applied computational method.

(S)-55  10 nM High throughput screening structure-based drug design. 2010, [182]	**A3Z10**  2 μM Fragment-based drug discovery. 2011, [124]	**43**  4 nM QSAR and QM. 2012, [183]
16	**12c**	**KMI-1027**

 11 nM Structure-based optimization and docking. 2012, [184]	 20.75 µM Fragment-based molecular design and virtual screening. 2012, [185]	 50 nM Conformational structure-based drug design. 2012, [128]
KMI-1303  9 nM Conformational structure-based drug design. 2012, [128]	**12**  26.9 µM *De novo* fragment-based inhibitor design, QSAR, and virtual high throughput screening. 2013, [125]	**Triptofordin B1**  Virtual screening, molecular docking, ADMET prediction, and molecular dynamics simulation. 2014, [186]
8  5.96 µM Docking and ADME prediction. 2014, [153]	**NCI0262634**  11.1 µM Virtual screening and molecular docking. 2015, [151]	**ZINC30028065**  Per-residue-based pharmacophore modeling, structure-based virtual screening, docking, and MD simulations. 2016, [133]
26  0.7 nM Pharmacophore modeling and docking analysis. 2016, [187]	**26**  Fragment-based drug discovery. 2017, [126]	**Narirutin**  pIC_{50} = 11.1 µM Structure-based and QSAR-based screening, QSAR antioxidant, and anti-amyloidogenic prediction. 2017, [138]
8e	**ZINC000002010548** 	**33**

 2.84 µM QSAR and molecular docking. 2017, [188]	Virtual screening and molecular docking. 2018, [152] **4b**  12 µM Docking, molecular dynamics simulations, quantum theory atom in molecules calculations. 2019, [177]	 K_d = 10 nM A statistical approach, *in silico* planting method, molecular dynamics, and free energy perturbation. 2019, [180]
C000000956  4.301 µM QM/MD assisted docking, free energy calculation, ADMET, and autoQSAR. 2019, [140]	**11**  15 µM Fragment-based inhibitor design, pharmacophore-based virtual screening, and docking. 2020, [155]	**NPC469686**  **NPC262328, NPC29763, NPC86744** pIC_{50} for the 4 compounds is 5.3 to 8.1 nM Molecular docking, free energy calculation, ADMET, and autoQSAR. 2020, [139]
TGN2  Structure-based drug design and molecular docking. 2020, [135]	**B3**  Structure-based drug design and molecular docking. 2020, [136]	**20—SCH_3**  DFT and ONIOM calculations. 2021, [170]

4. Authors insight on the topic

Presently, the fate of BACE1 targeting in AD treatment is attracting more debate due to the several failed clinically tried BACE1 inhibiting compounds. Some researchers are beginning to question the existence of BACE1 in AD emergence due to this repeated failure [189]. However, the enormous research studies on BACE1 inhibition, structural-functional activity using experimental and theoretical methodologies have indicated the relevance of

BACE1 in Alzheimer's disease. The framework required for designing active/approved BACE1 inhibitors seems more complicated than the envisaged features often considered by researchers. An investigator is mindful of the small molecular-sized BACE1 inhibitors, specific high selectivity for BACE1 inhibition, BBB permeation, and the existence of many amino acid bonds and interactions at the active site in designing potent inhibitors. In BACE1 and other aspartic proteases, the active site plays significant roles in ligand binding because of the open and close dynamic changes, which regulate incoming substrates or ligands access [159]. Inhibitors should have appreciable resident time within the active site of the enzyme for optimal interaction and inhibition. Computational modeling of BACE1 has enabled researchers to monitor structural fluctuations indicating huge conformational transitions from open to closed or vice versa. Studies showed that the measurement of Cα atoms and the apex of the β-hair loop (flap) distances relative to one of the aspartate dyads is the most popularly used metric [159, 190-194].

For effective BACE1 inhibition drug design, small molecules should be present with hydrogen bonding potentials to the dyad [195]. S3 sub-pocket is another conserved feature of BACE1 located close and opposite to the active site. S3 sub-pocket plays a significant role in BACE1 inhibition properties as viable, effective, and potential drug molecule that engages S3 subsite, thereby establishing direct interactions [196]. Also, hydrophobic moiety at the meta- position of the aromatic ring enables better interaction with the S3 sub-pocket. A highly electronegative substituent like fluorine will enhance affinity for the S2 pocket [197-201]. Using the Merck compound MK8698 as an example, a study of the crystallized drug molecule predicted that the molecule established a closer and interacting link with the S3 sub-pocket with four coordinated water molecules. This interaction resulted in pocket narrowing, enhanced binding affinity, and increased entropy [184, 202].

More so, a better insight into the structural determinants behind BACE1 selectivity is crucial in designing BACE1 inhibitors with clinical usefulness. It is also practical to investigate the existing charges on the non-peptide BACE1 inhibitors as they play a vital role in forming electrostatic interactions with Asp32/228. It is, however, widely accepted that Asp32/228 has a net charge of -1, but this is still subject to further studies to determine the correct protonation state for BACE1 inhibitors. It is, therefore, a prerequisite to study the protonation state of the BACE1- ligand complex as there are no hard and fast rules in assigning the protonation states [170]. Authors have recommended further research to unravel the favorable protonation state of BACE1 inhibitor in complex with the enzyme [203]. Lately, we applied the ONIOM model to unveil the binding affinity of AM-6494 and CNP-520 towards BACE1 [170]. For the mono protonated BACE1 system, the calculation gave binding free energy of -62.849 and -
33.463 kcal/mol for these inhibitors, respectively. In the unprotonated model, the calculated binding free energy is
-59.758 kcal/mol for AM-6494. These values show that the protonated model is slightly better than the unprotonated form [170]. Understanding the detailed molecular interaction of known inhibitors has long served as a basis for pharmacophore exploration towards improved inhibitor design.

Although multi-directed ligand design for BACE1 and other AD targets are available, no BACE1 MTDL has made it to preclinical trials as the approach comes with complications, time, and resource-intensive. However, there is a need

to push BACE1 MTDLs to preclinical and clinical trials to establish this approach [204]. Researchers have explored single targeting compounds with specificity and selectivity for the BACE1 active site. We noticed little attention on targeting allosteric regions or exosites [205-211]. We recently reviewed allosteric site inhibition and exosite antibody for BACE1 [212]. The survey enabled us to embark on a current study to establish allosteric inhibition at the molecular level. The outcome would likely show that inhibiting BACE1 in other regions and not just the catalytic active domain is feasible and tenable. This computational protocol will also involve designing the possibility of one ligand targeting dual or multisite on BACE1 for better inhibition, like MTDL. We hope that the extensive application of computer-aided drug design approaches to unravel other binding sites might be a way forward to inhibit BACE1 in AD treatment. Such investigation might help in reducing the associated challenges with designing BACE1 inhibitors of improved anti-AD properties. We also envisage that detailed computational exploration would assist preclinical and subsequent clinical trial advancement of BACE1 inhibitors development.

The application of computational methodologies in BACE1 inhibitor design is quite fascinating. However, using the QM method, including the DFT calculation, is scarce in designing potential BACE1 inhibitors. We expect less QM application because the pipeline to computer-aided inhibitor design requires interacting identified potent molecules with the enzyme, which often have many atoms. Hence, QM methods with a high level of precision cannot handle large biomolecules. Advanced force field development and QM/MM methods are tenable approaches to determine the binding affinity of biomolecules to a target with comparable output to experiment [168-174]. However, DFT QM methods apply to designing BACE1 inhibitors through electronic fine-tuning, molecular electrostatic potential mapping, and quantitative prediction of electronic properties of small molecules [170]. We propose more application of QM methods in identifying potent BACE1 inhibitors. National governments and multilateral organizations should assist with financial support for computational resources to enable quality studies on BACE1 inhibitor design.

Finally, the multifactorial nature of AD might also be an impediment to identify the activity of BACE1 inhibitor in clinical trials. Further investigations to unravel the exact mechanism of AD emergence will facilitate designing more potent molecules targeting BACE1. Investing time and resources to unveil the actual network involved in AD pathology will assist profoundly in ascertaining some pathological concepts, targets and facilitate better understanding towards designing more potent anti-AD drugs.

5. Conclusion

Although there is no approved BACE1 inhibitor for AD treatment, the status of BACE1 inhibitor design and development is promising. The probability of identifying potent candidate(s) is high. With the volume of computational and theoretical studies on BACE1 inhibitors design, it is suggestive as a goal and not a joke. Researchers have used *in silico* methods to identify compounds with better activity/affinity than the failed and clinical trial BACE1 inhibitors. Many of these compounds, if further analyzed, might be good anti-AD agents, and research in this direction should be encouraged. For an efficient and effective BACE1 inhibitor design at the theoretical level, the investigator should augment screening and docking with improved modeling methods such as

MD and QM/MM simulations or *in vitro* assay to provide details on the binding mechanism of such inhibitor. Based on the existing support from X-ray crystallography, *in vitro*, and *in silico* studies on the feasibility of allosteric inhibition and exosite modulation in BACE1 [205-211], we suggest more computational investigations in this direction. Applying computational methodologies to unveil non-catalytic BACE1 binding sites could enable MTDL design and allosteric inhibition. Exploring the non-catalytic region on BACE1 could present a unique approach to overcome failed clinical inhibitors. Due to the applications of computational procedures in screening physicochemical, ADMET, and drug-like properties, experimentalists should consider exploring computational techniques before running off with synthesis and *in vitro* studies to save time and resources.

Conflict of interests

The authors declare no conflict of interest.

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INTERLINKING PAGE TWO

Chapter three is a review article on BACE1 exosites-binding antibody and allosteric inhibitor development as therapies. It also covers BACE1 biological roles, the associated disease mechanisms, and the enzyme's conditions for amyloid precursor protein (APP) sites splitting with a little overview on BACE1 gene properties and substrates. We give a detailed review of the possible allosteric binding and exosite of the BACE1 inhibitors. Chapter three suggests advanced computational modeling of BACE1 intra-atomic properties to further investigate allosteric sites and exosites. This suggested study promises to contribute to the advancement in BACE1 targeting and antibody development. The next chapter briefly discusses some of the computational techniques employed in the subsequent two reviewed chapters above.

CHAPTER THREE

MANUSCRIPT TWO

AN OVERVIEW OF β-AMYLOID CLEAVING ENZYME 1 (BACE1) BIOLOGICAL FUNCTIONS – ELUCIDATING ITS EXOSITE-BINDING ANTIBODY AND ALLOSTERIC INHIBITOR

Samuel C. Ugbaja,[1] Monsurat M. Lawal,[1]* and Hezekiel M. Kumalo.[1]*

[1]Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Durban 4001, South Africa.

*Corresponding Authors: Dr. H. M. Kumalo (email: kumaloH@ukzn.ac.za, Tel: +27312604940) and Dr. M. M. Lawal (lawalmonsurat635@gmail.com)

Abstract

Over decades of its identification, numerous past and ongoing research has focused on β-amyloid cleaving enzyme 1 (BACE1) therapeutic roles as a target in treating Alzheimer's disease (AD). Although the initial BACE1 inhibitors at phase-3 clinical trials tremendously reduced β-amyloid-associated plaques in patients with AD, the researchers eventually discontinued the tests for lack of potency. This discontinuation has resulted in limited drug development and discovery targeted at BACE1, despite the high demand for dementia and AD therapies. It is, therefore, imperative to describe the detailed underlying biological basis of the BACE1 therapeutic option. Herein, we highlight BACE1 bioactivity and genetic properties. We review papers on BACE1 exosite-binding antibody and allosteric inhibitor development as therapies. The review also covers BACE1 biological roles, the disease-associated mechanisms, and the enzyme conditions for amyloid precursor protein sites splitting. We suggest advanced computational modeling of BACE1 intra-atomic properties to investigate allosteric sites and exosites. Such a study will contribute to the advancement in BACE1 targeting and antibody development.

Keywords: BACE1 biological properties, Alzheimer's disease, BACE1 exosites antibody, BACE1 substrates, Gene expression, Allosteric inhibitors.

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1. INTRODUCTION

1.1 Description of BACE1 aspartic properties

Generally, scientists classified proteases as serine (Ser) proteases, cysteine (Cys) proteases, aspartate (Asp) proteases, and metalloproteases. There is a characteristic exhibition of similar peptide breaking process observed among the different protease classes. Studies show that β-amyloid cleaving enzyme 1 (BACE1) and the other Asp proteases like cathepsin, BACE2, pepsin, and renin preferably undergo their catalysis in acidic systems. Despite differing biological activities of the Asp family members, their residues

sequences are homologous and strictly conserved [1-4]. For instance, cathepsin has about 15 different categories grouped under Ser, Cys, and Asp proteases. These cathepsin classes are A and G (Ser protease); B, C, F, H, K, L, O, S, V, W, and Z (Cys protease); and D and E (Asp protease) with various bioactivities [5]. The Asp class forms a noncovalent intermediate (substrate transition state) between the protease and substrate [6].

BACE1, also known as Asp2 and memapsin2, and discovered over a decade ago, displayed all the known properties of β-secretase. BACE1 is an aspartic protease whose cells have a nucleus enclosed within a nuclear membrane of an eukaryotic cell. It has 501 amino acids sequence that is common to pepsin aspartic family. It has two lobes or terminal structures with a characteristic aspartate protease-like property. The N-lobe/terminus has residues 1 to 21, and C-lobe/terminus has residues 455 to 480 [7-9]. BACE1 is classified generally as a class-1 trans-membrane enzyme due to its amino group terminus region, a linking strand, and a liquid matrix around the membrane-enclosed organelles [10, 11]. BACE1 is a type- 1 membrane enzyme with luminal active site location that gives it an advantage inclination for the β-site splitting of amyloid precursor protein (APP). It is a common practice to take out its residues 22 to 45 (pro- peptide domain) to allow the evolution of the matured residues, which start at residue 46 (Glu) [12]. It also possesses 6 Cys residues engaged in the formation of disulfide bonding within the molecules and the N-connected site for attaching a hydroxyl or other groups from other molecules to a carbohydrate [13]. Sequential splitting of APP by γ- and β-secretase produces β-amyloid plaques and C99 polypeptides containing 99 amino acid residues (**Figure 1**) around the different regions of the transmembrane protein. Structural alteration of some genes in some amino acid residues, indicated in yellow (**Figure 1**) results in AD. The γ-secretase splitting of the wild type (WT) C99 residues phenotype species at residues 48 or 49 (green circle), and β-secretase subsequent splitting, results in shorter species released once they cross the site signified with cyan color in **Figure 1**. The amino acid residues in both C- and N-terminals are indicated with the short arrows in **Figure 1** [14].

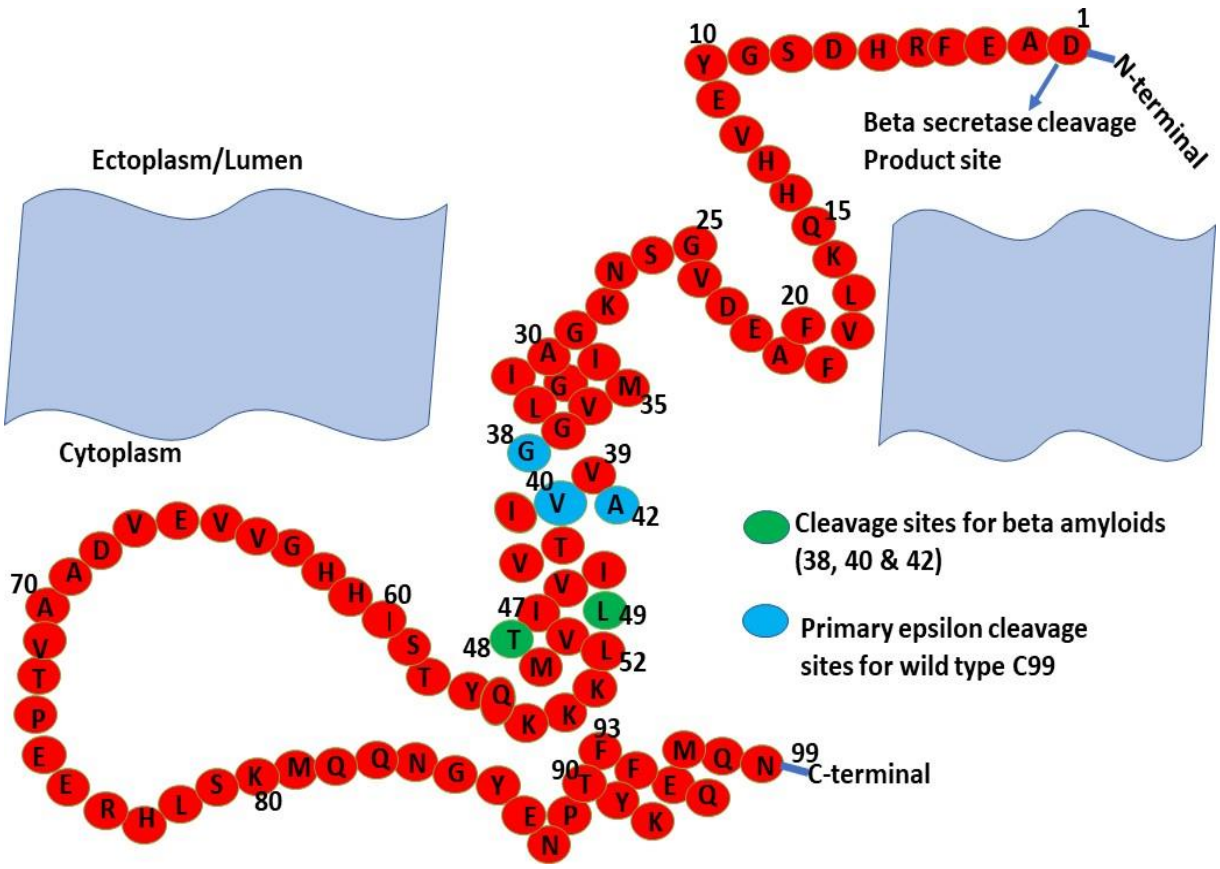

Figure 23. The structure of the human APP C99 re-drew from the literature [14].

1.2 BACE1 biological and genetic properties

Sequel to curiosity on the recent use of smaller ligand molecules as BACE1 inhibitors, Sauder *et al*. [15] carried out 3-D homology modeling of substrate-enzyme (human pepsin-pepstatin) complex crystallized at 2.0 Å. They [15] showed that BACE1 and pepsin share similar sequence alignment with a few differences, especially at the active sites. A notable similarity is residue Arg249 involvement in salt bridge formation with P1' of the Asp APP substrate, thereby validating BACE1 affinity for negatively charged (Asp) P1' residue [15]. At the active site of BACE1 there are Asp32/228, referred to as a catalytic dyad, which forms 4 hydrogen bonding with the substrate/inhibitor, besides 10 other hydrogen bonds from other residues. Therefore, the BACE1 active site is more hydrophilic and has a wide opening compared to other Asp proteases. Arg249, besides the active site, is very significant for effective substrate-enzyme or inhibitor-enzyme binding. Also, the entwined conformation from P2'–P4' of the inhibitor in complex with the enzyme is responsible for relatively low inhibitor-enzyme interconnection at P3'–P4'. This phenomenon results in the non-existence of S3' and S4' sub-pockets in BACE1. BACE1 has high specificity

for sequence splitting, whereby the replacement of amino acid with higher hydrophobic leucine (Leu) with methionine (Met) residue at P1 increases the splitting process.

On the other hand, the replacement of amino acids with lower hydrophobicity like valine (Val) at the P1 position hinders or slows down the splitting process. More so, other replacements within this position and its environment slow down the splitting process, thereby validating the specificity of BACE1 for a sequence in its cleavage or splitting function [7, 16, 17]. Genetic sequencing using radioactive tracer showed that β-amyloids extracted from the cultured cell and accumulated amyloids commonly start with Asp1 of β-amyloid [18]. However, some inconsiderable β-amyloids types start with Val3, isoleucine (Ile6), and glutamate (Glu11). BACE1 cleavage at Glu11 residue produces the non-amyloidogenic C89, which causes the truncation of the produced β-amyloid [19, 20]. Interestingly, Asp1 β-amyloid generated along with Glu11 indicates that BACE1 cleaves simultaneously at both sites, which are the common sites where APP splits [21, 22].

1.3 The intracellular transport of BACE1

Cellular synthesis (**Figure 2**) of BACE1 starts in the endoplasmic reticulum (ER), with proBACE1 (immature precursor protein) weighing approximately 60 kdaltons (kDa) [23]. The splitting that occurs between arginine (Arg45) and Glu46 helps in the removal of the proBACE1 from the Golgi region [24]. There is the tendency for proBACE1 to split APP in the biological synthesis resulting in β-amyloid production, which could be harmful to the neurons [25]. Acetylation of the 7 N-terminus lysine (Lys) residues is a prerequisite for the enzyme to transit from the ER to the secretion route [26]. At the Golgi apparatus and N-terminus, the already matured BACE1, weighing 70 kDa, undergoes sulfonation and glycosylation. The palmitoylation - covalently attaching fatty acids like palmitic acid to Cys - of the cytosolic Cys residues assists in the membrane-bound distribution of the matured BACE1 [27]. The already mature and stable BACE1, with a half-life above 9 hours, is situated inside the cholesterol-rich lipid rafts, while deacetylation of Lys takes place at the lumen side of the Golgi apparatus [26, 28].

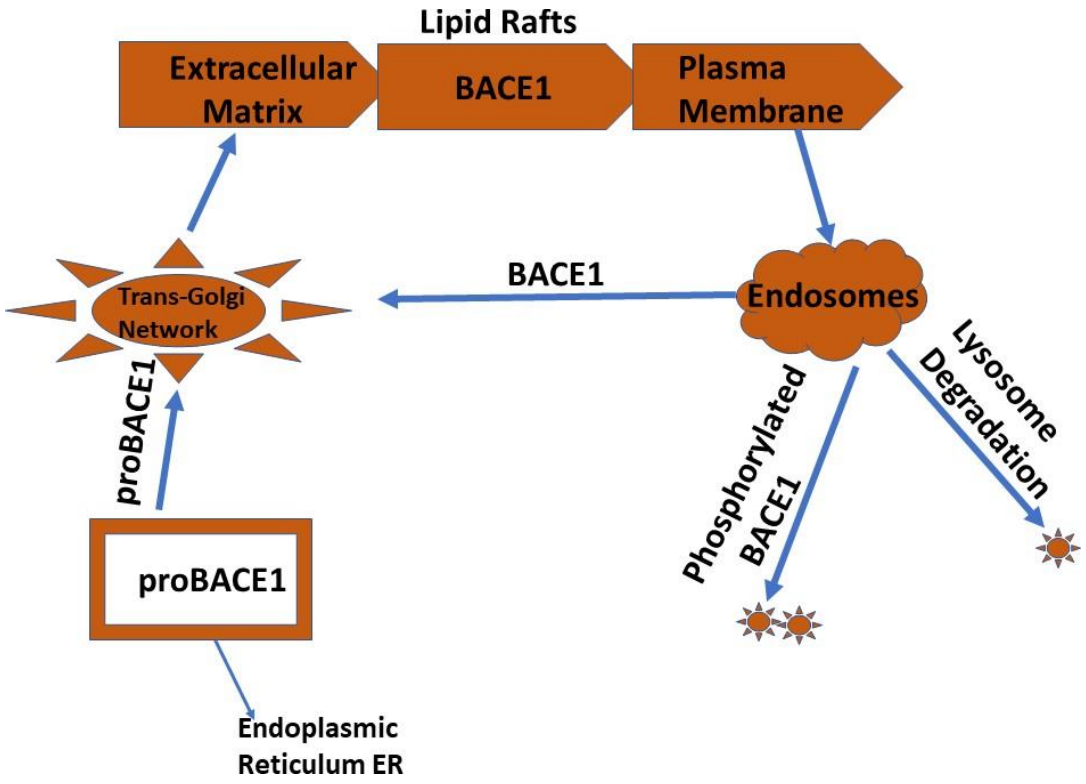

Figure 24. BACE1 cellular synthesis re-drew from the literature [7].

In vivo, BACE1 transportation occurs from openings between the axon passages to the axon terminal; this region is where β-amyloids are produced [29]. BACE1 has its peak efficiency in acidic (~pH 4.5) environments like the Golgi apparatus, trans-Golgi network (TGN), endosomes, and secretion passage [30, 31]. BACE1 production is presumably a type of membrane protein permanently linked to the biological membrane (hence integral membrane). A portion of the enzyme sometimes goes through an ectodomain shedding on the enzyme surface, which could cause its loss in the extracellular region [27]. This process does not affect the splitting of APP by BACE1 as the APP is co-expressed with the shedding product to add to the production of β-amyloid and favor the amyloidogenic APP pathway [32]. The recent detection of soluble BACE1, which is active in the human cerebral spinal fluid (CSF), has increased the possibility of organic liquids (biological fluids) usage for diagnostical analysis [7].

BACE1 has its highest deposit in the brain of healthy subjects and patients with Alzheimer's disease (AD). It also is abundantly expressed in the messenger ribonucleic acid (mRNA) of the pancreas and brain. Nevertheless, the BACE1 expressed in the pancreatic transcripted mRNA exhibits weaker BACE1

characteristics [33]. The BACE1 undergoes transfection, which involves nucleic acid introduction by alternative methods other than viral infection. This transfection produces a stable overexpressed APP that enhances spontaneous splitting by BACE1 to produce more soluble β-APP (sAPPβ) and additional C99 better than when the cell has not undergone transfection. Transfected BACE1 results in a decreased level of soluble α-APP (sAPPα), showing that there exists cellular competition by β- and α-secretase for APP substrates [21].

BACE1 (**Figure 3**) consists of an active site situated between the C- and N-terminals having the Asp 32 and 228 (catalytic dyad) centrally located within its binding region [34, 35]. The flap (hairpin loop) includes residues 67-75 located at the N-terminus, and it regulates the entrance of substrates into the active site through its dynamic conformations. The binding site also contains other pockets (subsites: S1, S2, S3, S4, S1', S2', S3', S4'). The hydrophobic residues are found within S3 and S1 (Leu30, Phe108, Ile110, Ile118, Trp115), while the hydrophilic (solvent-exposed) residues are within S4 and S2 (Lys9, Ser10, Thr72, Gln73, Thr231, Thr232, Arg235, Arg307, Lys321). The S4' and S3' (Pro70, Thr72, Glu125, Arg128, Agr195, Trp197) are other hydrophilic pockets, while S2' (Ser35, Val69, Tyr71, Ile126, Tyr198) situated closer to S4' is both hydrophobic and hydrophilic (amphipathic). Finally, the catalytic dyad is centrally located within the S1' (Ile226 and Val332) pocket [35, 36].

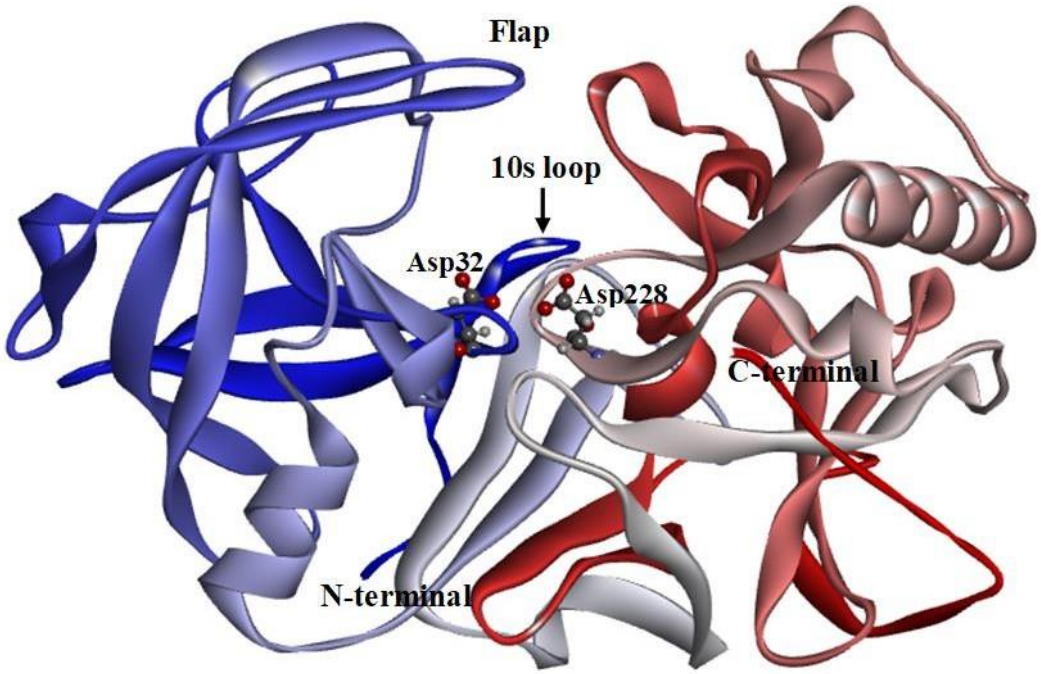

Figure 25. Three-dimensional structural details of BACE1 showing the N- and C-terminals, the catalytic dyad (Asp32 and 228), the flap (residues 67-75), and the 10S loop (residues 5-16).

1.4 Insight on BACE1 substrates

BACE1 is pivotal in the production of β-amyloid. The standard level of β-amyloid in the brain plays a beneficial role physiologically [37, 38]. Another study also shows that the endogenic generation of β- amyloid negatively affects the brain's sensitivity, regulating the physiologic pathway for potassium expression [39]. BACE1 also splits homologous APP, amyloid precursor protein-like proteins (APLPs) 1 and 2 (APLP1 and APLP2) [40, 41]. Although APLP1 and APLP2 do not share similar sequential arrangements with β-amyloid, they can be cleaved by γ-secretase and BACE1 internally, which results in a likely transcription mechanism [42, 43]. BACE1 often splits APP at the synapse's terminus with more BACE1 substrates located at the terminus and other non-APP substrates. As a result of this, specific site splitting of BACE1 may ease sufficient activity of the synapses [29, 44].

The neuronal transmission mode of charge impulses (action potential) propagates via an ion-rich pathway called the voltage-gated sodium channel (VGSC). The two subunits, β, and α make up the channels for action potential, while the β voltage-gated sodium channel subunits (VGSCβ) are essential supplementary subunits. For maximum VGSC performance, there is a need for all subunit expressions. Researchers regard VGSCβ subunits as the BACE1 substrates, and when BACE1 cleaves, it generates a β carboxyl-terminal fragment (β-CTF) [45, 46]. The VGSCβ subunits are processed more by γ-secretase similar to APP processing and produce β2-intracellular domain (β2-ICD) [46]. The β2-ICD controls how the α subunits are expressed. Additionally, there is a sustenance of the raised pool of VGSC intracellularly with cleaving of

β2 subunits results in loss of useful membraned channel, decrease in sodium ion, and changes in membraned excitations [46].

Lipoprotein receptor-related protein (LRP) is another supposed BACE1 substrate associated with the functional tasks of neurons. It also goes through the splitting of cell surface protein resulting in losing the extracellular domain (ectodomain shedding). It is a type-1 membraned enzyme that acts as a multi- dimensional endocytic receptor and plays signaling roles in the neurons [47]. A neuroinflammation is a pathological event associated with AD. There is a suggestion that neurotoxicity is associated with the inflammation of the central nervous system [7]. β-amyloid is responsible for triggering microglial activation thus, fostering the discharging of inflammatory cytokines, which include tumor necrosis factor- alpha (TNF-α), interleukin 6 (IL-6), and β-interleukin 1 (IL-1β). Notably, BACE1 substrates play significant roles in inflammation responses [48]. Presently, all the discovered BACE1 substrates are membrane- localized. According to Lichtenthaler and coworkers [49], BACE1 does not participate in splitting several other membrane enzymes like P-selectin, TNF-α, and a cluster of differentiation 14 (CD14), which suggests

that the protease family does not participate in membrane protein turnover [49]. Note that BACE1 also cleaves various residues or substrates to obtain different products and corresponding biological functions [50]. Researchers have discussed the known BACE1 substrate amino acid sequences in the literature [51, 52].

2. GENE EXPRESSION IN BACE1

BACE1 gene is responsible for protein-encoding in peptidase A1 family Asp proteases. Considering that the BACE1 gene plays significant causative roles in diseases like AD, the mechanisms involved remain unclear [53]. We briefly elucidate the genomic properties of the BACE1 gene and its roles on APP genes. Investigation of BACE1 gene expression *in vivo* is imperative to capture the BACE1 role in the cellular biological system and disease pathogenesis. A basic understanding of the specific roles BACE1 plays in the disease's pathway will assist in therapeutic selected inhibition devoid of off-targets. In some studies [54, 55] involving sequencing and analysis of rats and human BACE1 genetic promoters, the Authors observed that these promoters retained their conserved characteristic properties in both rats and humans. This shared expression forms a controlled mechanism to design disease treatment pathways [54, 55]. Note that the promoters could contain a sequence of deoxyribonucleic acids (DNA). Therefore, the enzyme binds to the DNA to start ribonucleic acids (RNA) transcription along the pathway [7, 56, 57].

The BACE1 gene estimates approximately 30 kbases of human chromosome 11q23.2 consisting of 9 exons. The BACE1 genetic promoter does not possess the usual TATA and CAAT boxes; it has distinct active terrains of 6 unique domains. Besides, it contains well-structured regions towards the ATG first (start) codon mRNA transcript. Note that the TATA box is a sequence of DNA enclosed in the core promoter gene domain, especially in membrane-bound organelles. The CAAT box signals the bonding region for RNA transcription and has a unique nucleotide sequence with GGCCAATCT [7, 58]. BACE1 possesses other transcript binding regions that enhance its transcription process, and these include - but are not limited to - cAMP response element-binding (CREB), GATA1 binding protein, and specificity protein1 (Sp1) [7].

The BACE1 enzyme has various control concepts at different phases of expression. Its adjustment and control occur at both negative (–ve) and positive (+ve) directions and involve varied processes. The binding region, contained in the promoter domain, is responsible for the controlled transcriptional activities of BACE1. When BACE1 transcripts, it generates different splicing species whose protein potentials are lower, such as template strand (antisense strand) that lacks the translatable codes in the 3' and 5' directions. The microRNA (binding sites) harboring 3'UTR and inhibitory 5'UTR regulate BACE1 translational activities as depicted in **Figure 4** [59].

Stephan and coworkers [60] demonstrated the practicability of the ultra-high density (UHD) whole- genome association technique in AD research. The prognosis reveals a futuristic approach for more gene recognition that could play significant roles in AD pathogenesis. They [60] showed that the apolipoprotein E gene (APOE4) is associated with AD. Their earlier scanning done with microsatellite markers did not reveal the association of this gene to AD. Interestingly, UHD single nucleotide polymorphism (SNP) assessment revealed its exact locus on chromosome 19 [60].

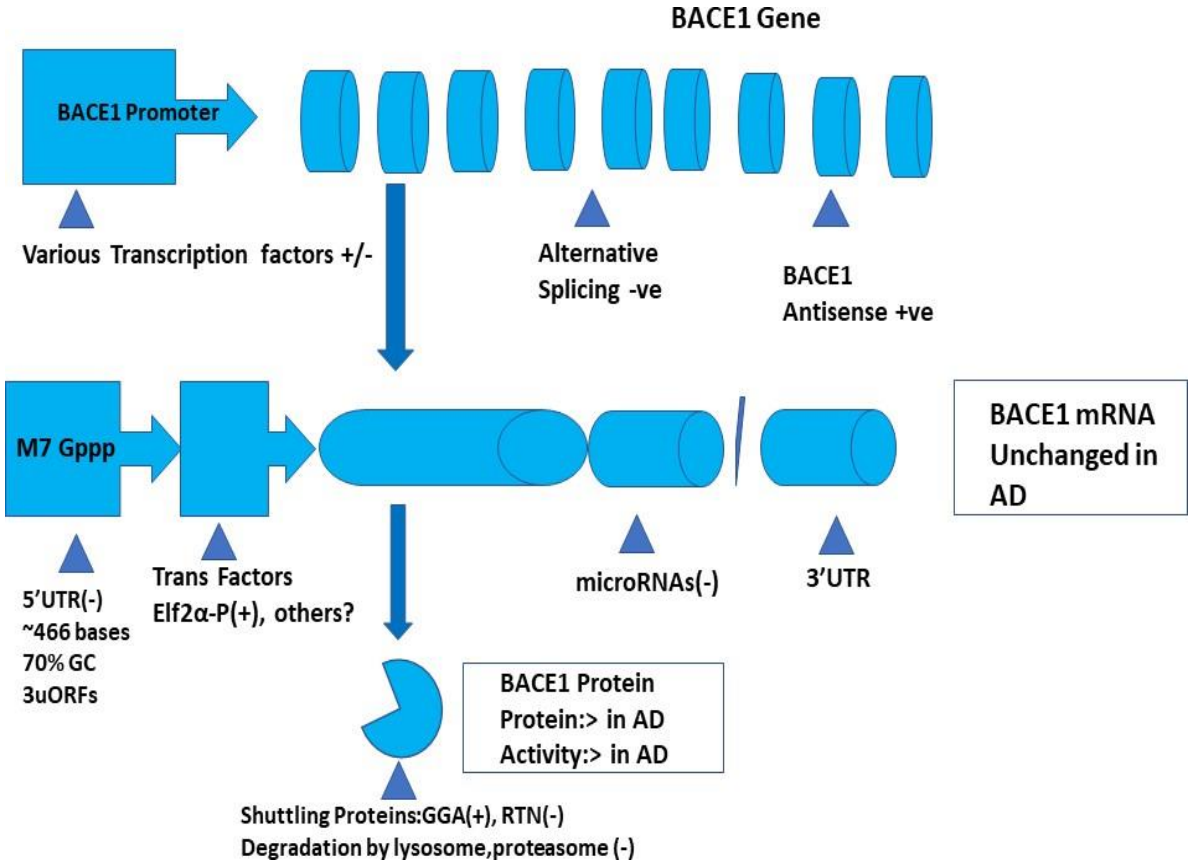

Figure 26. An illustration of BACE1 gene expression.

2.1 Presumed molecular mechanics controlling BACE1 complex expression

Detailed knowledge of BACE1 biological properties will potentially guide the design of improved inhibitory drug discovery processes. In addition to other factors that activate or repress the transcript of BACE1, other factors like modification of its half-life, post-translational changes, and different slicing activities can affect BACE1 protein behaviors. Moreover, BACE1 functionalities can also be affected by its molecular interactions with other enzyme constituents [7]. According to an *in vitro* study by Sun et al., an insufficient supply of oxygen in the tissues can elevate APP metabolic activities, while *in vivo* study ascertained

upregulated BACE1 mechanism in generating β-amyloids [61]. Temporary interruption of brain oxygen supply may result in a malfunction of mitochondria, and β-amyloid aggregation may result in an imbalance between generation and accumulation of oxygen free radicals and antioxidants in the body [62, 63]. Besides, BACE1 upregulation plays a significant role in traumatic brain injury and results in a corresponding increase in the level of BACE1 mRNA in the brain [64]. Ordinarily, neurons trigger β-APP and BACE1 expressions in the brain leading to AD. Study shows that β-amyloid and BACE1 elevation can also be contributed by astrocytes and glia, specifically when inflammation occurs [65].

3. BACE1 IN NEURODEGENERATIVE THERAPY

There has been much focus on BACE1 as a therapeutic target in managing AD. It is the most significant and slowest step (rate-limiting enzyme) in β-amyloid production, whose inhibition will stop the occurrence of β-amyloid-related AD. Besides the believed effects on specified memory roles resulting from the complete removal of BACE1, its shortage results in an increased untimely death rate [66]. The BACE1 enzyme-mediated splitting of APP is an unfavorable activity. Contrary to this notion, Ma *et al*. [67] disclosed that such a splitting enhances cognitive ability and changes the strength of existing synapses (synaptic plasticity) [67]. The researchers reported a spatially improved cognition in APP-overexpression rats, contrary to the AD common association with memory loss. Notably, there was an appreciable reduction in APP intracellular domain, which overrode the improved memory and synaptic plasticity. Their report, however, suggested that healthy brain activities are expedited by the controlled mechanism bound splitting of APP neurons by BACE1. They [67] noticed that the extraction of a gene from BACE1 appreciably decreased APP intracellular domain, while the level of produced species from the splitting was not affected. The result further reiterated the prerequisite for detailed preclinical research on BACE1 inhibition on both WT animal specimens and specimens from animal models for AD. The Authors [67] concluded that the deficiency of β-amyloid production in BACE1 lacking rats shows that therapy targeted to inhibit BACE1 will benefit brain β-amyloid reduction and AD patients at large.

The overall mechanism involving BACE1 relates to unpleasant reactions. Studies showed that the partial reduction of BACE1 enhances improvement in memory loss. It also improves β-amyloid reduction, especially in transgenic (Tg) models. There is a need for APP in the intracellular domain for functional cognitive ability; the accepted level of BACE1 to be inhibited, which will not pose an adverse effect on the level of β-amyloid, is subject to further studies [7, 68, 69].

The role BACE1 plays in AD is no longer questionable, however, the existence of other multifactorial causes can not be neglected. These include alterations in cells linked with deficiencies in energy, stress-

induced translational changes, inflammatory conditions, oxidative stress, and age-related causes [70]. Loss of neurons is a major disease-causing pathway to AD. Studies have shown AD connection with cardiovascular and cerebrovascular diseases; stroke and heart disease are parts of risks associated with AD [7, 71]. Advanced research on epidemiology and neuroimaging proved that these AD-related heart conditions contribute to dementia [72, 73]. Furthermore, chronic brain hypoperfusion (CBH) is one of the causes of AD as suspected in its association with dementia. Notably, CBH occurs during the early phases of mild cognitive impairment (MCI) as one of the early symptoms observed in AD [74, 75].

Another pathway involves the lack of adequate oxygen in the brain tissues and ischemia connection to CBH, which increases the incidences of AD [76, 77]. Recent research has linked β-amyloid production with heart diseases. The underlying heart-related conditions, such as deficiencies in energy, hypoxia, and stress on the cells, elevate BACE1 production and activities [78, 79]. The existence of dystrophic neurites around the aggregated Aβ results in neuritic plaques. Naturally, BACE1 location is at the synaptic bouton

(presynaptic axon terminal), but in the case of the AD brain, BACE 1 is at the dystrophic neurites [80-82]. The deficiency in axonal transportation results in the internal accumulation of APP cleaving enzyme1 and inflammatory axons [83].

Walker and colleagues [84] reported the implications of brain expression of Golgi localized gamma- containing ADP ribosylation factor binding protein 3 (GGA3). They study the GGA3 effect in the control of BACE1 degeneration located in the lysosome region. The GGA3 consists of 4 interconnected domains in their functions. A study showed a GGA3 deficiency link to the non-neuronal cell aggregation of BACE1 in early endosomes that hinders it from getting to the lysosome [85]. Furthermore, the removal of GGA3 escalates BACE1 level *in vivo*, thereby, worsens β-amyloid pathogenesis in the mouse model.

4. ALLOSTERIC BACE1 ANTIBODY WITH CHARACTERISTICS BLOOD-BRAIN BARRIER PERMEATION

The development of antibodies represents an alternative therapy to inhibit BACE1, that incorporates an immunotherapy mechanism to reduce APP processing by BACE1 [81]. Researchers have identified two strategic approaches to develop BACE1 antibodies: active site-directed antibodies and exosite-binding antibodies. The first is targeted against the BACE1 cleavage site of APP and could sterically hinder BACE1 catalytic site access to APP [86]. The second is non-catalytic site targeting which involves exosite/allosteric site antibody targeting on BACE1 surface, thereby allosterically regulating the enzyme activity [87, 88]. An Exosite-binding domain may appear on the structurally adjacent regions of the BACE1 loops C, D, and F [89]. When an antibody binds to these exosites, they induce conformational alteration to the BACE1. This

structural change affects the substrate-binding domain, preventing substrate recognition and binding [81, 90]. The detachment causes a decline in the efficient catalysis of APP and results in an allosterically inhibited reaction (**Figure 5**). The therapeutic application of antibodies thrives on their reduced characteristic toxicity, high selectivity, and specificity.

Further investigation of BACE1 inhibiting mechanisms in proteins and antibodies has unveiled that exosites can control protein mechanisms [89]. The compact cell-layered interior cell lining of the blood- brain barrier (BBB) permits a small number of selected molecules into the brain. This thick blockage hinders the easy flow of drug-like antibodies, thus preventing their useful therapeutic purposes on the CNS. Studies [87, 91] also showed the facilitation of BACE1 antibodies transport across the BBB through engineering one arm of the antibody to identify transferrin receptor (TfR) that shuttles transferrin across the BBB for iron delivery into the brain. Biochemically, the anti-BACE1 antibody maintains its integrity by attaching to an allosteric exosite within the BACE1. The antigen molecule to which the BACE1 antibody binds is the lobe of the C-terminus. This region consists of 12 peptide residues situated near the 3-D domain that comprises residues 254-257 (C-loop), residues 270-274 (D-loop), and residues 309-320 (F- loop) [89].

The first discovery of exosites was from the enzymes bound to BACE1 in a bacteriophage library by Kornacker *et al*. [92]. The enzyme showed an inability to non-competitively compete with OM99-2 for the active site despite being bound on sites with highly saturated inhibitors [93]. This discovery has led to proteolytic concentration-dependent BACE1-based and APP-based substrates. Investigations incorporated a crystallographic structural representation of BACE1-peptide complexes to unveil peptide binding between F- and D-loops [94]. The presence of shared proteins with predominant hydrophobic sequence matches the concentrated aromatic residues within F and D. We reiterate that additional studies to explore the mechanisms of exosite binding on proteins [89, 92] would assist in improved inhibitor/antibody design.

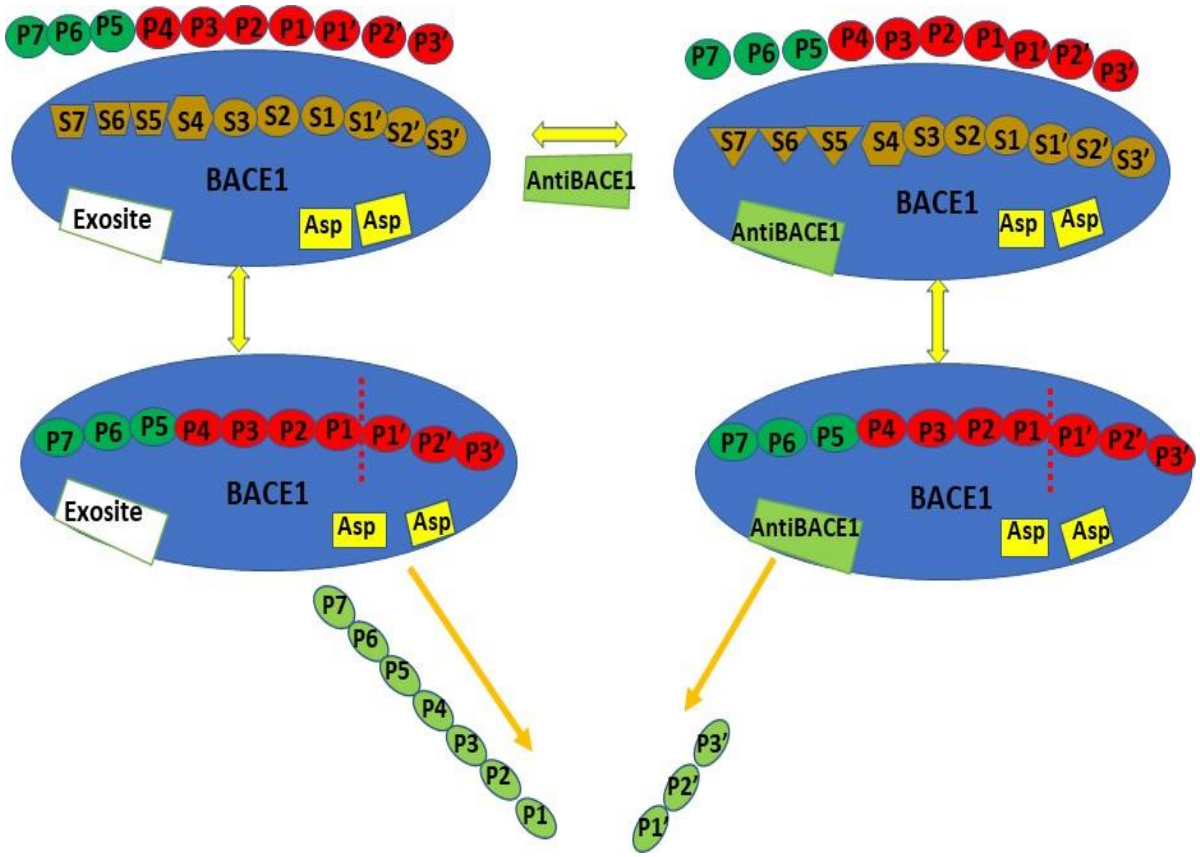

Figure 27. Diagrammatic representation of allosteric inhibition activity by the anti-BACE1 antibody. When substrates bind to BACE1, there are changes of conformations at S4 to S7 subsites.

Allosteric inhibition is a type of competitive inhibition occurring at another protein site different from the active site. In this mechanism, the inhibitor binds to a site(s) other than the active site thereby, rendering the active site unfit for the substrate to bind. In allosteric inhibition, it is a case in which moiety gets to the enzyme or protein first that blocks the other from binding. We review some identified allosteric inhibitors and exosite-binding antibodies of BACE1 over the last eight years (2013-2020) to put our discussion into perspective. Although some recent related reviews [36, 70, 95-99] on BACE1 inhibitor design are available, the most relevant review article on this perspective appears in 2013 from Wang *et al*. [89]: therefore, the topic is suitable for a revisit to put together research outcomes over the last eight years in this direction. Herein, we present allosteric BACE1 inhibitors and exosite antibodies identified using both experimental and computational methods.

Over the years, there is much dedication to designing and developing small molecules to inhibit the BACE1 active site as a potential cure for AD. Hence researchers have developed both failed and ongoing BACE1 inhibitors at the clinical trial [36, 95]. Some of the failed BACE1 inhibitors appear in **Table 1**. Although not an allosteric inhibitor, we briefly mention one of the latest developments on BACE1 inhibitors. Recently (2019), Authors [100] identified **AM-6494** as a potent BACE1 inhibitor and proposed it for preclinical development. Although analogous to verubecestat, **AM-6494** showed better selectivity for BACE1, unlike the discontinued verubecestat, which showed hair-color changes traced to BACE2 off-target inhibition [100, 101]. This discovery prompted recent studies [1, 2] from our group, where we explored **AM-6494** extensively at the molecular level (**Figure 6**). We used conventional and accelerated molecular dynamics (cMD and aMD) simulations, binding free energy analysis, and flap conformational analysis in the investigation [2]. The result provided a helpful understanding of the interatomic mechanical interactions of the BACE1-inhibitor complex. The outcome can also be useful in designing additional BACE1 inhibitors with specific selectivity [2]. Sequel to this study, we used density functional theory (DFT) and Our own N- layered Integrated Molecular Orbital and Molecular Mechanics (ONIOM) in predicting the binding free energy and electronic properties of BACE1-inhibitor complexes [1]. The two theoretical models [1, 2] successfully elucidated the potency of **AM-6494** over the discontinued **CNP-520** (**Table 1**) BACE1 inhibitors. We also identified a potentially active molecule (**AM-6494-SCH$_3$**, **Figure 6**) occupying the active site region optimally [1].

Table 8. Failed phase 3 trials small molecule BACE1 inhibitors

Name	**Terminated year**	**Targeted AD patients**	**Mode of action**	**Reasons for failure**
Verubecestat	2016	Mild-Moderate/prodromal	BACE1 inhibitor	Lack of efficacy
Umibecestat	2019	Preclinical	BACE1 inhibitor	Lack of efficacy
Lanabecestat	2018	Early/mild	BACE1 inhibitor	Lack of efficacy
Atabecestat	2018	Preclinical	BACE1 inhibitor	Toxicity
Umibecestat (CNP-520)	2019	Mild-Moderate	BACE1 inhibitor	Lack of efficacy

Despite its discontinuation, **CNP-520** is under a combined trial with a second-generation anti-Aβ vaccine **CAD106**, which recognizes Aβ1-6 and could appreciably clear amyloid aggregation in AD patients [102]. The investigators envisaged that this combination would reduce Aβ generation through **CNP-520** and clear

existing amyloid plaques through **CAD106**. They expected that this combination therapy might benefit late-stage AD patients as inhibiting BACE1 alone may be too late for the elderly AD patients [70]. The probable date for the outcomes of both generation 1 and 2 clinical trials is 2023 or beyond [103].

Figure 28. Three-dimensional structures of **AM-6494** [100] and **AM-6494-SCH$_3$** [1].

Implementing 3-D computer-aided drug design (CADD) modeling towards BACE1 inhibitors design has facilitated binding free energy predictions and new hit compounds identification. Literature reviews [36, 104] documented pharmacophore development and validation, docking and scoring, quantitative structure-activity relationships, quantum mechanics, MD simulation method for BACE1 inhibitors design. The wide-open flap of BACE1 still poses enormous limitations for effective inhibition by smaller inhibitor molecules. This challenge led to exosite uncovering through modulation study [36]. Experimental and computational studies showed that exosite targeting results in BACE1 enzyme inhibition [36, 105]. From the Web of Science [106] and Scopus [www.scopus.com] database, we retrieved 60 articles. The search criteria involve using the keywords BACE1 inhibitor, allosteric site, and exosite antibody. Streamlining this search to original research works from different authors and focusing on exosite-binding antibody and allosteric inhibitor design, we upheld 20 papers for discussion.

4.1 Experimental studies of BACE1 allosteric inhibitors and exosite-binding antibodies

John and coworkers [107] gave the first report of sAβPPα as a potent endogenous inhibitor of BACE1. Recall that sAPPα is a cleavage product of APP by α-secretase. Using *in vitro* assay, the Authors [107] recorded a half-maximal inhibitory concentration (IC_{50}) of approximately 25 nM for BACE1 cleavage by sAβPPα with a complete inhibition around 300 nM. The inhibition mechanism takes the form of anti-BACE antibody binding to an exosite on BACE1 (**Figure 5**). The peptide also acts as a BACE1 allosteric inhibitor. These investigators proposed that sAβPPα allosteric inhibition of BACE1 may be an evolutionarily

conserved mechanism. It protects β-amyloid overproduction, restores neuronal homeostasis, and performs neuroprotective functions [107].

John and coworkers [108] also identified peptide **65007** (Ac-Ala-Leu-Tyr-Pro-Tyr-Phe-Leu-Pro-Ile-Ser-Ala- Lys-NH_2) as the most potent allosteric inhibitor of BACE1, interacting at its loop F region and mimicking antibody inhibition. Computational modeling via molecular docking and MD simulation studies showed that **65007** interacts with loop F residues inducing distortion in the BACE1 enzyme backbone close to the distal subsites. Peptide **65007** showed selectivity for both BACE1 enzyme and APP substrate besides acting as an APP cleavage inhibitor in a cell model. This most potent peptide has a substrate cleavage mechanism akin to Genentech antibody and m-antibody 1A11 [109, 110], which inhibits long substrate cleavage, but not the short one [108]. For instance, they recorded an IC_{50} value of 2.8 μM for MBP-APPC125 substrate inhibition with peptide **65007** and more than 100 μM for P5-P5' substrate [108].

A study [111] showed that antigen-binding fragments from camelid heavy-chain antibodies (VHHs) exert notable BACE1 inhibition activity. In the investigation by Dorresteijn *et al*. [111], the generated VHHs against BACE1 incorporates an active immunization of *Lama glama* with the recombinant BACE1 protein. Sequencing the selected 12 independent clones, the Authors noted 3 different sequences represented by VHH B1a, B3a, and B5a. The binding of the immobilized BACE1 gave dissociation constant (K_d) values of 10.8, 0.3, and 9.3 μM for VHH B1a, VHH B3a, and VHH B5a, respectively, showing that VHH B3a has the best affinity. *In vivo* study of these clones with a transgenic AD-infected mouse showed a significant reduction of the β-amyloid plaque in the brain and plasma. The Authors [111] proposed that this inhibitory VHH may be considered a candidate molecule for the therapeutic reduction of β-amyloid aggregation, thereby preventing AD development. Finally, these researchers aimed to observe VHH binding near or at the BACE1 active site and not exosite or allosteric binding site. However, they did not probe in detail the exact mechanism of VHH B3a antibody reduction of plasma β-amyloid levels in the study [111].

Preparing drug or inhibitor candidates from natural sources, especially from plant materials of therapeutic implications, has proven advantageous due to low or no toxicity. A study showed that the active components of a wide variety of *Sargassum* species extracts have pharmacological properties such as anti-AD activity [112, 113]. Hence, Seong *et al*. [114] evaluated the active components, meroterpenoids of *Sargassum serratifolium* for potential anti-AD activities. In the study, they targeted acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and BACE1. The extracted meroterpenoids are sargahydroquinoic acid (**1**, **Figure 7**), sargachromenol (**2**), and sargaquinoic acid (**3**). These three compounds showed high inhibitory activity towards BACE1

with IC_{50} values 4.4, 7.0, and 12.1 μM, respectively. Although not explored in detail, they [114] observed a mixed inhibition mechanism in compounds **1** and **3** plus a non-competitive model in **2** from their kinetics study. Molecular docking analysis revealed dual-site interaction of **1** and **3** on BACE1 through the catalytic dyad and allosteric sites. Compound **2** interacted at an allosteric region on the enzyme. The study outcome showed the potential application of meroterpenoids from *Sargassum serratifolium* as anti-AD molecules [114].

Figure 29. Some identified potential allosteric inhibitors of BACE1.

Youn *et al*. [115-118] identified a couple of allosteric inhibitors in the quest to find therapeutic compounds for AD from natural sources. They [115] investigated the inhibitory activity of BACE1 by *Tenebrio molitor* larvae and noticed that this organism is composed of substantial unsaturated long-chain fatty acids such as oleic and linoleic acids. Oleic acid binds BACE1 non-competitively with an IC_{50} of 61.3 μM and an inhibition constant (K_i) of 34.3 μM. These authors reported that the identified fatty acids interacted with BACE1 at allosteric sites of BACE1 through Cys319, Tyr320, and Gln304 [115]. In another report [116], gamma-linolenic acid (GLA, **Figure 7**) inhibits BACE1 non-competitively with an IC_{50} and K_i values 76 μM and 35 μM, respectively. Docking analysis revealed GLA binding to the allosteric domain of BACE1 through residues Lys9, Ser10, Tyr14, Leu154, Gln304, Tyr305, Arg307, Glu339, His360, Val361, and Cys359 sharing hydrogen bond with the ligand OH group [116].

Youn and colleagues reported two other research investigations [117, 118] on allosteric binding inhibitors of BACE1 from plants. They observed that biochanin A - a dietary isoflavone available in legumes - exhibited substantial inhibition towards BACE1 through *in vitro* human recombinant assay, enzyme kinetics, and molecular docking analysis [117]. Enzyme-based assays showed a non-competitive inhibition mechanism of biochanin A to BACE1 having IC_{50} and K_i values of 28 μM and 43 μM, respectively. Although docking pose showed the interaction of this potential BACE1 inhibitor at the active site involving Asp32/228, the Authors proposed that biochanin A could interact at an allosteric site of the enzyme [117]. These researchers [117, 118] also identified some isoflavones candidates as potential naturally occurring compounds inhibiting BACE1. The investigation showed genistein as the best moiety that significantly inhibits with an IC_{50} value of 63 μM. Molecular docking revealed that genistein binds at the BACE1 allosteric site through residues Asn37, Gln73, and Trp76, and essentially forming hydrogen bonds with the ligand [118].

The development of molecules that could permeate the BBB led to the Brain Shuttle (BS) technology that could facilitate large molecules transport into the brain [119]. Ruderisch *et al*. applied experimental and computational methods to design BACE1 peptide inhibitors attached to different lipid chains and transported to the brain using the BS approach [119]. The BS design incorporates *in silico* protocol whereby they generated a conformational ensemble by fixing the peptide portion, screening, removing clashed conformations, and manually selecting some representative members. With further refinement and modeling, these researchers generated a BS antibody attached through the sortase recognition scaffold to the peptide N-terminus short of one glycine. They designed the C-terminal transmembrane helix by manually attaching it to the BACE1 [119]. These Authors later prepared active-exosite peptides of BACE1 with dual binding mode and enhanced potency. They also made the co-crystallized BACE1 complex (**Figure 8A**) containing an active site and exosite-binding peptide inhibitor available with 5MCQ code in the RCSB protein databank [https://www.rcsb.org/structure/5MCQ]. This crystal structure resolved at

1.82 Å has peptide **16** with sequence Gly-Gly-Gly-Tyr-Pro-Tyr-Phe-Ile-Pro-DLys-Gly-DLys-Gly-Glu-Val-Asn- Sta-Val-Ala-Glu-DPro-NH_2, and IC_{50} value of 6.4 μM for cell β-amyloid clearance in the mice brain. The administration mechanism was intravenous using the modeled BS via sortase coupling [119]. The results also showed substantial time- and dose-dependent lowered β-amyloid in the plasma. These outcomes indicate that the BS is critical to BACE1 peptide inhibitors for efficient delivery to the brain. The Authors

[119] proposed that the active-exosite design of BACE1 peptide inhibitors coupled with modified lipid may be of therapeutic application.

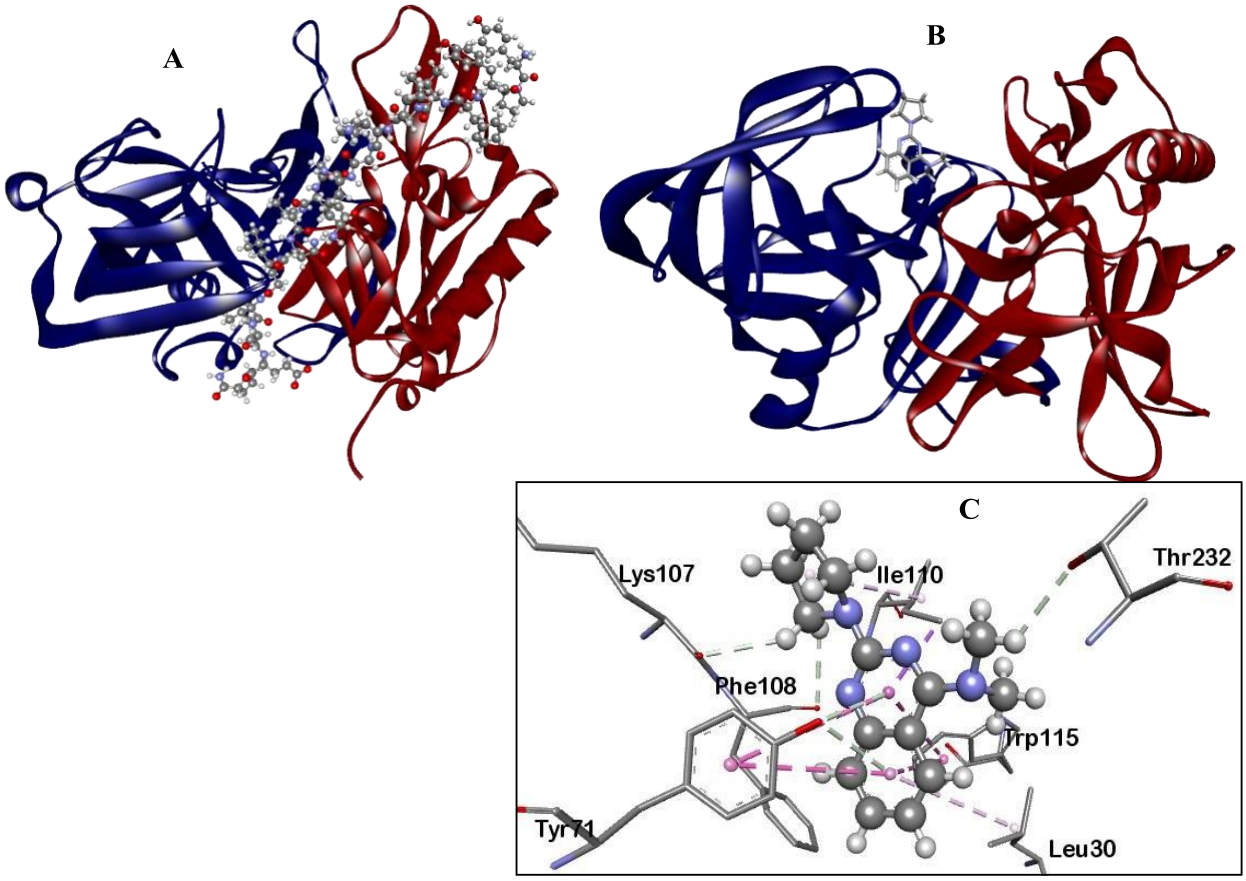

Figure 30. 3-D snapshot of BACE1 crystal structure complexed with (**A**) an exosite-binding peptide **16** inhibitor [119]; (**B**) compound **12** and (**C**) showing the interacting residues devoid of the catalytic Asp32/228 [120].

Gasse *et al*. [121] reported modified DNA aptamers that bind BACE1 and inhibit its cleavage activity. At higher inhibition concentrations, the modified DNA aptamer binds with a lower affinity to an allosteric site. They thus recorded the IC_{50} values for the various sequences without accounting for the high concentration of modulators. Overall, the studied aptamers displayed significant affinities for BACE1 with approximately 10 nM dissociation constant (K_d) and lower nanomolar IC_{50} values within 43.7 and 87.6 nM [121]. They also observed that the aptamers are best active with enhanced binding affinities at pH 4.5, similar to Shen and coworkers' report [122], and denoting that BACE1-mediated peptide cleavage activity is ideal at lower pH. The Authors [121] associate this feature with an allosteric exosite binding mechanism of the aptamers sequences. This proposal supported by crystal structure resolution [119] and continuous constant-pH MD analysis [122] is plausible. They also observed that the modified 5-chlorouracil proved to be a good structure supporting genetic transfer *in vivo* with appreciable bioactivity function [121].

Rombouts *et al*. [120] developed an approach to identify BACE1 inhibitors that do not form interaction through the catalytic Asp32/228 dyad of the enzyme. The investigation incorporates fragment screening, nuclear magnetic resonance (NMR), and X-ray crystallographic analysis. They [120] screened potential non-catalytic residue binding ligands using both surface plasmon resonance (SPR) and ThermoFluor (TF) with enzymatic fluorescence resonance energy transfer (FRET) assay. This integrated screening protocol yielded 6 hits that they confirmed with two or more assays, 1-D and 2-D NMR analyses. Further refinement and competition experiments with **OM99-2** [123] allowed for binding mechanism predictions of the soluble BACE1 with one of its catalytic Asp mutated (Asp32 Asn32). Among the 6 hits, compound **12** showed slight competition with **OM99-2** along with 3 other fragments. Other screening criteria including pH-dependent inhibition and IC_{50} profiling led to compound **12** (IC_{50} = 0.5 mM) selection for X-ray crystallization resolved at 2.52 Å [120]. This structure is available with PDB code 5MXD [https://www.rcsb.org/structure/5MXD], **12** occupies the active site region but devoid of interaction with the catalytic Asp32/228 (**Figure 8B** and **C**). Although the interaction pose of compound **12** occurs at the BACE1 active site domain, we propose it as a partial allosteric mechanism. The Authors [120] proposed applying this integrated approach involving fragment screening techniques and binding competition evaluation to other targets. Such a methodology will facilitate undesired interacting residues bypass and a hit-generation technique in a highly conserved intellectual property space [120].

Following the identification of melatonin (MLT) as a neuroprotective moiety [124], Panyatip *et al*. [125] reported its derivatives as potential BACE1 and AChE inhibitors. The Authors applied both *in vitro* and *in silico* methods to study inhibition properties, neuroprotective, neuritogenic, and binding conformation of the identified potent MLT-based molecules [125]. All the derivatives labeled compounds **1**-**5** exhibited appreciable inhibition for BACE1 with 88% inhibitory activity for compound **1** at 5 µM BACE1 concentration. Compound **1** (**Figure 7**) also showed the highest potency for neuron cells protection from oxidative stress through increased cell viability to 98% at 1 nM. The molecular docking analysis showed that compound **1** interacted at BACE1 allosteric site through residue Thr232 and the flap region residues. These Authors [125] suggested further exploration of the novel MLT derivatives as therapeutic molecules against AD and neuroprotective agents.

Juliano *et al*. [126] recently prepared and characterized various peptidomimetic analogs of BACE1 substrates with two unique stabilizing motifs. They probed the catalysis and inhibition profile of these substituted peptides using different assay strategies and considering competitive and non-competitive mechanisms. The results showed that incorporating β-amino acids at P1 scissile site position within known

peptide substrates are resistant to cleavage, and some replacements provoked a concentration- dependent stimulation of BACE1 [126]. This observation indicates a modulatory role of the native BACE1 substrates and a possible dual binding mechanism. They proposed that at low enzyme concentrations, the peptidomimetics bind to the active site. At high enzyme concentrations, they noticed allosteric or subdomain binding [126].

4.2 Computer aided-design of BACE1 allosteric inhibitors and exosites-binding antibodies

Gutierrez *et al*. [127] employed CADD techniques using binding free energy and MD simulations to reveal that exosite residues Glu225, Pro258, Phe261, Gly264 to Ala272, Asp311 to Ala313, Ser315, Asp317 to Tyr320 interact with protein modulator. These Researchers [127] noted that BACE1 exosite modulators exhibit allosteric enzyme inhibition from another *in silico* investigation [128]. Their study uncovered active epitopes situated inside 3 loops made of different residues (251 to 258, 270 to 273, 311 to 317) [127, 128]. Furthermore, in their recent research on peptide interaction with BACE1 modulators using DFT and MD simulations [105], the result revealed the interaction of residues Glu163, Glu255, Lys256, Phe257, Pro258, Asp259, Gly260, Phe261, Trp262, Leu263, Gly264, Glu265, Gln266, Leu267, Val268, Cys269, Trp270, Gln271, Ala272, Gly273, Thr274, Asp311, Val312, Ala313, Thr314, Ser315, Gln316, Asp317, Asp318,
Cys319, Tyr320, Lys321, and Phe322 with the modulators [105].

Authors [122] have reported the integration of free energy perturbation calculations with continuous constant pH molecular dynamics to unveil the selectivity of **LY2811376** (**Figure 7**) for BACE1. The molecule earlier studied to have an IC_{50} value of 0.9 nM [129] gave an affinity for BACE1 like cathepsin D at high pH, while at pH 4.6 its selectivity for BACE1 is –1.3 kcal/mol favorable [122] and showing similar trend with experiment [129]. Interestingly, these authors [122] linked the pH-dependent selectivity of **LY2811376** to the protonation of His45, which modulates the BACE1 113S loop for interaction. They [122] proposed that an allosteric moiety capable of distorting His45-Phe108 interactions within the BACE1 would increase inhibitor selectivity.

Di Pietro *et al*. [130] applied a multistep MD protocol to analyze the binding mode of novel hybrid huprine- rhein inhibitors of BACE1. Separately, huprine and rhein molecules have low inhibitory activity against BACE1, while an experiment showed an IC_{50} value of 80 nM for their hybrid compound **1** inhibitory activity [131]. An assessment of compound **1** (**Figure 7**) crystal structure showed no sufficient binding pocket for its interaction with BACE1 [130]. Therefore, the authors investigated the conformational plasticity of BACE1 with a focus on the highly fluctuating loop region 8-14, 154-169, and 307-318. The multistep

approach involves deliberate retrieval of apo- and holo-BACE1 PDB structures with the spatial arrangement of the selected loops, ensemble generation, MD simulations, principal component analysis (PCA), clustering, druggable pockets preparation, docking, conformational sampling, and post-simulation analysis [130]. One of the outcomes of this study is a transient secondary binding site detection in BACE1. Arg307 acts as stabilizing moiety at this site for small molecule binding at the edge of the catalytic pocket. With the identified druggable secondary pocket, these Authors [130] hypothesized that such a domain would facilitate multisite inhibitors binding to both catalytic and allosteric sites. Thus, MD simulations of the BACE1-huprine-rhein complex revealed the workability of their hypothesis. The results provide a basic description of the two enantiomeric forms of compound **1** high BACE1 inhibition despite their lengthy oligomethylenic linker (**Figure 7**). Taken together with the applied allosteric site modeling procedure, these findings [130] could apply to a wide range of enzymes binding to larger molecules. The study provided a direction to unravel novel functionalities in developing optimized BACE1 multisite inhibitors [130].

Chen *et al*. [132] also applied multiple short molecular dynamics (MSMD) simulations and molecular mechanics generalized Born surface area (MM-GBSA) approach to propose binding pockets of BACE1 and BACE2 for some inhibitors. They [132] also unravel the selectivity of inhibitors **DBO**, **CS9**, and **SC7** for BACE1 over BACE2. Using dynamics cross-correlation matrix (DCCM) analysis to probe the internal dynamics of the C-α atoms, the modeling protocol [132] is similar to the investigation by Di Pietro *et al*. [130]. Per-residue energy decomposition (PRED) analysis of the crucial residues interacting with the potent inhibitors showed 3 or 4 other secondary pockets beside the known active catalytic region. These sub-pockets houses the inhibitors for significant interaction with the enzymes. **CS9** (**Figure 7**) and **SC7** inhibitory potency showed IC_{50} values of 3 and 8 nM [133] and the calculated binding free energy value is approximately –16.5 kcal/mol for both inhibitors [132]. Besides other hydrophobic interactions, these two compounds formed hydrogen bonds on BACE1 subsite pockets through residues Thr72/232, Gln73, and Gly230. Surprisingly, they observed that the interactions of the catalytic Asp32/228 in BACE1 with **CS9** yielded unfavorable contacts [132], suggestive as an allosteric binding mechanism. Essentially, **CS9** interacted through residues Lys30, Tyr71, Trp115, and Ile118 in the first sub-pocket for hydrophobic group R1 binding; the second sub-pocket housing R2 (**Figure 7**) consists Thr33 and Ser35; the third binding subsite where R3 (**Figure 7**) occupies has residues Gln73, Thr231, and Ile110; with the interaction of Ile118 for M1 binding as the fourth sub-pocket [132].

Chen and colleagues [134] lately reported an advanced simulation protocol to probe the binding landscape of inhibitors **3KO**, **3KT**, and **779** with characteristic disulfide bond (SSBs) on BACE1. They applied multiple replicas accelerated molecular dynamics (MR-aMD) simulations, PCA, DCCM, and free energy analysis in the study [134]. The results from DCCM analysis showed that the cleavage of the SSBs has a substantial effect on the structural plasticity and internal dynamics of the selected inhibitors-BACE1 complexes. Apart from other detailed result analyses of importance to the research aim, the most relevant outcome of the study to our discussion is the observation of sub-pockets that house the inhibitors for optimum interaction with BACE1. From the PRED analysis, the Authors [134] identified Lys30, Ser35, Val69, Tyr71, Gln73, Trp76, Phe108, and Ile110/118 as the highest contributing residues to the binding of these inhibitors [134]. This phenomenal binding mechanism is similar to their previous research [132], and these subsites could be designated as potential allosteric regions if further explored.

Drug re-purposing represents a unique way of unraveling approved inhibitor/drug functionalities to target a different enzyme or pathway. This secondary targeting mechanism of known drugs has found applications in drug design through computational modeling. Kumar *et al*. [135] re-purposed some approved psychotic drugs as potential anti-AD candidates in a report. They targeted some enzymes connected to AD emergence using molecular docking and a web-based application. Although not the best inhibitor identified from their analyses, the interaction of anisoperidone (**Figure 7**) with BACE1 showed typical allosteric binding with hydrogen bonds formation through Tyr132, Trp137, Tyr259, and a salt bridge via Asp93. Besides, residues 71-74 of the flap region showed polar and glycine-like interaction, while Leu91, Val130, Ser96, Phe169, Ile171/179/187, and Trp176 formed hydrophobic interactions with the drug [135]. Interestingly, anisoperidone showed binding energy of −43.1 kcal/mol, which is more favorable than a known inhibitor **LY2886721** [136] with a value of −35.835 kcal/mol [135].

5. CONCLUSION

We reviewed BACE1 biological and genetic properties, its therapeutic option, and exosite binding activities. A detailed understanding of BACE1 biological roles could potentially provide better insight into AD-associated mechanisms toward its pharmacological treatment. We proposed further investigations on the identified peptidomimetics and non-peptide BACE1 allosteric inhibitors and antibodies. Such studies could guide improved anti-AD drug discovery and development. We recommend further experimental and theoretical investigations on BACE1 allosteric sites and exosites determination. The outcome would potentially stimulate a consensus on the secondary space available for binding on the enzyme. Extensive

computational approach usage would likely show the inter-atomic and molecular properties of allosteric BACE1 inhibition. Such detailed knowledge would assist preclinical and subsequent clinical trial advancement on BACE1 targeting.

CONSENT FOR PUBLICATON

Not applicable.

Conflict of interests

The Authors declare no conflict of interest.

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Abbreviations

1-D; One-dimensional 2-

D; Two-dimensional 3-D;

Three-dimensional

AD; Alzheimer's disease ADP;

Adenosine diphosphate

aMD accelerated molecular dynamics AMP;

Adenosine monophosphate

AP; Action potential

APLP1; Amyloid precursor-like protein 1

APLP2; Amyloid precursor-like protein 2

APOE4; Apolipoprotein E4 gene

APP; Amyloid precursor protein

BACE1; β-amyloid precursor protein cleaving enzyme 1

BBB; Blood-brain barrier

CADD; Computer-aided drug design CBH;

Chronic brain hypoperfusion CD14;

Cluster of differentiation 14 cMD;

Conventional molecular dynamics CNS;

Central nervous system

CREB; cAMP response element-binding CSF;

Cerebral spinal fluid

DCCM; Dynamics cross-correlation matrix

DFT; Density functional theory

DNA; Deoxyribonucleic acid ER;

Endoplasmic reticulum

FRET; Fluorescence resonance energy transfer

GGA3; Golgi localized gamma-containing ADP ribosylation factor binding protein 3 IC_{50};

Half-maximal inhibitory concentration

IL-1β; β-interleukin 1

IL-6; Interleukin 6 kDa;

Kilodaltons

LRP; Lipoprotein receptor-related protein

MCI; Mild cognitive impairment

MD; Molecular dynamics MLT;

Melatonin

MM-GBSA; Molecular mechanics-generalized Born surface area

MR-aMD; Multiple replicas accelerated molecular dynamics mRNA;

Messenger ribonucleicacid

MSMD; Multiple short molecular dynamics NMR;

Nuclear magnetic resonance

ONIOM; Our own N-layered Integrated Molecular Orbital and Molecular Mechanics PCA;

Principal componentanalysis

PRED; Per-residue energy decomposition

RNA; Ribonucleic acid

sAPPα; Soluble α-amyloid precursor protein

sAPPβ; Soluble β-amyloid precursor protein SPR;

Surface plasmon resonance

TF; ThermoFluor

TfR; Transferrin receptor

Tg; Transgenic

TGN; Trans-Golgi network

TNF-α;Tumornecrosisfactor-alpha

UHD; Ultra-high density

VGSC; Voltage-gated sodium channel

VGSCβ; Beta voltage-gated sodium channel subunits WT;

Wild type

β-CTF; Beta carboxyl-terminal fragment

β2-ICD; β2-intracellular domain

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INTERLINKING PAGE THREE

Chapter four deals with the introduction to the applied computational chemistry techniques mentioned in chapters two and three above. The chapter discussion encompasses quantum mechanical methods, molecular mechanical methods, and a hybrid of both methods. It briefly explains some of the contemporary computational methods reviewed in chapters two and three. It also presents the general computational methods employed in molecular modelings such as the gaussian application of density functional theory (DFT) and time-dependent density functional theory (TDDFT). It also gives a brief description of conventional and accelerated molecular dynamics simulations as succinctly applied in chapter six of this thesis. It gives a detailed description of the 2-layers and 3-layers ONIOM methods as applied and discussed in the subsequent chapter five. Furthermore, it gives a diagram illustration of mechanical and electrostatic embedding. Finally, it unravels the application of a hybrid of QM/MM in determining the interatomic properties of a novel potent (AM6494) BACE1 inhibitor in chapter five. In the next two subsequent chapters, we first explored the options of the conventional active sites in the study of BACE1 in complex with AM-6494 and CNP-520.

CHAPTER FOUR
INTRODUCTION OF THE COMPUTATIONAL SCIENCE TECHNIQUES

BACKGROUND

A course or subject which cuts across chemistry, physics, mathematics, and engineering called computational sciences was introduced in the latter part of the year 1960 (1). Two vitally important techniques of computational sciences are molecular mechanics (MM) and quantum mechanics (QM) (2, 3). In MM, interactions are depicted classically, employing molecular force fields to establish a multidimensional interpretation of biological and chemical systems (4). MM focuses on interactions within the nucleus rather than the electrons in the system, and this makes them useful in systems with numerous atoms at a significantly lower computational cost (5). Molecular mechanics also handles the effect of the electrons by using parametrization, which makes it computationally less costly (2). QM pays close attention to the structure of the electrons and links the parameters of the molecules as well as the energy through its attempt in proffering solutions to the Schrodinger equation (equation 1) (6)

Schrodinger equation $H\Psi=E\Psi$ ..... equation (1)

The equation (1) is based on approximation; hence, it is not ordinarily computationally feasible. The structure of the electrons (electronic structure) has broadly been stratified into two major groups (semi-empirical and ab initio) with the existence of a third group (density functional theory DFT) (7).

APPLICATIONS OF GAUSSIAN IN COMPUTATIONAL CALCULATIONS

With the recent introduction of the Gaussian 16, the application of DFT in the treatment and investigation of structures, energies, and other parameters of larger molecules in the ground and excited states are made easier on personal and high-performance computers (8). The overarching principle behind using the Gaussian computational technique (theoretical model) is in proffering a solution to the Schrodinger equation (9). This is done through the configuration and specification of structures of the electrons and the nuclei of atoms in molecules of heavy atoms. This is called theoretical model chemistry because it has no room for bias and results are verified and compared with existing results from experiments (10). It also combines computational (theoretical) techniques with a basis set as the specification of the chosen chemical molecular systems. Also, the computational model is always stated in Gaussian program applications (7, 11). Gaussian 16 offers an in-depth analysis of chemical or biological species of interest, focusing on the prediction of molecular transitional phases (states). It also handles the structural minimization with particular reference to the starting (stationary) positions through intrinsic reaction coordinate. It can also be used to perform other computational analyses such as nuclear magnetic resonance shielding effects,

chemical effects, coupling constants (spin to spin). Other operations include determination of ionization energies, optical rotations dipole/multipole moments, infra-red, visible, and ultraviolet spectra, optimization, frequency and energy calculations, etc. (12-15).

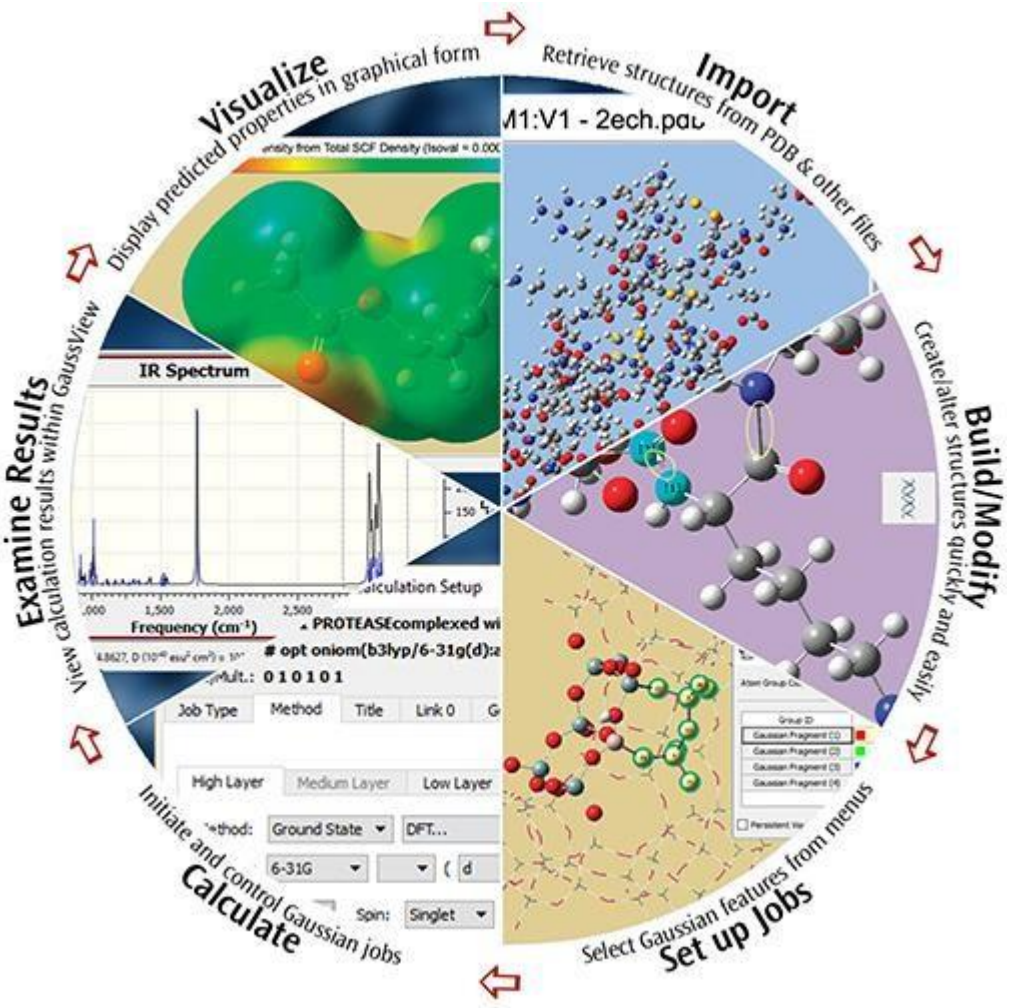

Figure 1. Schematic description of task gaussian software performs as adopted from an open-access image (16)

BASIS SET

The molecules inside molecular orbitals are usually mathematically depicted as a basis set (17). It is reported that a larger basis set gives electrons within orbital freer movement and a much correct approximation. Electrons are considered to have boundless spatial conformation within the concept of Quantum mechanics. There are mainly two basis set models: restricted or open, unrestricted, or closed (18). Applying the model chemistry concepts in illustrating different types of basis set representation, we have the following examples: split valence basis sets i. UHF/6-31+G (d, p), RHF/6-31+G (d, p) depicts using the

unrestricted U and the restricted R Hartree-Fock model with 6-31+G (d, p) basis set. Diffuse functions ++ and polarization functions d, p (d functions on all-atom but hydrogen while p functions on hydrogen) are usually added based on the nature and type of molecules involved. Other commonly used basis set include 6-31++G (d, p), 6-311G (d, p), 6-311++G (d, p), 6-311++G(3df,3pd) etc. There are also slater-type orbitals STO-3G which has been described as the minimum basis set (7, 19, 20).

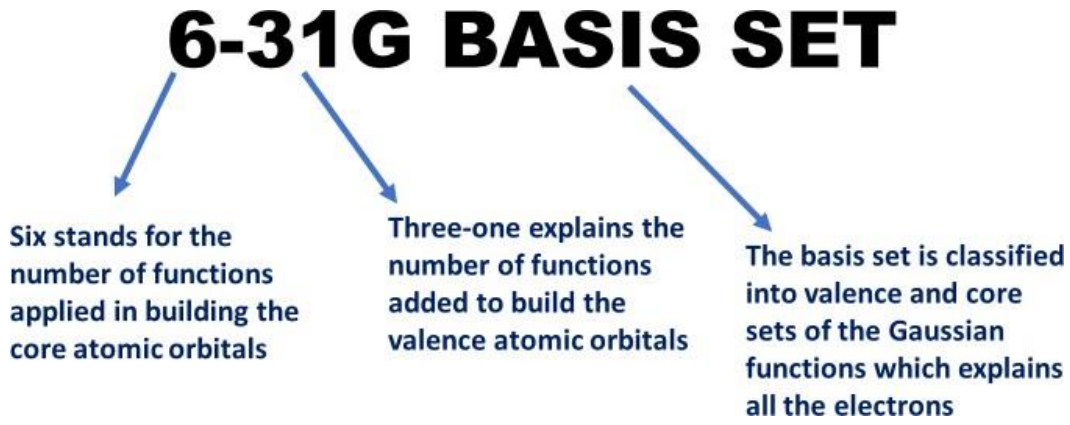

Figure 2. Descriptive Gaussian basis set per atom

DENSITY FUNCTIONAL THEORY AND TIME-DEPENDENT DENSITY FUNCTIONAL THEORY

Density functional theory DFT does not concentrate directly on just the wavefunction, instead, it optimizes the energy and molecular geometries by squaring the wavefunctions (21). It operates on the electron density as a function of time and space, focusing on just one body having three variables x, y, z rather than on multi-bodied wavefunctions. Kohn and Hohenburg postulated that ground state parameters of a body (system) are dependent on the density of that system, which means that the summation of the ground state of a multi-electron body depends on the density (22, 23). Computationally, the optimization of geometries, Raman, and infra-red scattering intensities and vibrational frequencies can quickly be done using DFT techniques (24). Over the years, improved techniques have evolved, which could satisfactorily compute, for example, the nuclear magnetic resonance spectra of biochemical and chemical systems. It has advanced further by integrating electron correlation effects, a vitally important component of analyzing the properties of nuclear magnetic resonance (7, 11).

Further advancement in quantum chemistry has given rise to the computation of molecular orbitals' electrons at excited states. Application of Gaussian Time-Dependent DFT (TDDFT) ensures a feasible computation of systems in excited states whose results can accurately be compared to the DFT results from ground state computation. Qualitative analysis of excited state electrons is successfully employed in natural

transition orbitals which are done by changing compacted particles in filled orbitals to empty or unfilled orbitals. Additional applications of TDDFT are in oscillational/vibrational frequencies and absorption spectroscopic analyses (25-28)

QUANTUM MECHANICS/MOLECULAR MECHANICS QM/MM ANALYSIS OF MECHANICAL AND ELECTROSTATIC EMBEDDING

Exploring QM/MM combination in studying and analysing larger biological and chemical systems has been proven to be successful (29). It involves the treatment of larger biochemical molecules, like proteins, in separate regions. On the other hand, the smaller active parts or sites are usually treated at the QM level, while the remaining components of the system are treated at the MM level (figure 3) (30).

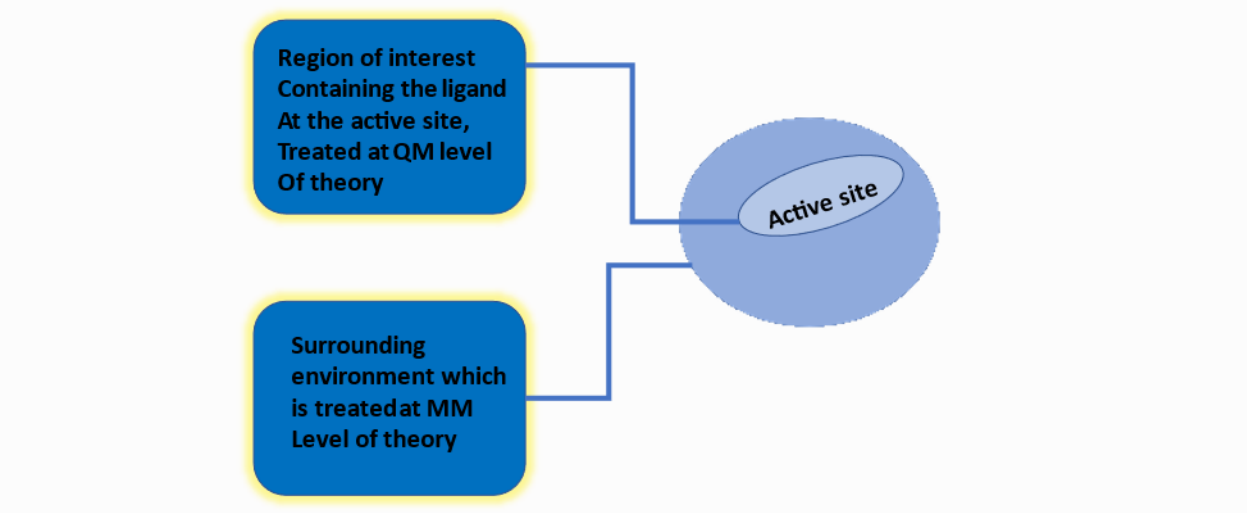

Figure 3. Illustrating the partitioning of the entire system where the entire system is a summation active site and the surrounding environment.

The combined or aggregate energy of the system can be expressed below in (equation 2)

$E(QM/MM) = E(QM) + E(MM) + E_{int}$ (QM – MM) .....equation (2)

Where E(QM) is the energy of the QM region, E(MM) energy of the MM region, and E_{int} (QM – MM) is the interaction energy that exists between the QM and MM (31, 32). The computation of E_{int} (QM – MM) is not a straightforward analysis as it involves calculations of electrostatic and bonding interactions and the nonbonding van der Waals interactions. A pseudo atomic system, localized orbital, and link atoms are used in the saturation of the swinging covalent bonds located at the boundary of the QM/MM region (33-36). The Van der Waals interactions are generally treated at the MM region, while electrostatic interactions are treated relative to the chosen combined QM/MM computational method (37). The classification of the

model to two or three systems, the QM and MM Hamiltonian resulted to subtractive and additive parts (38). Bakowie and colleague Thiel, further divided the QM/MM system interactions into electrostatic embedding and mechanical embedding (39). Electrostatic embedding incorporates mechanical embedding point charges into the operator of the Hamiltonian quantum mechanics and computes the electrostatic interactions existing between quantum mechanics (QM) and molecular mechanics (MM) at the quantum mechanics region (section). This incorporation, therefore, results in polarization of the QM section by the MM section, while on the contrary, there is no polarization of the molecular mechanic's section by the quantum mechanics section. In the Mechanical embedding system, the energy of the quantum mechanics is calculated at the gas state while using the Coulombic law and the atomic charges of the combination (QM/MM) to calculate the electrostatic interactions between quantum mechanics and molecular mechanics at the molecular mechanics' region. Although, computationally, the electrostatic interactions calculated using electrostatic embedding show better and correct results than those done with mechanical embedding and are more preferable (40-42).

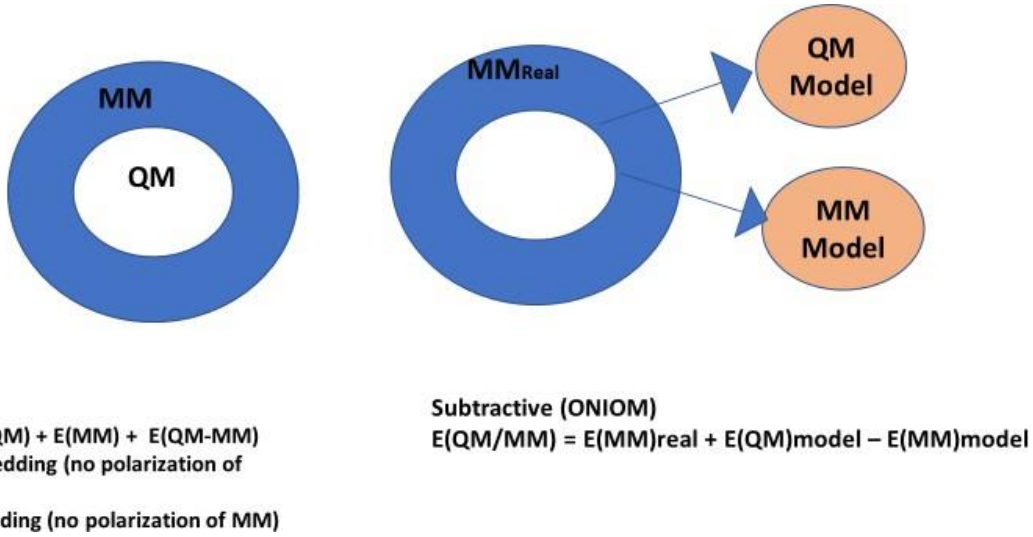

Figure 4. Diagrammatic representation of coupling method, mechanical and electronic embedding

ONIOM METHOD

Morokuma and colleagues originated Our own N-layered Integrated molecular Orbital and molecular Mechanics ONIOM, initially used in Gaussian 98 to handle larger biochemical molecules. However, it has a broader application in calculating electromagnetic and photochemical parameters, frequency of vibration, energy, and optimizing the conformation of structures and organometallic entities while using Gaussian 16 (43). It separates large molecules to be computed into two or three portions based on the desired level of precision (accuracy), thereby giving rise to 2-layered and 3-layered ONIOM. In the 2-layered method, the

smaller molecule of interest like the ligand in the active site is placed in the higher layer (where bond formation and breaking occurs) and computed with a more accurate level of theory. The remaining entire system is treated at the low layer (represents the whole environment in contact with the ligand or active system of interest) and computed at a less costly level (44). Other forms of ONIOM exist based on the level of model chemistry preferred, such as quantum mechanics/molecular mechanics (QM/MM), molecular orbital/molecular orbital (MO/MO), which involves the combination of two ab initio and molecular mechanics/molecular orbital (MO/MM). The combinations are interpreted based on where the small active molecule of interest is treated, such as QM/MM at QM, MO/MO at MO, and MO/MM at MO models of chemistry (44).

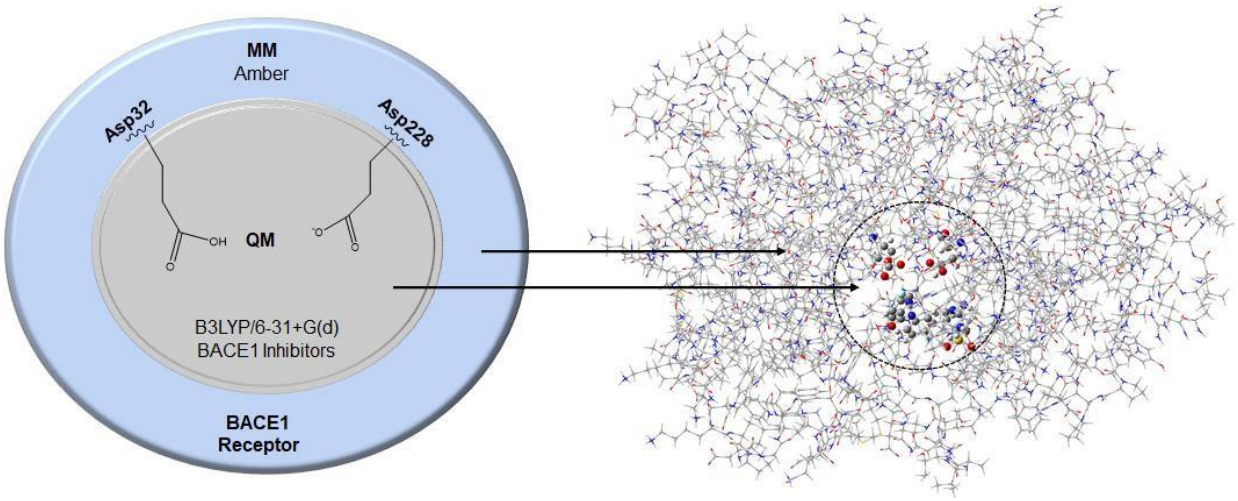

Figure 5. 2-layered ONIOM model QM (B3LYP/6-31+G(d): MM(Amber) of BACE1-Inhibitor complex.

A 2-layered ONIOM energy equation is represented below where R is the real system, which is made up of the entire atoms, computed at the molecular mechanics' region. SM is a small model system otherwise known as a model system and often computed on the high layer/expensive region (45).

$$E^{ONIOM}=E^{low}_{(R)}+E^{high}_{(SM)}-E^{low}_{(SM)} \quad \text{equation(3)}$$

$$E^{ONIOM} = \text{Amber} + \text{DFT} - \text{Amber} \quad \text{equation (4)}$$

DRUG DISCOVERY THROUGH VIRTUAL SCREENING AND HIGH THROUGHPUT SCREENING

The adoption of high throughput screening HTS, (filtering, identification, and selection of new lead drug candidates from multiple libraries of chemical/biochemical compound libraries) by the pharmaceutical companies paved the way for the emergence of computational structure-based drug design methods Fig. 6.

(46). Employing a combination of computational modeling and 3D structure-based drug design, the intricate atomistic/molecular interactions of enzyme-substrate binding activities from experiments (experimental results) are comprehensively investigated and interpreted (47). Virtual screening involves commercial deposition (libraries) of large quantities of molecules that have been filtered/selected (screened) theoretically using targeted investigated well-binding structures and testing them experimentally (48). It involves analysing the targeted 3D structures of the molecules that were experimentally generated through either nuclear magnetic resonance or x-ray crystallography. When no available generated crystal structures existed, homology modeling and subsequent molecular dynamic simulations are employed (47). Structurally, assigning the right tautomer, stereoisomers, and actual protonation states of the molecules is very imperative. Therefore, virtual docking of the small molecule into the receptor's binding site is essentially done with the selected program. Docking aims to predict the substrate-enzyme (ligand-protein) complex conformation by trying out the different structural orientations of the ligand in the enzyme's binding site (protein). This docking is subsequently followed by scoring the different pose (snapshot) and using it to determine the binding free energy of the complexes (46, 49).

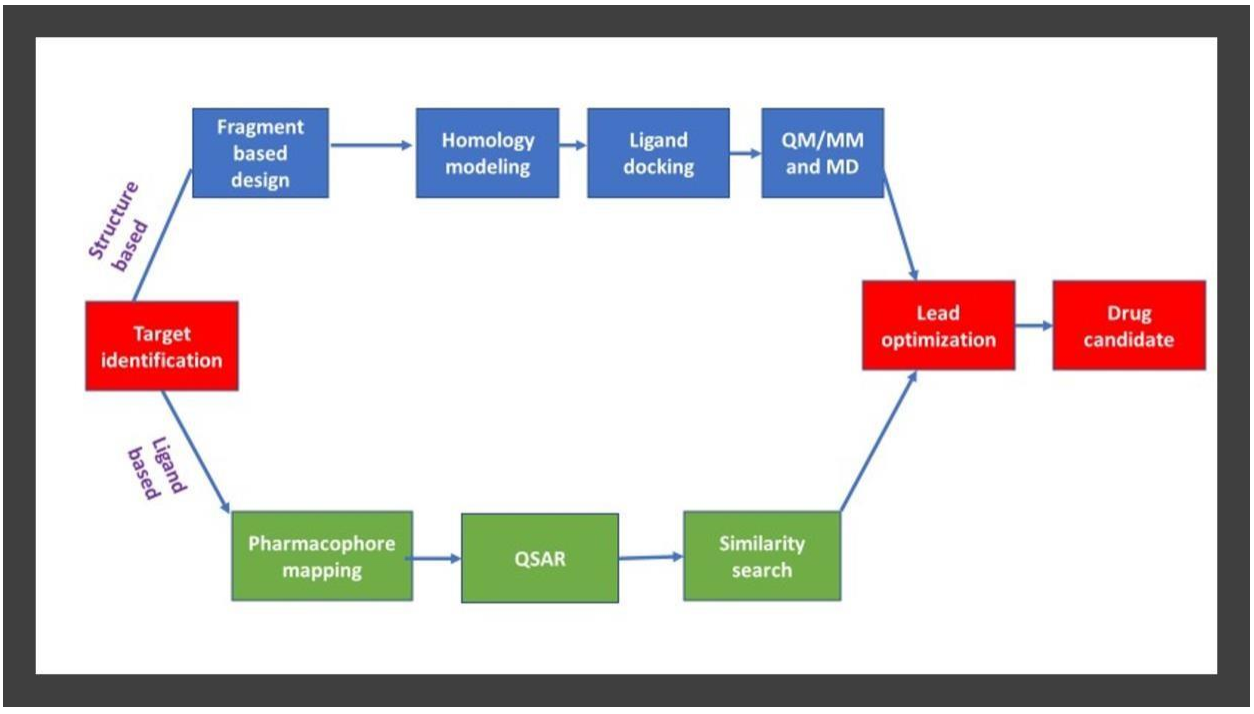

Figure 6. Diagrammatic representation of drug discovery processes

MOLECULAR DYNAMIC SIMULATIONS AND ACCELERATED MOLECULAR DYNAMIC SIMULATIONS

Molecular dynamics simulation is another technique in computational chemistry applications which analyses the movements of different atomic and molecular interactions of a given system at each time (50). It considers the molecules of interest to consist of multiple atoms which are linked with covalent bonding, where the molecules are taken as particles while the bonds, as springs. The atomic location (position) is often computed on time-based with Newton's second law of motion, as seen below (51, 52).

$F = Ma = mdv/dt = md^2x/dt$ ..... equation (5)

(where F is the force, M mass, v velocity, and t is time) (52).

For a productive MD, the simulations run properties such as time, boundary condition, solvation models, and bond/nonbonding are prerequisites. Furthermore, while the simulation is running, the pressure and temperatures must be constantly and adequately controlled. GROMACS, Amber, and CHARMM are the most used force field in recent MD simulations, while NPT, NVE, and NVT are the commonly used ensembles (51, 53, 54). When using assisted model building and energy refinement AMBER to run molecular dynamic simulations, molecular force fields are usually used to explain the interactions of every molecule under investigation (55). Sander is regarded as the main driving force for AMBER, while pmemd is the advanced form of the molecular dynamics driving force used on the graphic processing unit. Three essential files generated for running the molecular dynamics simulations are parm7 (for topology and parameters of molecules in a system), rst7 (for initial molecular coordinates), and mdin (explains the settings for molecular dynamics driving force) (56).

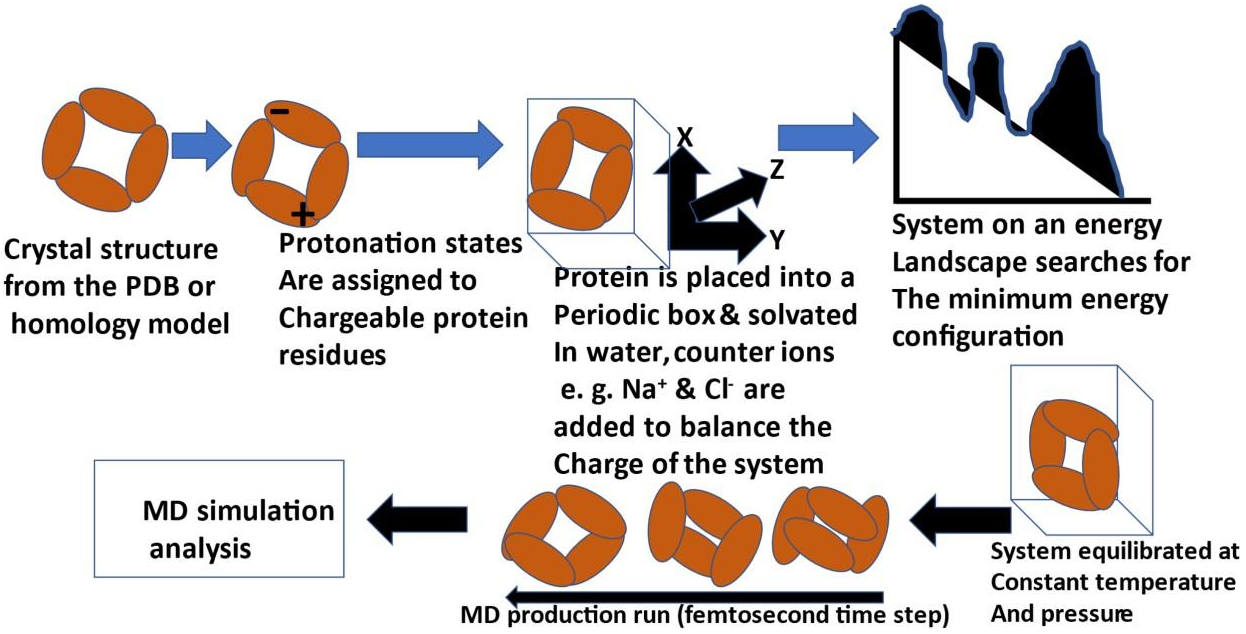

Figure 7. Schematic representation of the MD process image redrawn from an open-access source (57)

The three major phases of MD simulations are initialization, equilibration, and production (58). MD trajectory files are generated during MD production, which contains information on energy properties. Coordinates and topology files are generated as input files before the significant phases of MD simulation. The following properties (parameters) are analyzed from the MD output files; root means square fluctuation, root means square deviation, the radius of gyration, principal component analysis, secondary structures analysis, hydrogen bond, and free energy surface analyses (59, 60). The limited-time employed in conventional molecular dynamic simulations prevents adequate exploration of the energy landscape; hence, accelerated molecular dynamics simulations aMD recently overcame this obstacle. An improved (advanced) simulation method accelerates movements between diverse energy levels and makes available sufficient pathways to activate uncommon reactions that ensure enzyme structural changes (61, 62). Computing several nanosecond aMD ensures enough expression of the micro to split second interval activities in molecular dynamics, which are unbiased, and this ensures enough time to analyze the various ligand configurations and orientations at the enzyme's active site (63, 64).

SWISSDOCK WEB SERVER

This web server-based application differs from the other conventional docking software by automatically performing both the input and output tasks internally (65). The Swissdock (software

EADock GSS) carries out the automatic preparation of the ligand and enzyme structures before using the web-based server to dock them (66, 67). In Swissdock, the docking process is carried out via CHARMM 22/27 all-hydrogen force field; therefore, ligands and the protein files (in Mol2 and PDB) were automatically formatted in CHARMM format as soon as they were fed into the system (68). The automatic-based computation in Swissdock lowers human mistakes by applying the web interface in generating alternative input files and alternative parameters while interpreting the docking outcome (69). The application of the Swissdock for both docking and simulations, blind docking is usually done automatically, generating different binding modes or poses at the protein's binding site. The most favorable binding energies (CHARMM energies) calculation and ranking were concurrently carried out via the FACTS implicit solvation system (70, 71).

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INTERLINKING PAGE FOUR

This chapter focuses on applying the quantum mechanics of DFT and hybrid QM/MM ONIOM methods to investigate the chemical properties of AM-6494 (a novel drug) which showed high selectivity for BACE1 relative to CNP-520 (another BACE1 inhibitor). It further explores the use of molecular electrostatic potential (MESP) in the analysis of the effect of atomic charge distribution and natural bond orbital (NBO) on the studied BACE1 inhibitors. Further detailed interatomic investigation of the chemical properties of these two selected BACE1 inhibitors, were discussed in the next chapter (chapter six). Chapter six involves the application of the molecular mechanics method of accelerated and conventional molecular dynamic simulations in further probing into the properties of AM-6494 and CNP-520.

CHAPTER FIVE

MANUSCRIPT THREE

COMPUTATIONAL MODELLING OF POTENT β-SECRETASE (BACE1) INHIBITORS TOWARDS ALZHEIMER'S DISEASE TREATMENT

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Computational modelling of potent β-secretase (BACE1) inhibitors towards Alzheimer's disease treatment

Samuel C. Ugbaja [a], Zainab K. Sanusi [a], Patrick Appiah-Kubi [b], Monsurat M. Lawal [a,*], Hezekiel M. Kumalo [a,*]

[a] Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Durban 4001, South Africa
[b] Molecular Bio computational and Drug Design Research Group, School of Health Sciences, University of KwaZulu-Natal, Westville Campus, Durban 4001, South Africa

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ABSTRACT

Researchers have identified the β-amyloid precursor protein cleaving enzyme 1 (BACE1) in the multifactorial pathway of Alzheimer's disease (AD) as a drug target. The design and development of molecules to inhibit BACE1 as a potential cure for AD thus remained significant. Herein, we simulated two potent BACE1 inhibitors (AM-6494 and CNP-520) to understand their binding affinity at the atomistic level. AM-6494 is a newly reported potent BACE1 inhibitor with an IC_{50} value of 0.4 nM *in vivo* and now picked for preclinical considerations. Umibecestat (CNP-520), which was discontinued at human trials lately, was considered to enable a reasonable evaluation of our results. Using density functional theory (DFT) and Our Own N-layered Integrated molecular Orbital and Molecular Mechanics (ONIOM), we achieved the aim of this investigation. These computational approaches enabled the prediction of the electronic properties of AM-6494 and CNP-520 plus their binding energies when complexed with BACE1. For AM-6494 and CNP-520 interaction with protonated BACE1, the ONIOM calculation gave binding free energy of −62.849 and −33.463 kcal/mol, respectively. In the unprotonated model, we observed binding free energy of −59.758 kcal/mol in AM-6494. Taken together thermochemistry of the process and molecular interaction plot, AM-6494 is more favourable than CNP-520 towards the inhibition of BACE1. The protonated model gave slightly better binding energy than the unprotonated form. However, both models could sufficiently describe ligand binding to BACE1 at the atomistic level. Understanding the detailed molecular interaction of these inhibitors could serve as a basis for pharmacophore exploration towards improved inhibitor design.

1. Introduction

Alzheimer's disease (AD), which was discovered over a hundred years ago by Dr. Alois Alzheimer, a renowned Deutsch neurologist, pathologist, and psychiatrist, has been defined as a progressive neurodegenerative brain disorder [1,2]. A prevalent symptom of AD is dementia, and the Alzheimer's disease facts and figures reported in 2019 shows that approximately 5.8 million Americans have Alzheimer's dementia and predicted to increase to over 13 million by 2050 [3]. There is a record of high cases of AD with older people above 65 years in both Western Europe and America [4,5]. So far, there is no complete cure for this disease except for the development of a few treatment strategies [6–9].

AD has posed significant economic stress globally, accounting for approximately 600 billion dollars per year to care for over 35 million patients with dementia [10]. That is reportedly 1% of the global Gross Domestic Product (GDP) [11], hence, affecting the present expenditure and will potentially weigh down the future expenses. Therefore, investigations into the design and development of strategies to move beyond management to cure AD remained essential. Over the years, researchers have made efforts towards designing small molecules targeting specific pathway(s) within the multifactorial pathological network connected to AD emergence [12–14].

For instance, studies have shown that the β-amyloid (Aβ) precursor protein cleaving enzyme 1 (BACE1) or β-secretase in the pathogenesis of AD (Fig. 1) is a lucrative target for inhibitor design [14].

There are two steps involved in the cleaving of the amyloid precursor protein (APP) to produce Aβ. The first is cleaving by β-secretase resulting at the N-terminus to produce soluble β-amyloid precursor protein (sAPPβ) and C99 amino acid fragments (C99), subsequent cleaving at C-

* Corresponding authors.
E-mail addresses: lawalmonsurat635@gmail.com (M.M. Lawal), kumaloH@ukzn.ac.za (H.M. Kumalo).

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terminal by γ-secretase produce various lengths of peptides (from 38 to 43 amino acids) [15,16]. The amino acids produced by β-secretase are predominantly made up of Aβ-40 while those obtained through γ-secretase form about five to 10% Aβ-42 peptides. Despite the unpopular concentration of Aβ-42 amino acids, they have a higher tendency for the accumulation of amyloid plaques in Alzheimer's brain than Aβ-40 peptides [17,18].

There had been several therapeutic studies focused on designing drugs targeted at γ-secretase inhibition that led to the discovery of the first potent γ-secretase inhibitors (Semagacestat) that made it to phase three clinical trials [19]. However, this trial was terminated in April 2011 for lack of potency and accelerated cancerous (skin cancer) risk. Furthermore, similar and worsening side effects observed on other clinical trials targeted at γ-secretase showed off-target-effects related to notch receptors. Moreover, the elevation in the amyloid precursor protein γ-secretase substrates, which resulted in worsening of cognitive ability and other pharmacological and health hazards effects led to the shift from γ-secretase inhibitors [17,19].

Sequel to the above, BACE1 was given attention in the reduction of β-amyloids geared towards Alzheimer's disease treatment. In recent years, there had been increasing efforts in the design of β-secretase inhibitors with high selectivity and less off-target effects. Leveraging on the success in designing other aspartyl protease inhibitors such as HIV proteases, BACE1 inhibitors design become easier than γ-secretase [20]. Also, there had been tremendous advancement in the discovery of β-secretase inhibitors aimed at AD treatment at various clinical trials by the pharmaceutical companies when compared to γ-secretase [15].

Generally, extracellular Aβ plaques and neurofibrillary tangles (as supported by the amyloid cascade hypothesis) are reportedly the two main biological features of Alzheimer's disease [22,23]. However, similar (amyloid cascade) toxic contributions from soluble β-amyloid oligomers have been reported [24]. Oligomers also play an important role in diseases related to amyloid fibril production as well as reactions linked to protein accumulation [25,26].

There have been many concerns on the underlying *in vivo* mechanisms involved in the amyloid accumulation. Recent research by Sciacca et al. [27] unravelled the connection between IDPs (intrinsically disordered proteins) and amyloid aggregation processes. They also revealed that naturally disordered protein oligomers, which cause damaging of the membrane, are linked to β-amyloid aggregation, which results in AD [27–29]. However, after investigations [27,28,30,31] on the significant roles free lipids play in forming lipid-enzyme complexes, researchers proposed the consideration of lipid-protein chaperone [27,31,32] as a linking model for enzyme-membrane poration.

The accumulation of Aβ peptide stimulates physiological changes in the brain which later results in cognitive dysfunctions based on the amyloid hypothesis [33–35]. BACE1 is responsible for the formation and release of Aβ peptide in the brain [36], thereby serving as a pathological hallmark of AD [37–39]. Meanwhile, neuronal cell loss arises from the accumulation of insoluble and neurotoxic Aβ-42 as senile plaque, which is instigated by the sequential splitting of APP by BACE1 [40,41]. The β-secretase is an aspartate protease (Asp-PR) that has an N-terminal domain, a transmembrane region, cytosolic domain, as well as strand residues [42]. The active site of β-secretase consists of different potential groups, including the catalytic dyad, flap region, and 10s loop [43,44] as illustrated in Fig. 2.

The design and development of directed ligands to inhibit BACE1 as a potential cure for AD are quite attractive – its relevance is well studied and documented in the literature [15,21,45]. Although there is no approved inhibitor(s) targeting BACE1 towards AD treatment yet, it is, however, hoped that the relentless efforts of researchers in this direction would be fruitful sooner than later. It is imperative to mention that the presence of BACE1 at the blood-brain barrier (BBB) *in vivo* and *in vitro* in mice, bovine, and human brain endothelium and its regulation in an AD mouse model has been investigated and reported in the literature [12,14,46]. Due to the peculiarity of BACE1 at the BBB, inhibitors/drugs targeted at BACE1 are supposed to permeate the BBB sufficiently. Authors proposed [47] that many BACE1 inhibitors under development exhibit low BBB permeability or were quickly re-transported to the bloodstream by P-glycoprotein (P-gp) mainly because the wide-open BACE1 active site evolved to bind polypeptide substrates. Peptidomimetic BACE1 inhibitors do not possess optimum drug-like properties *in*

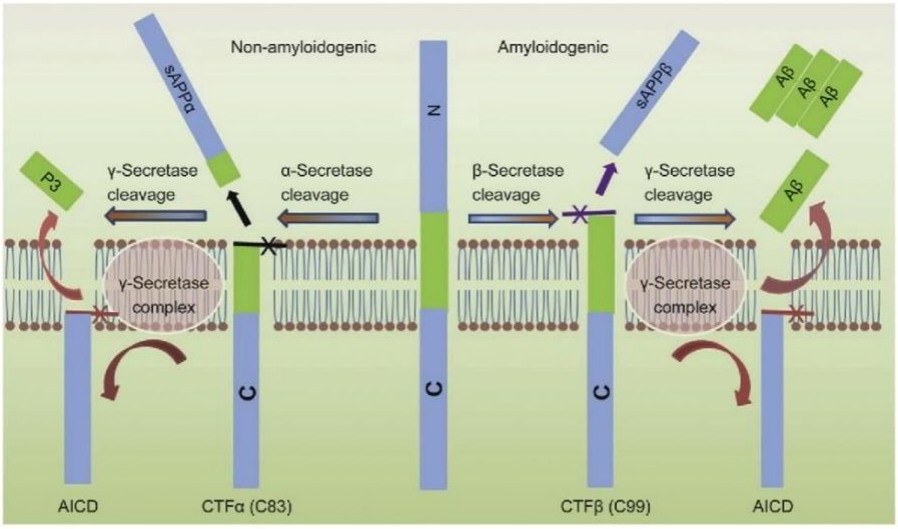

Fig. 1. Schematic structure depicting Aβ as the main aggregated component of amyloid plaques in the brain. The amyloidogenic pathway consists of the sequential cleaving of amyloid precursor protein (APP) by β-secretase, which releases the soluble ectodomain sAPPβ. The C99 fragment of APP is then cleaved by γ-secretase, resulting in the formation of the Aβ peptide. The Aβ peptide has a high tendency to accumulate, oligomerise, aggregate, and forms amyloid senile plaques, resulting in the substantiated alterations in Alzheimer's disease [21].

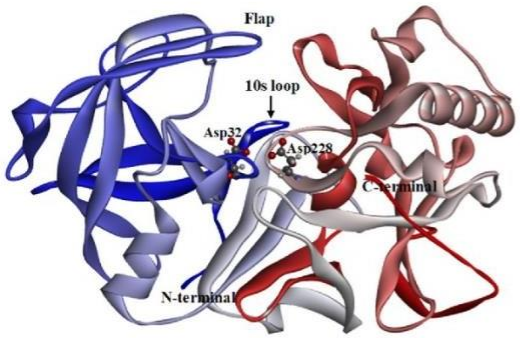

Fig. 2. 3D structure of β-secretase showing some essential domains within the enzyme.

vivo, such as oral bioavailability, long serum half-life, or BBB penetration. These have also made it uneasy to develop non-peptidic BACE1 inhibitors that are large enough to make sufficient contacts and bind with high affinity to the active site. Such inhibitors should also be appreciably lipophilic to cross the plasma and endosomal membranes [48]. These factors enable the inhibitors to reach the luminal BACE1 active site.

Recently, Pettus et al. [49] identified a potent BACE1 inhibitor, the compound was labelled AM-6494 (Fig. 3) and observed to be highly selective for BACE1. The discovery led to the selection of AM-6494 for preclinical considerations after showing appreciable *in vivo* inhibition with an IC_{50} value of 0.4 nM [49]. Among some earlier identified BACE1 inhibitors that attained phase II/III clinical trials are verubecestat (MK-8931) [50] and its analogue umibecestat (CNP-520) [51]. These two inhibitors were, however, discontinued lately in February 2018 [52] and July 2019 [53], respectively, as participants displayed worsened cognitive functions.

Despite the vast number of available research contributions on BACE1 inhibitors development, there exists a gap in terms of atomistic modelling of the potentially active compounds at the theoretical level. More interestingly, the inhibitory affinity of AM-6494 is not yet studied

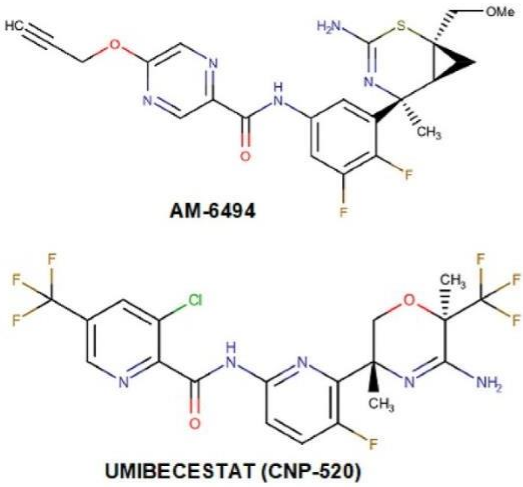

Fig. 3. 2D structures of the selected BACE1 inhibitors.

using a computational approach. The application of theoretical and computational methods in providing more insight into the interaction, chemical properties, and energetics of molecular systems remained viable [54–59]. Quantum mechanics (QM) approach including density functional theory (DFT) [60] has found usage in predicting the electronic properties of potent molecules targeting some enzymes [55,56,58,59]. Also, hybrid quantum mechanics/molecular mechanics (QM/MM) methods such as Our Own N-layered Integrated molecular Orbital and Molecular Mechanics (ONIOM) [61–64] has been used successfully for binding energy calculation [65,66]. Although the ONIOM approach has a simple calculation setup, one of its limitations lies in the number of input parameters and errors which makes achieving convergence more difficult and time-consuming.

This present research aimed at using a DFT method to explore the quantum chemical properties of AM-6494 in comparison to CNP-520, which have high selectivity for BACE1 (Fig. 3). Conceptual parameters evaluated within the DFT framework for these potent BACE1 inhibitors include local reactivity indices, charge distribution, and electrostatic potential (ESP) analyses. To provide a better insight into the binding and selectivity of these potent BACE1 inhibitors, hybrid QM/MM ONIOM approach was used to study each ligand interaction with BACE1. ONIOM hybrid approach involves treatment of an enzyme active region and ligand with a high level of theory, and the remaining residues with lower-level AMBER MM force field [67]. Like closely related investigations [65,66] involving HIV-1 protease, we compared the calculated binding free energies with the IC_{50} values of these ligands from literature. Results from the QM and QM/MM presented in this study showed that AM-6494 and CNP-520 are highly selective towards BACE1 with the former been slightly favourable than the latter candidate.

To broaden the scope of the present investigation, we proposed possible modification to the potent inhibitor (AM-6494) that could enhance its binding to the BACE1 enzyme. Based on our knowledge (ongoing research) of substrate-based modification *via* electronic calculation, we proposed selective *N*-methylation. Studies [68–70] have that *N*-methylation of amide nitrogen atoms increases the proteolytic stability/bioavailability of peptides, this might apply to these analogous inhibitors (Fig. 3) with amide bond. Some fragments (such as —SCH_3) are electron-donating and noted to strengthen the amide bond through a positive inductive effect, thereby increasing the stability of the substrate. This approach was therefore applied on AM-6494 to test this hypothesis. Note that detailed information on this procedure done using QM calculations of parameters such as force constant acting on the scissile bonds of the different structures is available in sequel research from our group.

2. Computational protocols

2.1. DFT calculations

The study initiates with DFT calculation of AM-6494 and CNP-520 to give a clearer picture of their potential quantum mechanics properties. Full geometry optimization of AM-6494 and CNP-520 potent BACE1 inhibitors were executed within the Gaussian 16 Rev. B01 program package [71] at B3LYP [72,73] DFT level and 6–31 + G(d) [74] basis set. The 3D structures of AM-6494 and CNP-520 with ID 6PZ4 [49] and 6EQM [51], respectively, were downloaded from the Protein Data Bank (PDB) [75]. Calculations set up for the respective conceptual parameters and post ligand properties analyses were evaluated in GaussView 6.0.16 [76].

2.1.1. Structural analysis and energetics

The selected BACE1 inhibitors were modelled with GaussView 6.0.16 [76] and geometries fully optimised at B3LYP/6–31 + G(d) level of theory in gas and implicit solvent. Also calculated, is the vibrational frequency to ensure that the structures are stable minima showing no negative Eigenvalue. Solvation free energy (ΔG_{solv}) was estimated for

the two ligands *via* the solvation model based on solute electron density (SMD) [77] approach using water implicitly. We estimate ΔG_{solv} from Eq. (1), where, SCF_{gas} is the total energy and $SCRF_{SMD}$ is the single point energy obtained from SMD calculation.

$$\Delta G_{solv} = SCRF_{SMD} - SCF_{gas} \quad (1)$$

Note that calculation in Gaussian software uses implicit solvent and not explicit, however, molecules could be modelled with solvent explicitly *via* some other modelling suites and exported to be calculated in Gaussian but using vacuum phase protocol.

2.1.2. Charge distribution and molecular electrostatic potential (MESP)

We did population analysis to study the effect of atomic charge distribution within each ligand through the natural bond orbital (NBO) analysis. Using the CHELPG [78] model, we plotted the calculated MESP surface in GaussView. Local reactivity of molecules can be estimated using the Fukui functions f_k^+, f_k, whereby atoms with nucleophilic and electrophilic properties could be predicted [79,80]. f_k^+ and f_k can be calculated using the finite difference approximation stated in Eqs. (2) and (3), respectively. N is the number of electrons in the original molecule with default charge (0), N + 1 corresponds to the anionic state, N–1 is the cation form, and the q_k is the charge on the atom *k* for the N total electrons. We execute this calculation using the Hirshfeld [81–83] population keyword for the potent molecules at neutral, −1, and +1 charged states. Their derived differences using Eq. (4) showed the potential behaviour of each atom.

$$f_k^+ = q_{k(N+1)} - q_{k(N)} \quad (2)$$

$$f_k^- = q_{k(N)} - q_{k(N-1)} \quad (3)$$

$$\Delta f = f_k^+ - f_k^- \quad (4)$$

2.2. ONIOM calculations

We used the retrieved BACE1 complexes of AM-6494 and CNP-520 with codes 6PZ4 [49] and 6EQM [51], respectively, for the ONIOM calculation. Modelling of the missing protein residues was done on MODELLER 9.19 [84] integrated within the Chimera software. Using the hybrid QM:MM ONIOM method, we analysed the binding affinity of these potent BACE1 inhibitors. Further analyses include the estimation of thermodynamic parameters, electrostatic, and hydrogen-bonding interactions of the selected inhibitor-enzyme complexes.

2.2.1. Thermochemistry study

Using a two-layered ONIOM (ONIOM2) approach, we calculate the binding free energies as well as other thermodynamic parameters of the selected BACE1—inhibitor complexes. Previous literature [65,66] has shown that the Becke3LYP algorithm is sufficient and gave relative energies that are in good trend with experimental data. Hence, we optimised the geometries of the ligands, enzyme, and ligand—enzyme complexes using ONIOM (B3LYP/6–31+G(d):AMBER) for the QM:MM level of theory. A schematic illustration of this ONIOM2 model is given in Fig. 4, whereby Asp32 is mono-protonated. Note that investigations [85–91] have shown that there are no hard and fast rules for protonation of the catalytic dyad. Herein, we chose both mono-protonated and unprotonated BACE1 models to provide better insight into the binding process of AM-6494 and CNP-520 and to give a detailed comparison. Based on charge distribution analysis of the enzyme with the H++ server [92], the low layer has a total charge of −10. The high layer is −1 for the protonated model and −2 for the unprotonated analogue.

The total in interaction energy, which represents the difference in the total energy components existing between the BACE1 and inhibitor, attained from the ONIOM2 calculation, is defined in Eq. (5). ΔE_{model} is the energies of the model system calculated at the high (QM) and low level (MM), respectively, ΔE_{real} represents the energy of the entire (real) complex at the MM level.

$$\Delta E_{ONIOM2} = \Delta E_{model,high} + \Delta E_{real,low} - \Delta E_{model,low} \quad (5)$$

We derived the standard Gibbs free energies (ΔG) of all the complexes from the frequency calculations at ONIOM (B3LYP/6–31 + G(d): AMBER) QM:MM level of theory. The Gaussian thermochemistry [93] algorithm implemented to estimate the Gibbs free energy incorporates both enthalpy and entropy functions (ΔG = ΔH − TΔS) at 298.15 K temperature. Thus, the corresponding ONIOM binding free energy value of each system calculated using Eq. (6) shows the contribution of each entity to the free energy change (ΔG_{bind}).

$$\Delta G_{ONIOM} \approx \Delta G_{bind} = G_{complex} - G_{protein} - G_{ligand} \quad (6)$$

Also, from the frequency calculation, we obtained the thermodynamics quantities (enthalpy and entropy) changes from the ONIOM calculations similar to the ΔG estimation. All reported energies and thermochemistry parameters are in approximately three decimal places after conversion from Hartree (default Gaussian output) to kcal/mol.

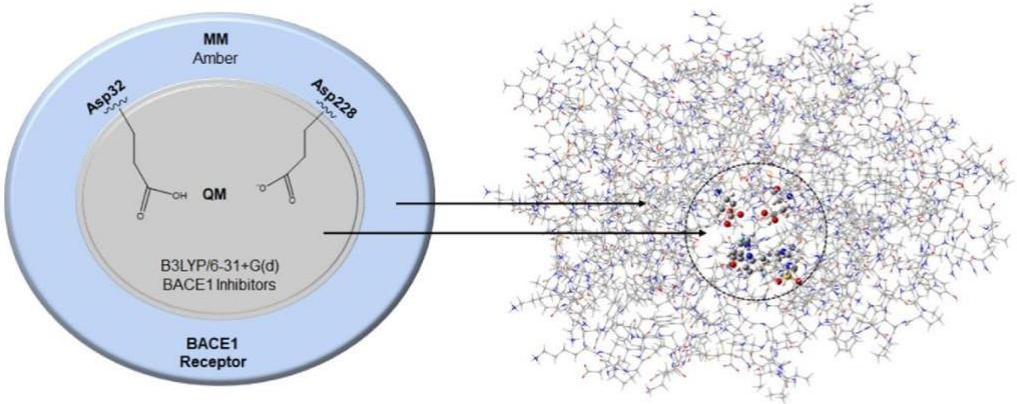

Fig. 4. Schematic representation of the two-layered ONIOM (B3LYP/6-31+G(d):AMBER) model for the BACE1—inhibitor complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Geometry optimisation

Complete geometry optimisation of the potentially active BACE1 inhibitors was achieved at B3LYP/6–31 + G(d) DFT level of theory in both vacuum and solvent phases to indicate structural stability. Frequency calculation also revealed no negative Eigenvalue (Fig. 5A) at both media for the optimised potent inhibitors to ascertain they are minima structures. There exists a remarkable effect in predicting the energy change from gas to the solvent phase in which the bulk solvent energy (ΔGsolv) contributions are −19.413 and −13.905 kcal/mol for AM-6494 and CNP-520, respectively. Also simulated to stable local minima are the BACE1—inhibitor complexes and the separated enzyme, and frequency calculation revealed no imaginary eigenstate values. Fig. 5B shows the eigenvalues (in cm^{-1}) plot associated with the first 100 modes for both protonated and unprotonated models. Note that the total atoms for the QM/MM calculation of each complex are >6000, and more than 18,000 modes with the respective eigenvalues showed no negative eigenvalues.

3.2. Charge distribution on AM-6494 and CNP-520 and their electrostatic potentials

Charges arising from the natural bond orbital (NBO) population approach are known as the natural atomic charges (NAC). Charge distribution on ligands plays a crucial role in perturbing electrostatic potentials [94,95]. Presented in Fig. 6 is the summary of charges across the atoms within the selected potent BACE1 inhibitors. The red colour indicates a negative atomic charge, black reflects neutral charge distribution, and the green colour indicates a positive charge on the atom. Analysis of the charge distribution on AM-6494 atoms shows large negative values compared to the positive pole in which the selected potentially active ligand gave up to −0.865 esu (esu). In contrast, CNP-520 produced the highest positive charge value of 1.094 esu in one of its atoms.

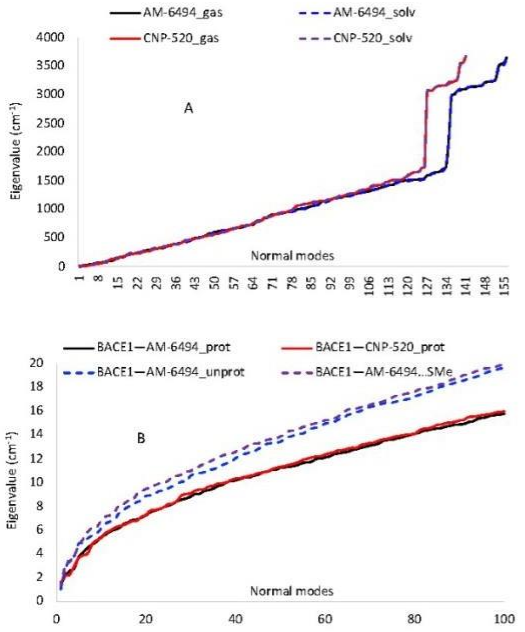

Fig. 5. Eigenvalues distribution for the fully optimised (A) potent BACE1 inhibitors in gas (B3LYP/6-31+G(d)) and solvent (SMD/B3LYP/6-31+G(d)) phases, and (B) BACE1—inhibitor complexes involving protonate`d and unprotonated analogues of the β-secretase at B3LYP/6-31+G(d):AMBER level of theory. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Plots from MESP surface analyses have been associated with binding sites prediction [55,96–99], the region of negative potential (red colour) denotes the site for possible proton attraction or nucleophilic attack. Areas of positive potential (blue colour) are indications of prospective electrophilic addition at such regions. The MESP surface provided in Fig. 6 reflects the availability of the ligands for binding – they have an even distribution of all potentials. The oxygen atoms are rich regions for possible hydrogen bond formation with BACE1 active residues while most of the nitrogen atoms are available for electrophilic attack. The alkyne group of AM-6495 also has positive potential with scantier yellow colour distribution as an indication of a weak nucleophilic region. Population analysis and electrostatic potential study of these molecules show their availability for possible interaction with the selected enzyme (BACE1).

Electrophilic and nucleophilic regions within a molecule can be predicted in quantitative terms using the Fukui indices approximations. Fukui functions or indices are used to estimate the atoms in a molecule that tend to either lose or accept an electron [100]. Sites with the highest Fukui indices value in a given molecule have been noted to have high selectivity for the corresponding attacks [79]. Provided in Table 1 is the estimated Fukui indices calculated at B3LYP/6-31+G(d) level of theory for the studied inhibitors with their highest values in bold print. Electrophilic addition (E+) sites are widely distributed compared to the nucleophilic regions (Nu–) in both AM-6494 and CNP-520 and this could be related to the MESP prediction (Fig. 6).

For the prospective E+ sites in AM-6494, C20 (Fig. 6) has the highest value of −0.064 to denote its availability for the highest electrophilic addition (Table 1). Atom N27 of AM-6494 has the largest nucleophilic site, and C12 is the most nucleophilic region in CNP-520 (Table 1). These two inhibitors are predictably active with both nucleophilic and electrophilic distributions across their atoms. To further analyse the selectivity and binding of these molecules, we carried out a two-layered ONIOM2 [61,101] calculation to examine the binding landscape of AM-6494 and CNP-520 as potent inhibitors of BACE1 towards Alzheimer's treatment as predicted from experiments [49,51].

3.3. ONIOM study

Binding free energy (ΔG_{bind}) calculations were performed on the inhibitor—enzyme complexes using the ONIOM computational model. After a complete optimisation at the selected ONIOM2 (B3LYP/6-31+G(d):AMBER) level of theory, a visual inspection using GaussView showed that the drugs remain inside the active pocket of the BACE1 enzyme. Table 2 showed the ONIOM ΔE and other thermochemistry components, and we compared the ΔG_{bind} values with the available experimental IC_{50} [49,51] data of the considered inhibitor—enzyme complexes. The lower the IC_{50}, the better the inhibition performance while highly negative theoretical binding energy denotes a favourable process. Ligand interaction network shows the nature of the interactions between these selected inhibitors and BACE1 active residues. All possible non-bonded interactions were measured at 3.0 Å neighbour distance from the inhibitor using the Accelrys Discovery Studio [102].

3.3.1. The interactions and energetics of AM-6494 and CNP-520 binding with BACE1

Analysis of the inhibitor—enzyme contact map after a complete optimisation showed that AM-6494 actively interacted with BACE1 through residues Lys9, Gly11, Gly13, Tyr14, Leu30, Gly34, Tyr71, Thr72, Phe108, Trp115, Ile118, Val170, Gly230, Thr231, Thr232,

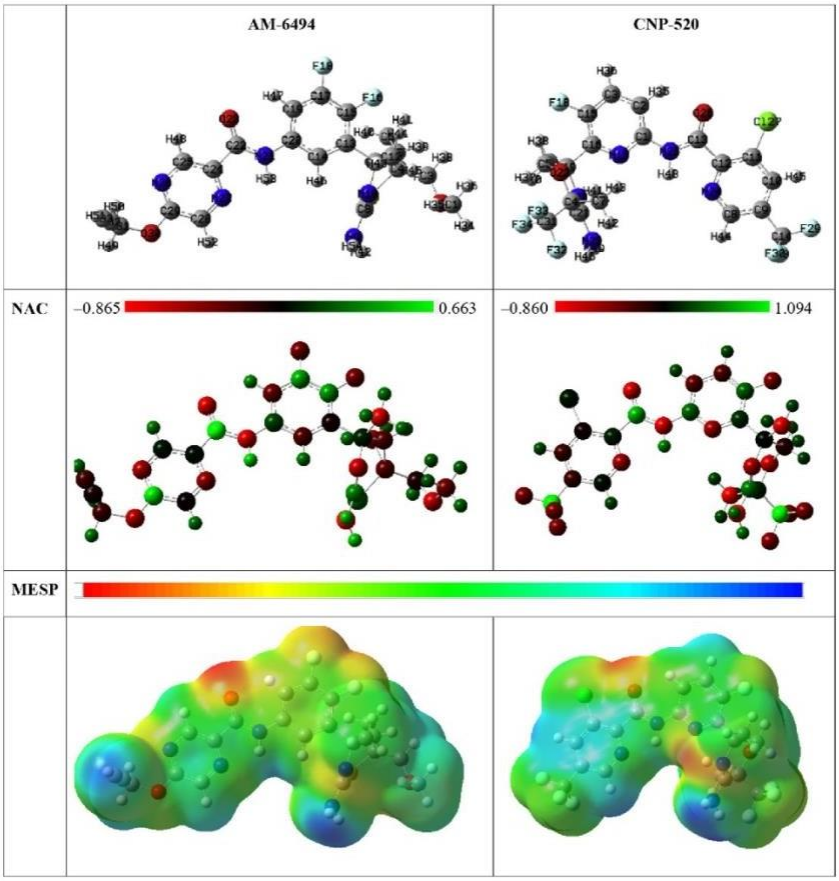

Fig. 6. Natural atomic charge (NAC) distribution and molecular electrostatic potential (MESP) plots for AM-6494 and CNP-520 at B3LYP/6-31+G(d) DFT level.

Arg235, Arg307, Ala335. These residues are in addition to the protonated Asp32 (Ash32) and Asp228 catalytic dyad at the QM layer level in the ONIOM calculation. Twenty residues partake in the binding of BACE1—AM-6494 system forming van der Waals (vdW), conventional hydrogen bond (HB), carbon HB, alkyl and pi-alkyl among others (Fig. 7A). Asp32/228 and Thr72 formed conventional HB (an interaction occurring between H and N/O/F atoms of a non-bonded complex) through the proton on Asp32 to the N in the hexacyclo base. The oxygen molecules in Asp228 interacted with the H_2 of the side NH_2 fragment through HB as well. Thr72 also formed a classical HB while Thr232 formed carbon HB together with Lys9, Gly11 and Gly13 residues. Carbon HB is an interaction occurring between carbon and hydrogen within a non-bonded molecular system. There exists an amide-pi stacked interaction through Thr231 while Tyr71 formed three different interactions, namely, pi-sigma, alkyl and pi-alkyl with AM-6494. The inhibitor gave vdW interactions through Leu30, Gly34, Trp115, Gly230, Arg235, and Arg307 (Fig. 7A).

Due to the structural similarity of the BACE1 inhibitors studied herein, we noticed comparable non-bonded interactions in AM-6494 and CNP-520 binding mode at the active site of BACE1. 19 residues interacted actively with CNP-520 in which 8 residues (Ser35, Thr72, Ile110, Trp115, Thr231, Thr232, Arg235 and Ala335) formed vdW interactions (Fig. 7B). Gly34 and Lys107 formed halogen interaction through the fluorine atoms of the CNP-520 while Gly34 also formed unfavourable donor-donor interaction. These appreciable interactions of BACE1 active residues with both AM-6494 and CNP-520 could be attributed to the presence of all possible electrostatic potentials within these inhibitors (Fig. 6). Overall, all the interacting residues are critical for BACE1 inhibitor binding [103].

Highlighting the zoomed-in interaction of AM-6494 and CNP-520 with BACE1 at the QM region (Fig. 7C and D), we noticed that the same interaction exists between both ligands and the catalytic aspartate dyad. Classic HB exists between Asp32 proton and the N in the hexacyclo base was at 1.58 and 1.59 Å for AM-6494 and CNP-520, respectively. The unprotonated inner oxygen of Asp32 also formed conventional HB with one H molecule of the attached hexacyclo NH_2 at distances 1.94 Å for AM-6494 and 1.96 Å for CNP-520. Asp228 inner oxygen also formed the same interaction to this fragment at 2.68 and 2.86 Å for AM-6494 and CNP-520, respectively. Similarly, the outer oxygen of Asp228 interacted with the second H of this hexacyclo NH_2 at bond length distances 1.67 Å (AM-6494) and 1.66 Å (CNP-520). This NH_2 was predicted to be available for electrophilic attraction through the MESP plot (Fig. 6) and the Fukui analysis (N9 in AM-6494 and N23 in CNP-520; Table 1).

Table 2 depicts the thermodynamic properties binding free energy

Table 1
Fukui function analyses indicating the sites for electrophilic addition (E+) and nucleophilic attack (Nu−) for atoms within AM-6494 and CNP-520 (See Fig. 6 for atom labels).

AM-6494				CNP-520			
ATOM	f+	f−	Δf	ATOM	f+	f−	Δf
C1	−0.01845	−0.01036	−0.00809	C1	−0.03951	0.000862	−0.04037
O2	−0.00679	−0.00468	−0.00211	C2	−0.07315	−0.02258	−0.05057
C3	−0.01943	−0.0115	−0.00793	C3	−0.05202	−0.03531	−0.01671
C4	−0.00609	−0.00337	−0.00271	C4	−0.00847	0.000865	−0.00933
C5	−0.00736	−0.00433	−0.00303	C5	−0.05629	−0.01247	−0.04381
C6	−0.00779	−0.0033	−0.00449	C6	−0.06294	−0.0226	−0.04034
S7	−0.031	−0.00832	−0.02268	C7	−0.03852	−0.00163	−0.0369
C8	−0.00647	−0.00072	−0.00575	C8	−0.01488	−0.07151	0.056637
N9	−0.04151	−0.00547	−0.03604	C9	−0.01753	−0.07408	0.056545
N10	0.003266	0.00417	−0.0009	C10	−0.02479	−0.082	0.057207
C11	0.0008	0.000432	0.000368	C11	−0.0103	−0.02969	0.019387
C12	−0.03304	−0.01689	−0.01616	C12	0.00863	−0.07217	**0.080799 Nu−**
C13	−0.0231	−0.01423	−0.00887	C13	−0.02507	−0.05174	0.026664
C14	−0.07226	−0.01791	−0.05435	C14	−0.00398	−0.02409	0.020111
C15	−0.07957	−0.02766	−0.05191	C15	−0.05536	−0.0299	−0.02546
F16	−0.06927	−0.02539	−0.04388	C16	−0.02073	−0.01605	−0.00468
C17	−0.04147	−0.01476	−0.02671	N17	−0.01005	−0.01651	0.006464
F18	−0.05178	−0.02219	−0.02959	F18	−0.05343	−0.02867	−0.02476
C19	−0.05675	−0.02273	−0.03402	C19	−0.01394	−0.00032	−0.01361
C20	−0.06068	0.003786	**−0.06447 E+**	N20	−0.05684	0.000832	−0.05767
N21	−0.07572	−0.0388	−0.03691	C21	−0.02637	−0.00105	−0.02532
C22	−0.02931	−0.05008	0.020766	O22	−0.02908	−0.00127	−0.02781
O23	−0.06457	−0.07333	0.008766	N23	−0.08971	−0.00654	**−0.08317 E+**
C24	0.005811	−0.06152	0.067326	N24	0.001427	−0.0595	0.060924
C25	−0.0384	−0.063	0.024592	N25	−0.04965	−0.03662	−0.01302
N26	−0.01656	−0.07138	0.054818	O26	−0.05314	−0.06741	0.014265
N27	−0.00573	−0.09583	**0.090097 Nu−**	Cl27	−0.03797	−0.08986	0.051893
C28	−0.03433	−0.09197	0.05764	F28	−0.01006	−0.0178	0.007736
C29	−0.02561	−0.08127	0.055666	F29	−0.0122	−0.0592	0.046995
O30	−0.02574	−0.04634	0.020601	F30	−0.00954	−0.0478	0.038255
C31	−0.02994	−0.05688	0.026939	C31	−0.00598	−0.00218	−0.0038
C32	0.001965	0.001276	0.000689	F32	−0.01603	−0.00536	−0.01067
C33	−0.03309	−0.0655	0.032417	F33	−0.01912	−0.00811	−0.01101
				F34	−0.01341	−0.00855	−0.00485

Table 2
Thermodynamic components and interaction energies (in kcal/mol) for the binding of AM-6494 and CNP-520 with BACE1 using hybrid ONIOM (B3LYP/6–31 + G(d):AMBER) model. Also provided are the available IC_{50} data from the literature.

	Protonated model		Unprotonated model	
	AM-6494	CNP-520	AM-6494	AM-6494 (—SCH_3)
ΔE_{ONIOM2}	−87.604	−60.833	−81.394	−107.689
ΔH	−92.227	−63.553	−81.873	−110.245
TΔS	−29.378	−30.091	−22.115	−38.414
ΔG_{bind}	−62.849	−33.463	−59.758	−71.830
IC_{50} (nM)	0.4 [49]	11 [51]		

ΔE Total interaction energy, ΔH Enthalpy change, TΔS Entropy contribution, and ΔG_{bind} Gibbs binding free energy.

(ΔG_{bind}), enthalpy (ΔH), entropy (TΔS) and interaction energy (ΔE), of the inhibitor—enzyme complexes. All the studied energetic parameters gave very high negative values to denote favourable interactions of AM-6494 and CNP-520 with the BACE1 enzyme. For the protonated BACE1 model, the relative interaction energy values are −87.60 and −60.83 kcal/mol for the binding of AM-6494 and CNP-520, respectively. The change in enthalpy, which denotes the energy quantity in a thermodynamic process, were recorded as −92.23 and −63.55 kcal/mol for AM-6494 and CNP-520, respectively. These values indicate that the overall thermodynamic energy during the binding of these inhibitors to BACE1 is exothermic. From these large negative ΔH values, approximately −29.4 kcal/mol (AM-6494) and −30.1 kcal/mol (CNP-520) connect with random activities during the BACE1 inhibition. Therefore, the accrued negative change in entropy (TΔS) values reflect that the BACE1—inhibitor systems tend towards stability during the binding process. The calculated ΔG_{bind} values are −62.85 and −33.46 kcal/mol for AM-6494 and CNP-520, respectively, thereby reflecting that the inhibition of BACE1 with these molecules are thermodynamically favourable. In total, AM-6494 showed better bioactivity and selectivity as a BACE1 inhibitor compared to CNP-520. Note that the calculated ONIOM binding free energies (Table 2) are in good trend with the available experimental data [49,51].

3.3.2. *The interactions of AM-6494 with unprotonated BACE1*

To provide a better understanding of the binding of this newly identified molecule to the active region of BACE1, we calculated the binding affinity of AM-6494 when the enzyme is unprotonated. That becomes necessary to ensure that the obtained result from the Asp32 monoprotonated model is not an artefact. Note that this protease enzyme can exist in more than four different protonated forms [85–91]. For this calculation, we have selected only AM-6494 because AM-6494 and CNP-520 are analogues (Fig. 3) and its binding affinity reflects better inhibition. Also, pursuing the theoretical properties of an already discontinued molecule (CNP-520) might seem less logical, especially, if not directed towards enhancing such inhibitor for improved bioactivity. Hence, we investigated the unprotonated BACE1 interaction with AM-6494.

The binding landscape of BACE1—AM-6494 system revealed through the contact map (Fig. 8A) after the simulation was the same as the protonated model (Fig. 7A). When the active Asp dyad and the inhibitor at the QM level during the ONIOM calculation examined (Fig. 8B), three HBs (conventional) were formed between these catalytic moieties and AM-6494. The thermodynamic quantities gave large negative values to denote a favourable binding of this inhibitor to the unprotonated BACE1 (Table 2). ΔG_{bind} value of approximately −60

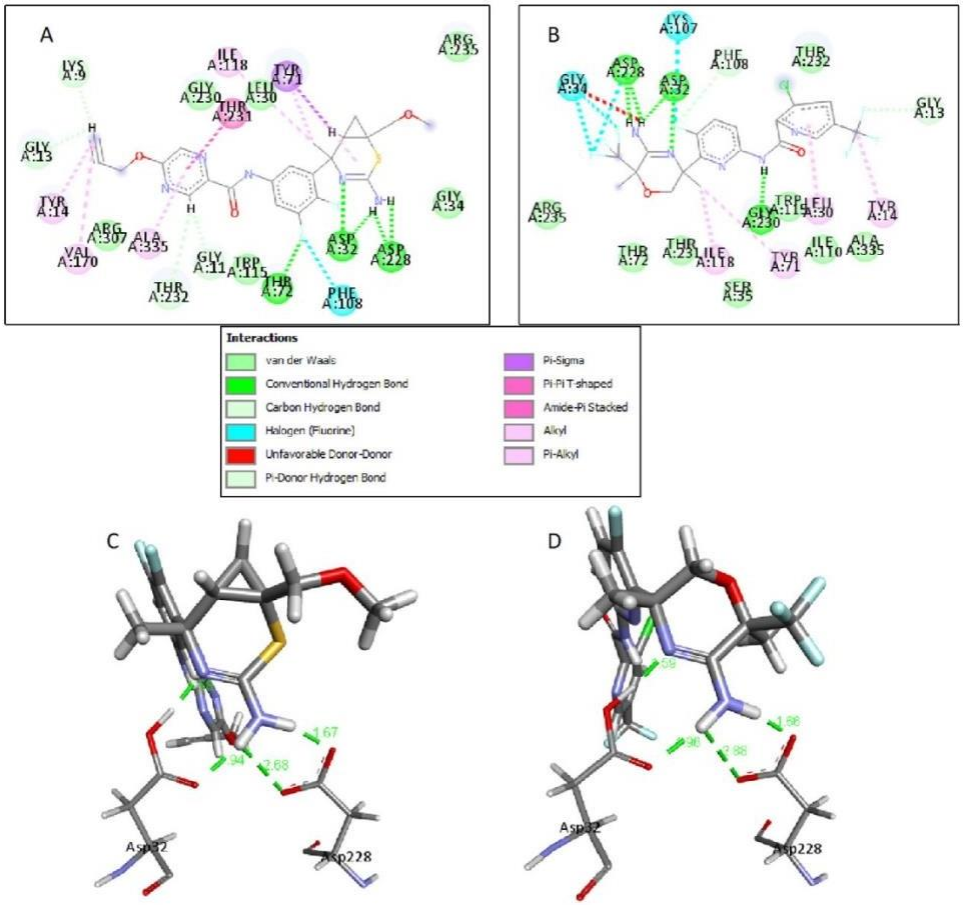

Fig. 7. Molecular interactions after the ONIOM calculation of the protonated BACE1 with (A) AM-6494 and (B) CNP-520; C and D are the respective 3D representation of the QM level.

kcal/mol was recorded for the binding of AM-6494 to the unprotonated BACE1. This value is about +3 kcal/mol difference compared to the protonated Asp32 model to reflect that Asp32 protonation improved the thermodynamic properties of AM-6494 binding to BACE1. Other energetic parameters of the unprotonated model are around +7 to +10 kcal/mol difference compared to the monoprotonated BACE1 (Table 2).

Within the context of the present investigation, we also explored improved inhibitor design *via* electronic calculation. Based on an ongoing experience with peptide-based inhibitor design whereby some substituents were attached on the N of the amide bond to strengthen the C—N bond, we proposed a new inhibitor (Fig. 9). Using a DFT method and a small model system, —SCH_3 addition to the amide nitrogen gave the most favourable output among the studied fragments. The study is currently at the experimental phase to confirm our theoretical calculation. Applying this electronic approach on the amide NH of AM-6494 (Fig. 9), we predicted the theoretical binding affinity of the —SCH_3 derivative in complex with unprotonated BACE1 (Table 2). The AM-6494—SCH_3 binding at the active site of BACE1 gave similar nonbonded interaction with the catalytic residues as in the parent molecule (Fig. 8). All the thermodynamic parameters showed improved values with interaction energy of −107.69 kcal/mol, which is −26.29 kcal/mol favourable over AM-6494. The calculated binding free energy and enthalpy values are −71.83 and −110.25 kcal/mol for the proposed structure in complex with unprotonated BACE1. This study has therefore identified a new potential lead with a methyl-thio unit on AM-6494 for BACE1 inhibition which would be confirmed through high-resolution experimental studies in a subsequent investigation from our group. Note that CNP-520 would likely pass this modification with improved binding energy.

4. Conclusion

In this study, we used both QM and QM/MM methods to study two potent BACE1 inhibitors. These methods allowed the investigation of the quantum mechanical properties and binding affinities of AM-6494 and CNP-520 as therapeutic molecules for BACE1 inhibition aimed at AD management. The study involves DFT calculations using B3LYP/6-31+G (d) combination and a two-layered ONIOM modelling at B3LYP/6-31+G (d):AMBER level of theory. A solvation energy value of −19.3 and −14 kcal/mol was estimated for AM-6494 and CNP-520, respectively. We

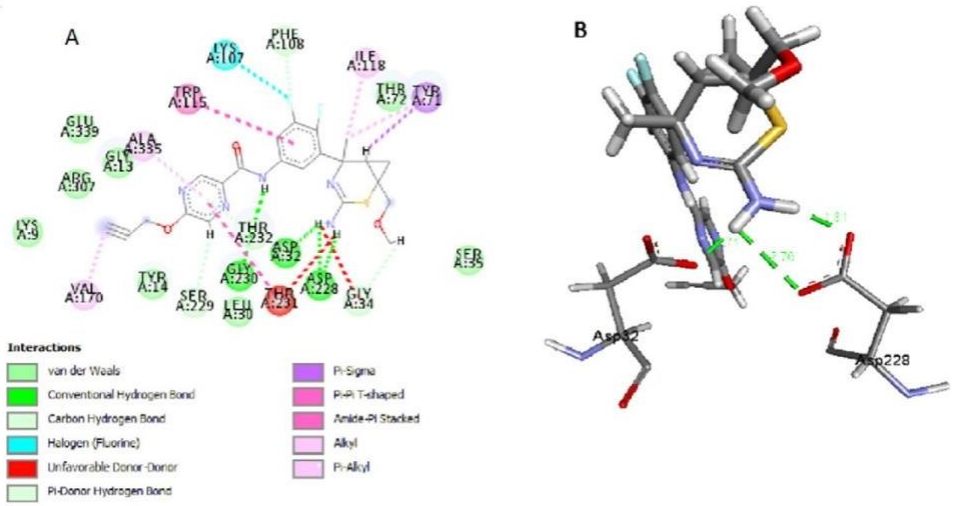

Fig. 8. Contact maps showing the interaction network of the unprotonated BACE1 with (A) AM-6494 and (B) 3D representation of the high layer fragments.

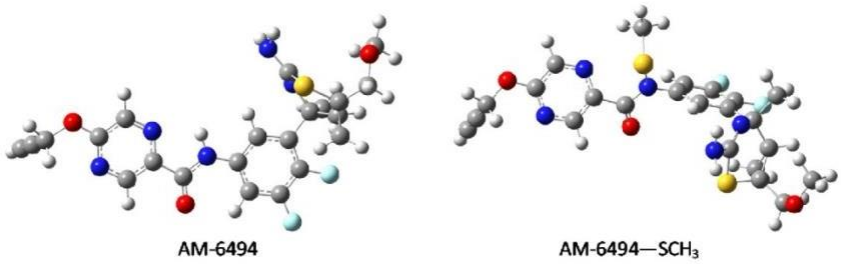

Fig. 9. Optimised (at B3LYP/6-31+G(d) level) AM-6494 and the proposed derivative (AM-6494—SCH_3) based on amide bond strengthen *via* electronic fine-tuning.

also evaluate inhibitor binding sites through the MESP plot (Fig. 6) and the Fukui function analysis (Table 1). The QM calculation binding sites prediction showed N9 and N23 (Fig. 6) of AM-6494 and CNP-520, respectively, availability for electrophilic attraction through the MESP plot and the Fukui analysis. These regions (N9 and N23) interacted with the Asp active units forming classical hydrogen bonds (Figs. 7 and 8).

We calculated the interaction of AM-6494 and CNP-520 in the active pocket of the enzyme using the ONIOM method when Asp32 is mono-protonated and unprotonated in the BACE1 structure. Analysis of the results showed that both AM-6494 and CNP-520 actively interacted with BACE1 through residues Lys9, Gly11, Gly13, Tyr14, Leu30, Asp32, Gly34, Ser35, Tyr71, Lys107, Phe108, Trp115, Ile118, Val170, Asp228, Ser229, Gly230, Thr231, Thr232, Arg307, Ala335, and Glu339. These residues are crucial to inhibitor binding in the active pocket of BACE1 [103]. As shown in Figs. 7 and 8, Asp32/228 residues at the QM high level of theory and other active residues at MM level formed favourable non-bonded interactions with the studied inhibitors to enable the complexes stability.

Analysis of the binding thermochemistry (Table 2) revealed that AM-6494 produced higher values for both its interaction energy ($\Delta E = -87.6$ kcal/mol) and enthalpy ($\Delta H = -92.23$ kcal/mol). The result suggests that AM-6494 exhibited a better binding affinity and selectivity towards the BACE1 enzyme compared to CNP-520. Asp32 mono-protonation in BACE1 gave slightly better energy than the unprotonated form. Thus, indicating the possibility of at least one proton on the aspartate dyad for a favourable binding. Our calculation also revealed that electronic fine-tuning of the amide link in an inhibitor could yield a more selective and active inhibitor (Fig. 9). Understanding the detailed molecular interaction of these inhibitors could serve as a basis for pharmacophore exploration towards improved inhibitor design.

Declaration of Competing Interest

Authors declare no financial and intellectual conflict of interests.

Data availability

No data was used for the research described in the article.

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Author statement

S. C. Ugbaja: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft.

Z. K. Sanusi: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing.

P. Appiah-Kubi: Conceptualization, Investigation, Project administration, Validation, Visualization, Writing - original draft.

M. M. Lawal: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Validation, Writing - review & editing.

H. M. Kumalo: Formal analysis, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

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INTERLINKING PAGE FIVE

This chapter further elucidates the structural and binding dynamisms of AM-6494 in comparison with CNP-520. It deals with the application of the computational instruments of conventional molecular dynamic simulations (cMD) and accelerated molecular dynamic simulations (aMD) in the study of the chosen inhibitors. It covers the conformational monitoring of the flap covering of the active site of the studied inhibitors when bound to BACE1. Comparison of the binding free energy of the studied inhibitors as well as principal component analysis (PCA) was also covered in this chapter. Hitherto, researchers have focused so much on the BACE1 active site in the design of BACE1 inhibitors. In the next chapter, we shifted our focus from the conventional BACE1 active site to allosteric and exosite as suggested from the review article in chapter three.

CHAPTER SIX

MANUSCRIPT FOUR

UNRAVELING THE MOLECULAR BASIS OF AM-6494 HIGH POTENCY AT BACE1 IN ALZHEIMER'S DISEASE: AN INTEGRATED DYNAMIC INTERACTION INVESTIGATION

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Unravelling the molecular basis of AM-6494 high potency at BACE1 in Alzheimer's disease: an integrated dynamic interaction investigation

Samuel C. Ugbaja[a], Patrick Appiah-Kubi[b], Monsurat M. Lawal[a], Nelisiwe S. Gumede[a] and Hezekiel M. Kumalo[a]

[a]Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Durban, South Africa; [b]Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

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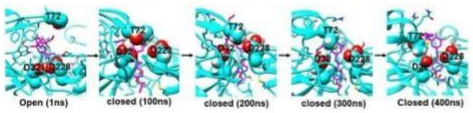
ABSTRACT

β-amyloid precursor protein cleaving enzyme1 (BACE1) has prominently been an important drug design target implicated in Alzheimer's disease pathway. The failure rate of most of the already tested drugs at different clinical phases remains a major concern. Recently, AM-6494 was reported as a novel potent, highly selective, and orally effective inhibitor against BACE1. AM-6494 displayed no alteration of skin/fur colour in animal studies, an adverse effect common to previous BACE1 inhibitors. However, the atomistic molecular mechanism of BACE1 inhibition by AM-6494 remains unclear. To elucidate the binding mechanism of AM-6494 relative to umibecestat (CNP-520) as well as the structural changes when bound to BACE1, advanced computational techniques such as accelerated MD simulation and principal component analysis have been utilised. The results demonstrated higher binding affinity of AM-6494 at BACE1 with van der Waals as dominant energy contributor compared to umibecestat. Conformational monitoring of the β-hairpin flap covering the active site revealed an effective flap closure when bound with AM-6494 compared to CNP-520, which predominantly alternates between semi-open and closed conformations. The observed effective flap closure of AM-6494 explains its higher inhibitory power towards BACE1. Besides the catalytic Asp32/228 dyad, Tyr14, Leu30, Tyr71 and Gly230 represent critical residues in the potency of these inhibitors at BACE1 binding interface. The findings highlighted in this research provide a basis to explain AM-6494 high inhibitory potency and might assist in the design of new inhibitors with improved selectivity and potency for BACE1.



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1. Introduction

Alzheimer's disease (AD) is an age-related degeneration of the neurons in the brain which gradually happens in stages. It is implicated in dementia which may start with memory loss, difficulties in decision making, speaking or writing, mood swings and other behavioural problems (Cummings et al., 2018; Reitz et al., 2011). AD has multiple disease-causing pathways, extracellular Aβ plaques and NFTs (neurofibrillary tangles) are predominantly the two main typical biological features of AD. This is followed by impaired communication, resulting from the disconnection of neurotransmission network in the brain of the person with the disease (dos Santos et al., 2018; Efthymiou and Goate, 2017). The fundamental reasons for reduced sound memory functions are due to the collapse of some neurons and synapses, and this greatly affects the communications network (Das and Yan, 2019).

However, β-amyloid Aβ brain aggregation has also been reported to be responsible for the imbalance of free radicals and antioxidants (oxidative stress) as well as an inflammatory response which result in neurotoxicity and impairment of cognitive functions. The deadness and damaging of neurotransmitters, imbalance of free radicals and antioxidant, soreness, lack of calcium balance, pressure on the endoplasmic reticulum, disorder of mitochondria and synapses are all resultant effects of the accumulation of Aβ (Herrup, 2015; Keskin et al., 2019).

CONTACT H. M. Kumalo kumaloH@ukzn.ac.za Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Westville Campus, Durban 4001, South Africa; P. Appiah-Kubi appiahpat@gmail.com Discipline of Pharmaceutical Sciences, School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa

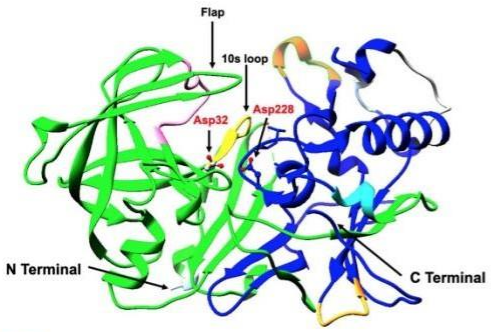

Figure 1. Structure of BACE1 showing significant domains within the enzyme. The two catalytic Asp32 and Asp228 dyad are shown in ball and stick.

The β-amyloid precursor protein cleaving enzyme 1 (BACE1) also known as β-Secretase (Figure 1), is an aspartyl protease which spans from one side of a membrane through the other side (transmembrane) and splits amyloid precursor protein (APP) at the β-site. β-Secretase is expressed mainly in the central nervous system (CNS) and brain neurons. The systematic proteolytic splitting of APP is carried out by β and γ secretases, respectively and results in the accumulation and aggregation of the β-amyloid peptide in the human brain. Consequently, amyloidogenic secretases are principal therapeutic focus investigated for AD-modifying mediation (Das and Yan, 2019). The structure of BACE1 has common features with other aspartyl proteases in the same family which include cathepsin E, BACE2, pepsin, cathepsin D and renin. The diagram of BACE1 shown in Figure 1 has many similarities as the closest member of the family BACE2.

Recent research findings posit that BACE1 inhibitors possess great prospect as a potential approach in reducing Aβ brain concentrations, thereby restraining the amelioration of AD (Koelsch, 2017). It is, therefore, imperative to develop effective BACE-1inhibitors that have high and effective BACE1 inhibition while minimising off-target side effects (Scott et al., 2016). A healthy course of treatment with regards to BACE1 inhibition must show high specificity towards BACE1 function and should be geared towards minimising BACE1 to acceptable level rather than suppressing it completely. It has been reported that total suppression or elimination of BACE1 could lead to myelin sheath disorder in the brain (Gedeon et al., 2015; Salvadores et al., 2017).

There are several BACE1 inhibitors which have been successfully tested at the various clinical trials stages, verubecestat stood out amongst them as the first to progress to stage 3 clinical trial. The clinical trials of this drug were, however, terminated (February 2018) as there was no appreciable recovery signs on the subjects (Merck, 2018). Saravanan et al. (2019) recently elucidated the structural properties and binding mechanism of verubecestat at BACE1 active site using molecular dynamic simulations and quantum mechanical methods (Saravanan et al., 2019). Also, Jannat and colleagues have recently reported the interactions of several pterosins scaffolds at BACE1 and cholinesterases using both experimental and molecular docking simulation techniques (Jannat et al., 2019).

Umibecestat denoted as CNP-520 (Figure 2) is an orally available small-molecule BACE1 inhibitor which was among some earlier identified BACE1 inhibitors that attained phase II/III clinical trials along with its analogue verubecestat (MK-8931). Sequel to an extensive study, CNP-520 displayed favourable selectivity and safety features with a three-fold selectivity towards BACE1 (IC_{50} = 11 nM) over BACE2 (IC_{50} = 30 nM). CNP-520 was reported to have no effect on melanin and hair pigmentation on the mice dosed with it for 8 weeks (Thaisrivongs et al., 2018). Umibecestat and verubecestat were, however, discontinued lately in February 2018 and July 2019, respectively, as participants displayed worsened cognitive functions (Hampel et al., 2020; Merck, 2018; Neumann et al., 2018; Thaisrivongs et al., 2018).

Pettus et al. (2020) recently identified AM-6494 as a novel potent and orally effective BACE1 inhibitor that has advanced to preclinical development. AM-6494 molecule demonstrated high selectivity for BACE1 (IC_{50} = 0.4 nM) over BACE2 (IC_{50} = 18.6 nM) (Pettus et al., 2020). The administration of AM-6494 in an experimental study recorded improved β-amyloid reduction and no alteration of skin/fur colour in the mammals (monkey and rat) tested with it (Pettus et al., 2020). However, the atomistic molecular recognition mechanistic and conformational dynamics of AM-6494 have not been elucidated.

The conformational dynamic behaviour of proteins can be studied using classical molecular dynamics (cMD) (Lindorff-Larsen et al., 2011). Detailed understanding of how proteins behave upon ligand binding is imperative for rational drug design. Despite the application of sophisticated high-performance computers, cMD simulations are greatly disadvantaged in exploring the full energy landscape due to limited simulation time (Baker et al., 2013; Shaw et al., 2010). Therefore, accelerated molecular dynamics (aMD) is an advanced and modified simulation technique that speeds up transitions between different low-energy states (Hamelberg et al., 2004, 2007), provide an efficient path to trigger uncommon activities necessary for protein conformational change (Markwick and McCammon, 2011), and strengthens the conformational sampling of biomolecules (Hamelberg et al., 2004; Markwick and McCammon, 2011). Performing multiple nanosecond aMD simulations are sufficiently adequate to express micro to split second interval events of a process of molecular dynamic simulations which are not biased (Gedeon et al., 2015; Hamelberg et al., 2004; Markwick and McCammon, 2011). Accelerated MD simulations, therefore, represent an active and efficient method of exploring different ligand orientations and conformations at BACE1 binding site.

In this work, aMD simulation has been employed to elucidate the atomistic level interactivity between BACE1 and its inhibitors (AM-6494 and CNP-520) as well as associated conformational changes upon inhibitor binding. Binding site residues that drive the potency of these inhibitors at BACE1 active region was identified. The findings highlighted in this study may provide useful insights to comprehend the

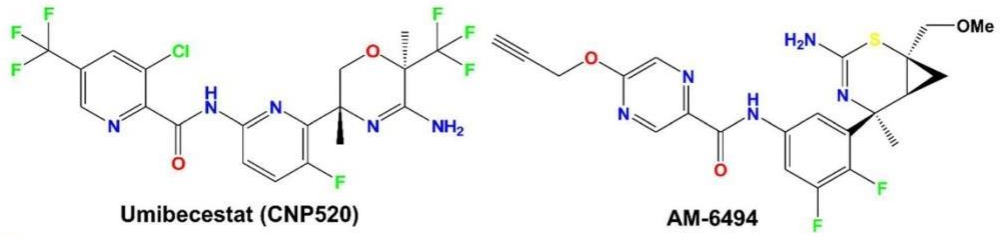

Figure 2. Two-dimensional structures of the studied BACE1 inhibitors.

interaction mechanism of AM-6494 relative to CNP-520 with BACE1, as well as assist in the design of more potent and selective inhibitors.

2. Materials and methods

2.1. System preparation

The molecular dynamic simulations were carried out using crystallographic structures of BACE1-ligand complexes. AM-6494 and CNP-520 BACE1 complexes were fetched from the Protein Data Bank (RCSB PDB, http://www.rcsb.org/) with PDB codes 6PZ4 and 6EQM, respectively. The protein structures were pre-treated using Maestro protein preparation wizard package (Schrödinger, 2019). The protein preparation step involved the addition of hydrogens, assigning bond orders as well as the removal of water and non-ligand molecules. Missing residues and side chains were also modelled and fixed. The protonation states of the receptors were assigned using PROPKA at pH 7. The protein was further energy minimised with the root mean square deviation set to 0.50 Å using the OPLS3e force field.

The protonation of Asp32 and deprotonation of Asp228 of this catalytic dyad have been reported in earlier studies (Cascella et al., 2005; Park and Lee, 2003). The simulations in this study were performed with Asp32 protonated and Asp228 unprotonated (Cascella et al., 2005; Park and Lee, 2003; Shimizu et al., 2008). The structural optimisation of the two selected BACE1 inhibitors was carried out using Gaussian 16 (Frisch et al., 2016) package at B3LYP (Becke, 1993; Lee et al., 1988)/6-31G(d) (Hehre et al., 1972; Pople et al., 1992) level of theory. Restrained Electrostatic Potential method was employed to assign charges based on optimal conformations (Burger et al., 2013). The General AMBER Force Field (GAFF) of antechamber (Sprenger et al., 2015) was finally employed in generating force field parameters of the two BACE1 inhibitors. The addition of appropriate sodium ions was based on coulombic potential grid to maintain the systems' neutrality. Solvation of the systems was achieved using the TIP3P water model (Jorgensen et al., 1983) at 12 Å distance from the solute in an octahedron water box. The protein parameters and the water molecule were described with AMBER 14 force field (ff14SB) (Maier et al., 2015). The AMBER Leap module was then used to generate the coordinate and topology files for molecular dynamics simulation.

2.2. Conventional molecular dynamics (cMD) simulation

BACE1 in complex with AM-6494 and CNP-520 was energy minimised and simulated using AMBER 18 (Bergonzo and Cheatham, 2015). The minimisation steps consisted of an initial partial minimisation of 10000 steps with 10.0 kcal/mol.Å^{-2} harmonic restraint on all heavy atoms to relax the system and to remove potential atom clashes. This was followed by additional steepest and conjugate gradient descents of 5000 steps each without restraint. Afterwards, the heating of the systems was from 0 K to 300 K for 300 ps using Langevin dynamics, a 1 ps-1 collision frequency and a 5 kcal/mol.A^{-2} applied harmonic restraints at a constant volume. A subsequent 500 ps equilibration step was carried out at 300 K under constant pressure and temperature (NPT) ensemble. Conventional MD simulation of 100 ns was finally performed with a time step of 2 fs without any restraints at 300 K and 1 atm in the NPT ensemble. The Langevin temperature scaling (Izaguirre et al., 2001) and Berendsen barostat (Berendsen et al., 1984) were used to maintaining the temperature at 300 K and the pressure at 1 atm during the production phase, respectively. The molecular dynamic simulation was done using particle mesh Ewald method for the periodic boundary conditions (Darden et al., 1993; 1993) assigned for long-range electrostatic interactions (cut off =12 Å) and SHAKE algorithm holding fix bonds involving hydrogen (Ryckaert et al., 1977).

2.3. Accelerated molecular dynamics (aMD) simulation

Accelerated molecular dynamics (aMD) simulations were performed on the BACE1 systems using the "dual-boost" function (Hamelberg et al., 2007) in boosting the entire potential with an extra boost to the torsions implemented in AMBER 18. A non-negative potential boost was added to both the total energy of all the atoms and the dihedral angles of the systems. The total energy and the dihedral acceleration boost parameters were calculated in terms of Equations (1) and (2).

$$E_{dihedral} = V_{avg_{dihedral}} + 3.5 \times N_{res},\ \alpha_{dihedral} = 3.5 \times \frac{N_{res}}{5} \quad (1)$$

$$E_{total} = V_{avg_{total}} + 0.2 \times N_{atoms},\ \alpha_{total} = 0.2 \times N_{atoms} \quad (2)$$

Where $V_{avg_{total}}$ and $V_{avg_{dihedral}}$ are the averaged total potential energy and the dihedral energy values derived from the 100 ns cMD simulations. The total number of atoms and residues are denoted as N_{atoms} and N_{res}, respectively. We

performed 400 ns aMD simulation for each BACE1 system restarting from the 100 ns cMD simulation final structures at 300 K with random atomic velocity initialisation.

2.4. End-point binding free energy calculations

The most common MD-based binding free energy techniques include Free Energy Perturbation (FEP) (Deflorian et al., 2020; Lenselink et al., 2016), Thermodynamic Integration (TI) (Bhati et al., 2017), Linear Interaction Energy (LIE) (Rifai et al., 2018), and Poisson-Boltzmann/Generalized Born Surface Area (MM-PB/GBSA) molecular mechanics (Aldeghi et al., 2017; Miller et al., 2012). However, compared to MMGBSA, methods such as TI, FEP are quite computationally expensive, whereas the MM/GBSA method provide a balance between speed and accuracy (Hou et al., 2011). In computing absolute binding free energies, MM/PBSA is more effective, whereas MM/GBSA computes relative binding energies efficiently. Therefore, MM/GBSA appears to be the most effective choice to be utilised in the correct inhibitor ranking compared to MM/PBSA (Hou et al., 2011). The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method of the *MMPBSA.py* program implemented in AMBER 18 was used to estimate the relative binding free energies of the studied BACE1 complexes. Equation (3) illustrates how the *MM/PBSA*.py program computes the binding free energy (ΔG_{bind}):

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{sol} - T\Delta S \quad (3)$$

Where, ΔE_{MM} and ΔG_{sol} denote the molecular mechanics interaction and the solvation free energies, respectively, while $-T\Delta S$ denotes the entropy contribution. The sum of the van der Waals (ΔE_{vdw}) interaction and the electrostatic (ΔE_{ele}) interaction energies constitute the molecular mechanics interaction energy (ΔE_{MM}) between the receptor and ligand (Equation (4)).

$$\Delta E_{MM} = \Delta E_{vdw} + \Delta E_{ele} \quad (4)$$

The solvation free energy contribution is broken down into the polar (ΔG_{pol}) and the nonpolar ($\Delta G_{nonpolar}$) terms (Equation (5)).

$$\Delta G_{sol} = \Delta G_{pol} + \Delta G_{nonpolar} \quad (5)$$

The nonpolar term of the solvation energy was determined based on the solvent-accessible surface area (SASA), as shown in Equation (6):

$$\Delta G_{nonpolar} = \gamma(SASA) + \beta \quad (6)$$

where β and γ set at -0.5692 kcal/mol and 0.0378 kcal/mol.Å^{-2}, respectively. A water probe radius of 1.4 Å was used to determine the SASA. The external and internal dielectric constants were set at 80 and 1, respectively. The relative binding energies were computed using 500 snapshots from the last 100 ns. The conformational entropy contribution was also computed using normal mode analysis of AMBER NMODE module (Pearlman et al., 1995; Zoete and Michielin, 2007) by extracting 50 snapshots from the last 100 ns.

Table 1. MM/GBSA binding free energy calculation for AM-6494 and CNP-520.

	Compound	
Energy terms	AM-6494	CNP-520
E_{vdW}	-59.66 ± 0.18	-54.05 ± 0.30
E_{ele}	-10.10 ± 0.20	-6.93 ± 0.11
G_{pol}	29.30 ± 0.15	28.79 ± 0.12
G_{nonpol}	-7.37 ± 0.02	-7.19 ± 0.03
ΔG_{gas}	-69.76 ± 0.27	-60.98 ± 0.32
ΔG_{sol}	21.94 ± 0.15	21.60 ± 0.11
ΔH	-47.82 ± 0.20	-39.38 ± 0.27
$-T\Delta S$	20.43 ± 0.44	22.86 ± 0.56
ΔG_{bind}	-27.39 ± 0.24	-16.52 ± 0.29
Expt.	0.40	11

E_{vdW} = van der Waals energy; E_{ele} = Electrostatic energy; G_{pol} = Polar solvation energy; G_{nonpol} = Nonpolar solvation energy; ΔG_{gas} = Gas phase energy; ΔG_{sol} = Solvation energy; **ΔG_{bind}** = Total binding energy; Expt. = Experimental IC_{50} (nM).

2.5. The decomposition of the total energy on a residue basis

To estimate the contributions of active site residues to the overall inhibitor binding (ΔG_{per}), the overall relative binding free energy was estimated as outlined in Equation (7).

$$\Delta G_{per} = \Delta E_{vdW} + \Delta E_{ele} + \Delta G_{pol} + \Delta G_{nonpolar} \quad (7)$$

2.6. Analysis of the molecular dynamic trajectories

The generated aMD trajectories were utilised to evaluate BACE1 stabilities and conformational changes. The Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Radius of Gyration (Rg), and distances were analysed with CPPTRAJ module (Roe and Cheatham, 2013) of AMBER 18. The principal component analysis cluster plots and the dynamic cross-correlation matrix were generated using Bio3D (Grant et al., 2006) software in R programming.

3. Results and discussion

Molecular dynamics simulations have been performed to obtain atomistic binding interaction and conformational dynamics of AM-6494 and CNP-520 with BACE1. The generated molecular dynamics conformational ensembles were analysed using clustering and principal component analysis, binding free energy analysis, protein–inhibitor interaction, and structural stability analysis (RMSD, Rg, RMSF).

3.1. Binding energetics of AM-6494 and CNP-520 with BACE1

The calculated relative binding free energies by MM/GBSA approach for AM-6494 and CNP-520 binding with BACE1 are summarised in Table 1. The predicted relative binding free energies for AM-6494 and CNP-520 were -27.39 ± 0.24 kcal/mol and -16.52 ± 0.29 kcal/mol, respectively. These predicted energy values were in good correlation with their experimental binding results (IC_{50} of 0.40 nM and 11 nM for AM-6494 and CNP-520, respectively). The computed binding energies can be decomposed into components such as electrostatics,

van der Waals, polar and nonpolar contributions, to provide a further understanding of the complex binding process. In both AM-6494 and CNP-520 complexes, the electrostatic interactions, the nonpolar solvation energies and van der Waals interactions were observed to favour the binding free energies. These favourable interactions were more towards AM-6494 binding to BACE1 compared to CNP-520. The average sum of the hydrophobic (nonpolar) and van der Waals (vdW) interaction energies ($E_{vdw}+ G_{nonpol}$) which are responsible for the embedding of inhibitor's hydrophobic groups upon binding were −67.03 kcal/mol and −61.24 kcal/mol, respectively. The mean entropic contribution (−TΔS) values were 20.43 kcal/mol and 22.86 kcal/mol for AM-6494 and CNP-520, respectively. Thus, conformational entropy was more favourable for AM-6494 compared to CNP-520. The van der Waals interactions were observed to be the dominant contributor to the binding of AM-6494 and CNP-520.

3.2. Identification of hotspot residues in AM-6494 and CNP-520 binding at BACE1

The estimated binding free energies were further decomposed to identify key binding site residues that make substantial intermolecular interaction contributions to the binding of AM-6494 and CNP-520. The active site residue total energies, the van der Waals and electrostatic interaction energy contributions for AM-6494 and CNP-520 were calculated and the results (in kcal/mol) presented in Figure 3. The amino acid residues Gly13 (−0.936), Tyr14 (−2.008), Leu30 (−1.347), Val31 (−1.004), Tyr71 (−0.727), Trp115 (−0.806), Leu152 (−0.75), Leu154 (−0.868), Ser229 (−1.282), Gly230 (−0.843), Thr231 (−0.724) favoured AM-6494 binding at BACE1 active region with average energy contributions greater than −0.7 kcal/mol (Figure 3(A)). Similarly, Tyr14 (−2.137), Leu30 (−1.823), Tyr71 (−1.003), Phe108 (−0.713), Trp115 (−0.938), Leu154 (−0.688), Ser229 (−0.828), and Gly230 (−1.176) displayed stronger interactions with CNP-520 with interaction energies higher than −0.7 kcal/mol (Figure 3(B)).

It can be observed that Tyr14, Leu30, Tyr71, Trp115, Leu154, Ser229, and Gly230 residues make significant contributions to the binding of both AM-6494 and CNP-520 at BACE1 binding site. In contrast, amino acid residues Gly13, Val31, and Leu152, and Thr231 make significant contributions towards AM-6494 binding to BACE1 which may be attributed to the observed higher affinity of AM-6494 compared to CNP-520. Van der Waals interactions dominate electrostatic interactions of the inhibitor contacts with the active site residues making significant contributions to inhibitor binding. The contributions of the Asp catalytic dyad to the binding of both inhibitors was prominent through the unprotonated Asp228 which gave substantial electrostatic energy values of −0.98 and −1.36 kcal/mol for AM-6494 and CNP-520, respectively (Figure 3).

3.3. Diversity in the conformational stabilities of bound and unbound BACE1

To discover the relative structural stability of the studied complex systems, the structural properties such as the root mean square (RMSD), the radius of gyration (Rg), and the root mean square fluctuation (RMSF), were estimated (Figure 4). The RMSD represents the structural deviations of biomolecules relative to their starting structures, which assesses the equilibration and dynamic stability of a system. The average RMSD distribution of the entire protein for Apo, AM-6494 and CNP-520 were 3.75 ± 0.44 Å, 3.08 ± 0.33 Å and 2.84 ± 0.33 Å, respectively (Figure 4(A)). The results suggest that the bound conformations of the BACE1 induced a more stable conformation than the unbound conformation over the entire simulation. However, the computed stability of the binding site within 8 Å of the inhibitors indicates that the binding pocket of the BACE1 was more stable when bound to AM-6494 relative to CNP-520 with observed RMSD of 1.61 ± 0.21 Å and 1.86 ± 0.52 Å, respectively (Figure 4(B)). The Rg is an estimate for protein structure compactness (Sinha and Wang, 2020). The active site Rg was measured and observed to display marginal average value difference of 5.07 ± 0.14 Å and 5.12 ± 0.18 Å for BACE1—AM-6494 and BACE1—CNP-520, respectively (Figure 4(C)).

The RMSF was also evaluated for Cα atoms of each amino acids of BACE1 for the studied complex systems (Figure 4(D)). The RMSF highlights overall lower residue fluctuations in the bound conformations compared to the unbound conformation. Reduced mobility was highly prominent around residues of the 10 s loop (9–14), the flap region (67–77), the catalytic dyad (28–33; 225–230), the D-loop (270–273) and F-loop (311–318) in the bound conformation compared to the unbound conformation. Thus, the RMSF results showed that the binding of AM-6494 and CNP-520 reduce the overall residue mobility in the loop regions of BACE1.

3.4. Conformational dynamics of the flap tip and the two catalytic dyads

To have a better insight of ligand binding terrain, flap dynamics in addition to the overall conformational flexibility of enzymes, are vital features (Cai et al., 2012; Kumalo and Soliman, 2016). It has been reported that when considering aspartic proteases, the flap(s) covering the active sites play a significant function in ligand binding as a result of conformational variations that regulate access of incoming ligand or substrates (Hornak et al., 2006; Narang et al., 2019). Different conformations have been observed in crystal structures of diverse proteases, especially the open and closed conformations. The frequently used parameter in measuring the various flap(s) conformations is based on the Cα atom distance between one of the Asp residues in the catalytic dyad and the tip of the flap (Gorfe and Caflisch, 2005; Kumalo et al., 2016; Wall et al., 2003). In this present research, the flap movements, as well as interatomic distances, were carried out for a further understanding of the BACE1 flap dynamics of bound AM-6494 relative to CNP-520 throughout the 400 ns simulation. The Cα atom distances between the catalytic aspartates and the Cα atom of the flap tip residue Thr72 of BACE1 protease in complex with AM-6494 and CNP-520 were measured, as these distances play essential roles in the substrate recognition (Gorfe and Caflisch, 2005).

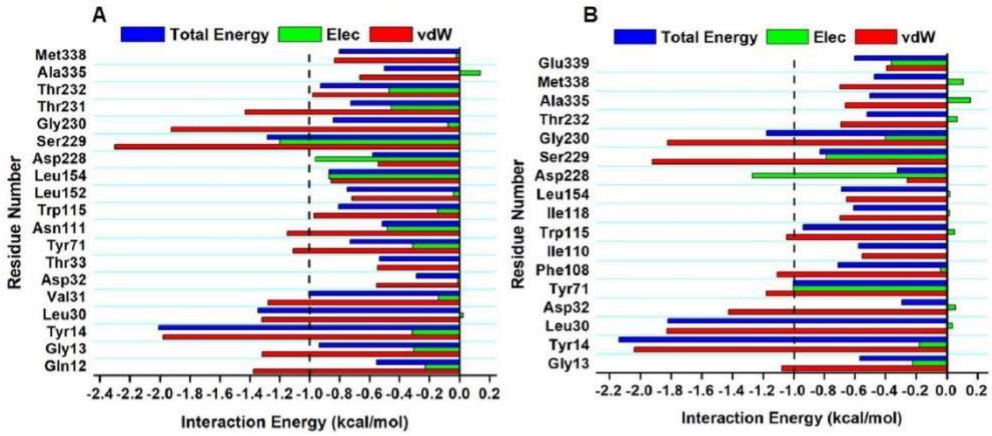

Figure 3. Decomposition of the binding energy on per residue for (A) AM-6494 and (B) CNP-520.

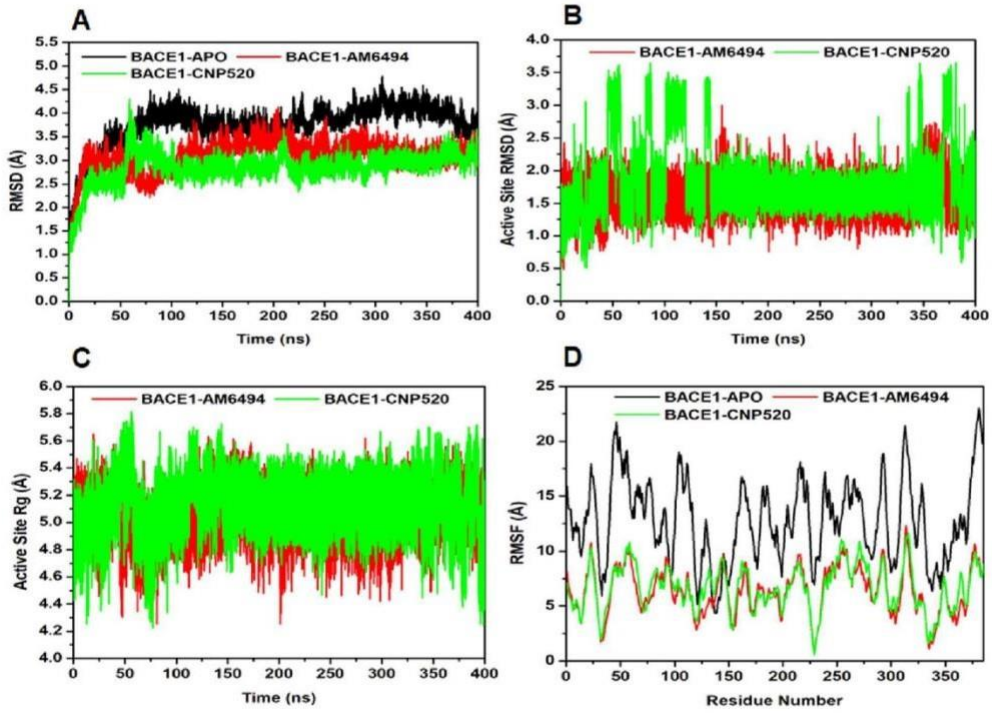

Figure 4. Time evolution for (A) the RMSD of the entire system (B) RMSD of the active site, (C) the active site Rg, and (D) the RMSF of the amino acids.

The observed average distances between CαAsp32-CαThr72 (d1) atoms were 11.21 ± 2.19 Å and 12.59 ± 2.20 Å for BACE1—AM-6494 and BACE1—CNP-520 complexes (Figure 5(A)), respectively. Similarly, the average distances between CαThr72-CαAsp228(d2) were also observed to be 11.27 ± 2.32 Å for BACE1—AM-6494 and 12.36 ± 1.96 Å for BACE1—CNP-520 complexes (Figure 5(B)). The minimum d1 and d2 distances for BACE1—AM-6494 were 5.55 Å and 5.93 Å, respectively, whereas 7.18 Å and 7.79 Å were the observed minimum d1 and d2 distances for BACE1—CNP-520, respectively. Previous studies have reported distances above 13.0 Å to correspond to the open conformation, whereas distances below 9.0 Å correspond to the closed flap conformation (Gorfe and Caflisch, 2005; Patel et al., 2004; Xu et al., 2012).

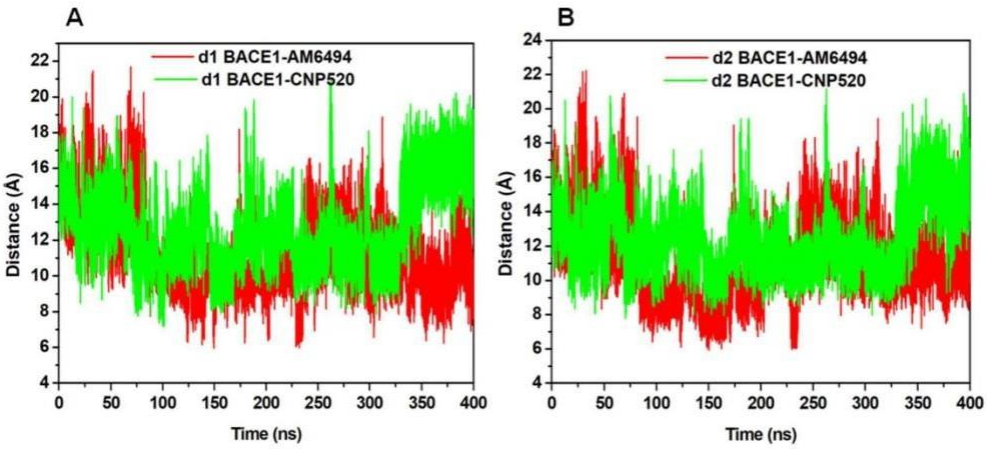

Figure 5. Time evolution distance between (A) Asp32 (CαD32) and the flap tip (CαT72) and (B) Asp228 (CαD228) and the flap tip (CαT72).

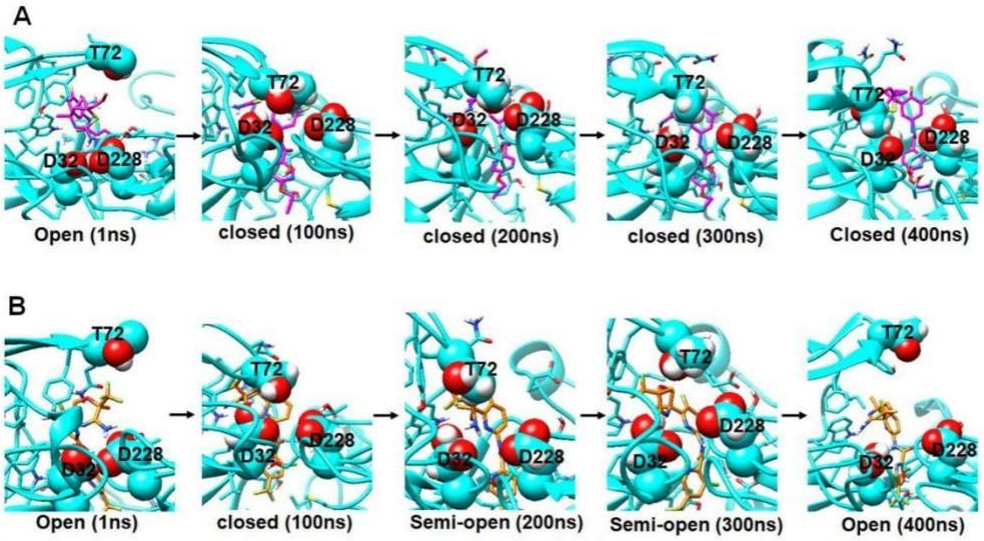

Figure 6. Time evolution of flap tip and catalytic dyad conformational dynamics for (A) AM-6494 and (B) CNP-520 at BACE1 active site.

To further visualise the flap conformation of BACE1 inhibitor bound, 3 D snapshots were extracted at 100 ns intervals through the entire simulations and displayed in Figure 6. An open conformation was observed for both BACE1—AM-6494 and BACE1—CNP-520 at the start of the simulation. The binding of AM-6494 thereafter was observed to maintain closed flap conformations at100 ns, 200 ns, 300 ns and 400 ns (Figure 6(A)) while CNP-520 was observed to induce a closed (100 ns), semi-open (200 ns and 300 ns) and open (400 ns) conformations (Figure 6(B)). The dominant closed conformation of the flap tip and the catalytic aspartic dyad residues (Asp32 and Asp228) of BACE1—AM-6494 complex may explain the stronger binding of AM-6494 in the BACE1 active site compared to CNP-520.

3.5. Internal dynamics of BACE1-induced inhibitor binding revealed by dynamic cross-correlation matrix (DCCM) and principal component analyses

Proteins undergo conformational changes in their different functional state due to their collective motions of the protein backbone atoms (Ichiye and Karplus, 1991). The difference in protein conformations is widely believed to play a significant role in the design of inhibitors towards the inhibition of the

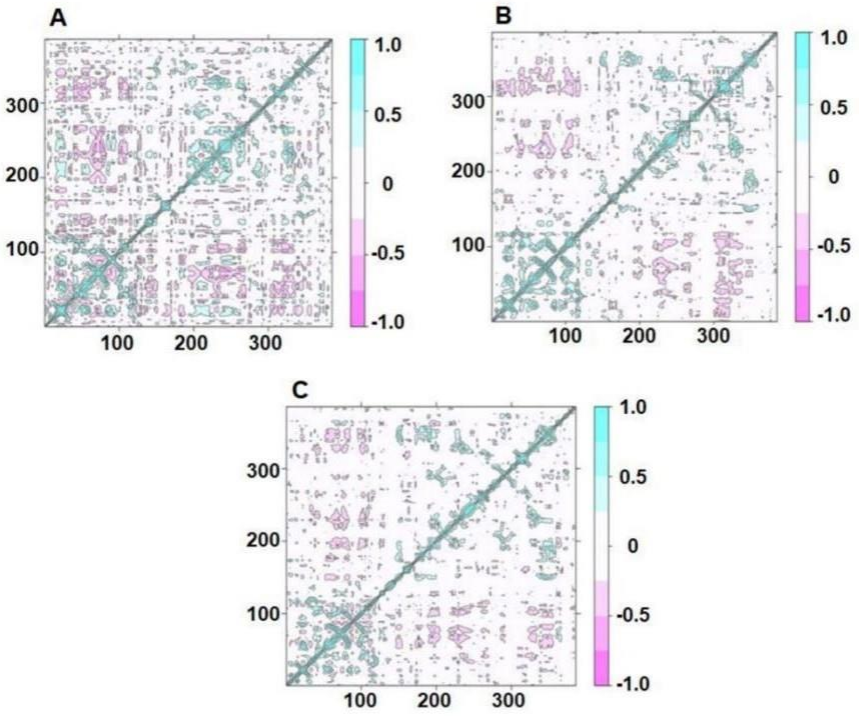

Figure 7. Computed cross-correlation matrix analysis of BACE1 residue fluctuations for (A) BACE1—Apo, (B) BACE1—CNP-520, and (C) BACE1—AM-6494.

activity of diverse protein target (Chen et al., 2018). To gain further insight into the dynamics of the bound and unbound BACE1, principal component analyses (PCA) and dynamic cross-correlation matrix (DCCM) were performed for the trajectories of the different systems.

To investigate the internal dynamics alterations of BACE1 induced by the binding of AM-6494 and CNP-520, the cross-correlation matrices of Cα atom fluctuations were computed (Figure 7). The DCCM was used to evaluate the negative and positive correlation effect difference of amino acids upon inhibitor binding. The positive (white to cyan; a scale of 0 to 1) and negative (white to pink; a scale of 0 to −1) shows residue regions reflecting correlated and anti-correlated motions, respectively, between specific residues (Figure 7).

The unbound BACE1 mainly displayed intensely mixed (both negative and positive) correlation spectrum in the entire residue (Figure 7(A)). The bound BACE1, however, showed significant alteration with reduced positive and negative correlated residual motion (Figure 7(B,C)). The bound conformation of AM-6494 and CNP-520 induced significant anti-correlated residue motion between residues 200-385 (D-loop and F-loop regions). The observed reduction in the positive and negative correlation of the bound BACE1, as opposed to the unbound conformation, suggests a substantial conformational change upon inhibitor binding.

To further support the conformational rearrangement of BACE1 induced by the binding of CNP-520 and AM-6494 inhibitors, the first two principal components (PCs) were analysed for all Cα atoms and results presented in Figure 8. The PCA allows the projection of protein dynamics on a set of patterns, which is diagonalised to obtain a set of eigenvalues (magnitude) and eigenvectors (direction) of the coordinated movement of the protein (Hosen et al., 2019). The top 20 principal components (PCs) of the Apo, CNP-520 and AM-6494 systems accounted for 77.3%, 76.5% and 73.8% of the total proportion of variance of the simulation, respectively. The first two PCs also accounted for 39.6%, 37.9% and 29.9% of the total variance for Apo BACE1, CNP-520 and AM-6494 systems, respectively. The first few PCs are enough to describe the majority of the atomic fluctuations in a sample system conformation (de Groot et al., 2001). The conformational ensembles of the studied systems were further clustered into two-dimensional (2 D) subspace of the first two principal components, PC1 and PC2 (Figure 8). The conformational changes of Apo BACE1 displayed larger conformational distribution in phase space than the bound state. Thus, the inhibitor bound state results in a lower conformational change, which is more prominent in AM-6494 binding.

3.6. Dynamic interactions and binding modes of AM-6494 and CNP-520 at BACE1 binding site

To explore the protein-inhibitor interactions of the selected inhibitors, three averaged representative binding modes AM-

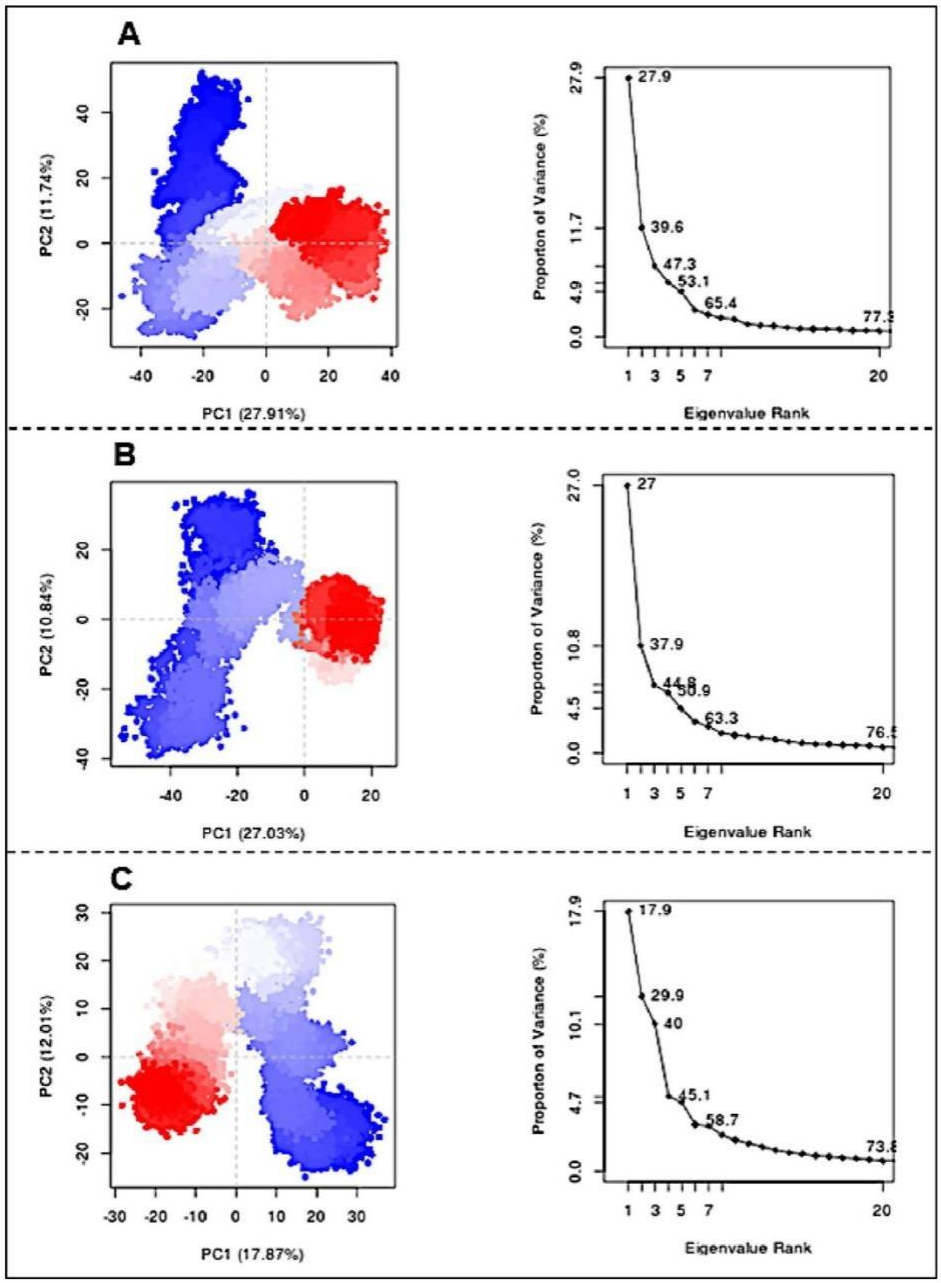

Figure 8. Clustering and principal component analysis plots result from the MD simulation for (A) BACE1—Apo, (B) BACE1—CNP-520, and (C) BACE1—AM-6494 trajectories.

6494, and CNP-520 at BACE1 active site were extracted over the simulation. As shown in Figure 9, AM-6494 formed seven hydrogen bonds with Thr232, Trp115, Ser229, Gly11, Asp32, Asp228, and Tyr14. Also, Tyr14 and Trp115 further formed π-π stacking interactions with the pyrazine ring and the 1,2-difluorobenzene ring of AM-6494, respectively.

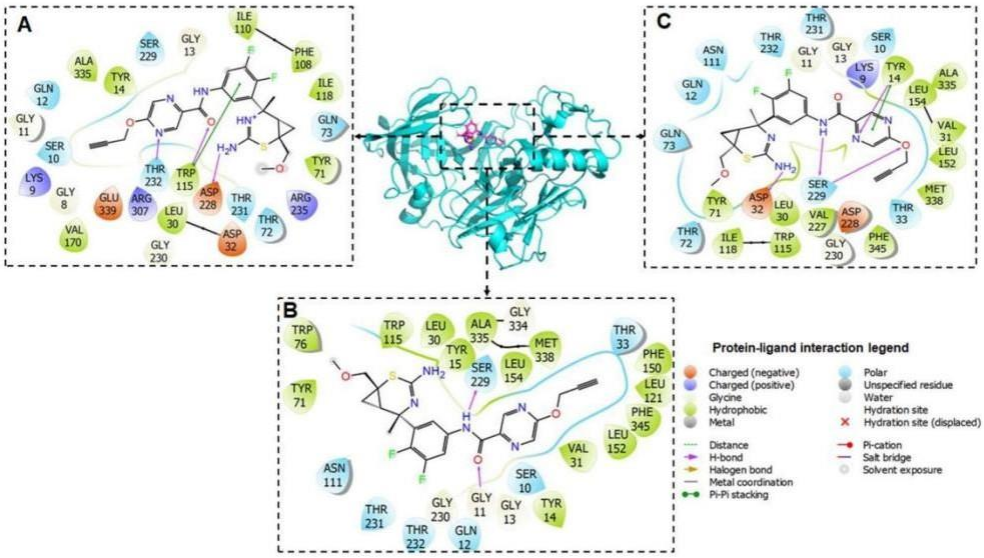

Figure 9. Representative binding interaction modes of AM-6494 with BACE1 extracted from the (A) initial, (B) intermediate, and (C) final stages of the simulation.

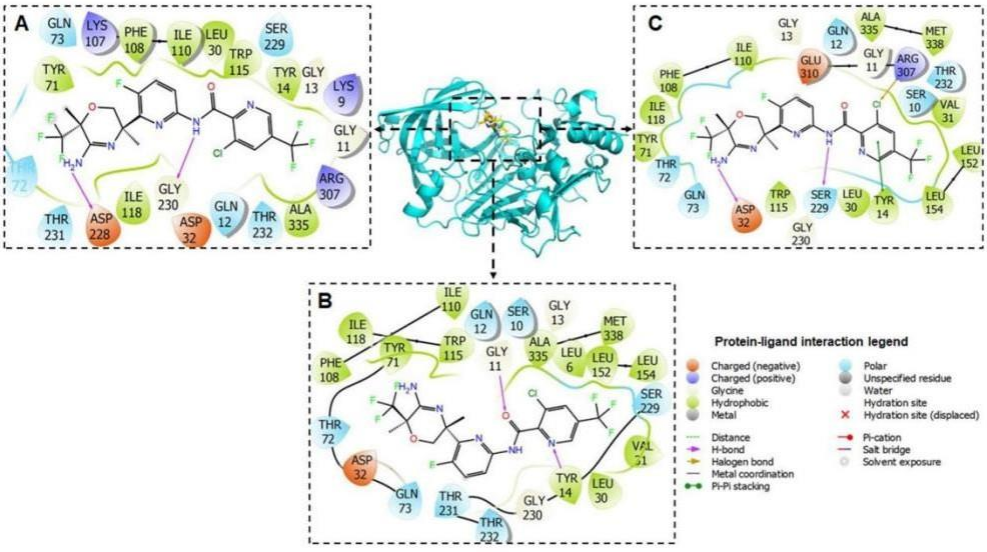

Figure 10. Representative binding interaction modes of CNP-520 with BACE1 extracted from the (A) initial, (B) intermediate, and (C) final stages of the simulation.

In CNP-520, six hydrogen bonds were observed to be formed with Gly230, Asp32, Asp228, Ser229, Gly11, and Tyr 14 (Figure 10). Similarly, Tyr14 engaged in π-π stacking interaction with the 3-chloro-5-(trifluoromethyl)pyridine moiety. Additionally, Arg307 was observed to form a halogen bond with the chlorine atom of 3-chloro-5-(trifluoromethyl)pyridine moiety. Active site residues such as Gly230, Tyr71, and Tyr14 were observed to be critical residues common to the studied inhibitors; similar to the per-residue energy decomposition analysis (Figure 3).

4. Conclusion

This study employed a series of computational techniques to investigate the binding mechanism of the newly identified preclinical inhibitor AM-6494, which has shown high potency

and selectivity towards BACE1 relative to umibescestat. The study combined cMD simulations, aMD simulations, binding free energy analysis and diverse computational analytical approaches. Results from the binding free energy analysis revealed that AM-6494 displayed a higher binding affinity, which is mainly controlled by van der Waals and electrostatic interaction energies compared with umibescestat. The dynamic binding conformations of AM-6494 are characterised predominantly by the closure of the flap tip-dyad maintaining high residency of AM-6494 compared to umibescestat, which alternates between closed and semi-closed conformation. Thus, AM-6494 ability to induce an effective flap closure is associated with its inhibitory activity compared to CNP-520. Conformational variations revealed by PCA indicate that Apo BACE1 displayed larger conformational distribution in phase space than the bound states which were more prominent with AM-6494 binding.

Furthermore, the residue-based decomposition of the free energy identified Tyr14, Leu30, Tyr71, Trp115, Leu154, Ser229, and Gly230 to make substantial contributions towards the binding of both AM-6494 and CNP-520 at BACE1 binding site. On the other hand, the higher affinity of AM-6494 compared to CNP-520 at BACE1 may be attributed to the differential contributions of Gly13, Val31, Leu152, and Thr231 residues. Taken together, the results highlighted in this study offer atomic mechanistic insights into the preferential binding interaction mechanisms of the preclinical BACE1 inhibitor AM-6494 relative to CNP-520. The findings highlighted in this study provide a basis to explain AM-6494 high inhibitory potency and structural changes to BACE1 binding compared to CNP-520, which may assist in the future therapeutic design of BACE1 inhibitors.

Disclosure statement

The authors declare no conflicts of interest in this work.

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CHAPTER SEVEN
CONCLUSION

Conclusion and Recommendation

Alzheimer's disease, as a progressive multifactorial neurodegenerative abnormality, tremendously affects the memory of persons living with it. The mechanism involved in neurodegeneration is often related to the malfunctioning of the neurons situated in the brain cerebrum (significant for cognitive functions). This malfunction resulted in impaired neurotransmission and failed communication network, which manifests as clinical dementia. In addition, the aggregation of misfolded β-amyloid oligomeric plaques is majorly responsible for neurotoxicity in Alzheimer's disease. These accumulated β-amyloid plaques are products of the subsequent splitting of amyloid precursor protein (APP) by γ-secretase and β-secretase. The β-secretase, also known as BACE1 (β-amyloid precursor protein cleaving enzyme1), has been a primary ongoing treatment focus in Alzheimer's disease-modifying therapies since its discovery in 1999.

Having well-informed knowledge of BACE1 inhibition mechanisms is imperative in achieving effective and efficient drug design and development. Unfortunately, the Food and Drug Administration (FDA) has approved only five drugs (donepezil, galantamine, rivastigmine, memantine, and tacrine that have been withdrawn due to hepatoxicity) for AD treatment. Sadly, none of these drugs is BACE1 inhibition focused. Therefore, scientists and pharmaceutical companies are making relentless efforts in designing some small drug molecules capable of good BACE1 inhibition. Some of the initially discovered BACE1 inhibitors include verubecestat, lanabecestat, atabecestat, and umibecestat. Sadly, these inhibitors at phase 3 clinical trials significantly lowered β-amyloid plaques-associated in patients with neurological AD but were terminated at the clinical trials for lack of potency and some drug-related side effects. This termination has resulted in insufficient drug development and discovery targeted at BACE1 despite the high demand for neurological dementia and AD therapies.

Sequel to the failed BACE1 inhibitors at the clinical trials, many debates and doubts have been sparked off on the critical and authentic roles BACE1 plays in AD remediation (1). However, several studies on BACE1 inhibition focusing on structure-based functionalities using experimental and corresponding computational methods have proven the importance of BACE1 in AD management. The process involved in designing active BACE1 inhibitors appears more complex than the researchers had envisaged. The small molecule size compounds to overcome the BBB, compounds with high specificity and selectivity for BACE1 and, the numerous peptide bond interactions at the binding sites are essential properties to be considered in the design of the BACE1 inhibitors. When in contact with the ligand (open and close dynamics), the active site

critical roles are also significantly considered. Several computational methods and interventions have

helped researchers monitor the structural dynamics, different conformational orientations, and the open and close dynamics of the flap. It has also been reported that measuring the Cα atoms and the tip of the flap (β-hair loop) distances relative to one of the aspartate dyads is the most popularly used metric (2-4). This measurement was substantiated in our study on flap dynamics of the AM6494 and CNP520 in chapter five. Another significant feature close to the active site is the S3 sub-pocket. It has the vital characteristic function of engaging the ligand with the BACE1 thereby resulting in higher selective and potent inhibition (5). More so, hydrophobic species of an aromatic ring at the meta position enhance the interaction with the S3 sub-pocket. When a strong moiety (for example, fluorine) with higher electronegativity is present within the active site, it establishes a good interaction with the S2 sub-pocket.

In this study, we also carried out a thorough, intensive, and advanced computational investigation of a novel potent, orally effective, and highly selective AM-6494 BACE1 inhibitor discovered recently. This novel BACE1 inhibitor exhibited no fur coloration and common skin alteration, as observed with some initial BACE1 inhibitors. AM-6494 with an IC50 value of 0.4 nM *in vivo* is presently selected and at the preclinical phase trials. Before this study, the inhibition properties of this novel BACE1inhibitor at the atomistic and molecular level of BACE1 inhibition remained very unclear. We extensively explored the computational tools of QM and QM/MM techniques in the investigation of the novel AM-6494 and the recently discontinued CNP-520 BACE1 inhibitors. The computational investigations were performed using DFT (B3LYP/6-31+G) and a two-layered model of ONIOM at B3LYP/6-31+G (d): AMBER level of theory. The results gave estimations of - 19.3 kcal/mol (AM-6494) and -14kcal/mol (CNP-520) solvation energy values, respectively. The inhibitor binding site evaluation was also done using the molecular electrostatic potential (MESP) plot and the Fukui function analysis for electrophilic attraction availability. The QM computation binding sites predicted N9 for AM-6494 and N23 for CNP-520 interaction with the Asp active units while establishing classical hydrogen bonding. The further calculation for the interaction of both inhibitors (AM-6494 and CNP-520) in the active pocket of the enzyme was done using the ONIOM model with both protonated and unprotonated states of Asp32 in the BACE1 structure. From the analysis of the results, both AM-6494 and CNP-520 established active interaction with BACE1 using residues Lys9, Gly11, Gly13, Tyr14, Leu30, Asp32, Gly34, Ser35, Tyr71, Lys107, Phe108, Trp115, Ile118, Val170, Asp228, Ser229, Gly230, Thr231, Thr232, Arg307, Ala335, and Glu339. There is a strong indication that these residues are vitally important to inhibitor binding in the active pocket of BACE1. The BACE1-inhibitors complexes were studied when Asp 32 and Asp 228 residues were treated at the QM high level while the other active site residues at the MM level of theory. It was observed that both inhibitors established favorable non-bonding interactions with BACE1, which enabled the complexes' stability. Further investigation of the binding thermochemistry showed that AM-6494 recorded higher interaction energy ((ΔE = - 87.6 kcal/mol)) as well as higher enthalpy ((ΔH = - 92.23 kcal/mol)) values. These findings

suggest that AM-6494 showed a better binding affinity and selectivity towards the BACE1 enzyme than CNP-520. We also observed that the mono protonation of Asp 32 produced slightly better energy when compared to the unprotonated system in BACE1. This observation elucidates that having a protonation or un-protonation of one of either Asp 32 or Asp 228 would enhance a better binding affinity with the inhibitors. Additional computational analysis showed that electronic fine-tuning of the peptide connections in an inhibitor could result in a better selective and potent inhibition. As a result, we suggest that good knowledge of interatomic ligand-BACE1 complex interactions, established through computational investigations would assist in pharmacophore development.

In another separate study, we employed advanced computational methods to investigate the binding dynamism of the novel AM-6494, which has been proven to have high potency and selectivity relative to CNP-520. These computational methods include conventional and accelerated molecular dynamics simulations, binding free energy analysis, and diverse computational analytical approaches. The investigation results substantiated that AM-6494 again showed higher binding affinity, predominantly made of van der Waals and electrostatic interaction energies relative to CNP-520. In addition, AM-6494 also maintained mainly a closed flap related to its inhibition potential compared to CNP-520. The principal components analysis (PCA) also unveiled that the BACE1 apo showed more excellent conformational distribution in phase space than the bound state, especially when bound with AM-6494. More so, the residue-based decomposition of the free energy revealed Tyr14, Leu30, Tyr71, Trp115, Leu154, Ser229, and Gly230 to make substantial contributions toward the binding of both AM-6494 and CNP-520 at the BACE1 binding site.
On the other hand, the higher affinity of AM-6494 compared to CNP-520 at BACE1 may be connected to the differential contributions of Gly13, Val31, Leu152, and Thr231 residues. Altogether, the findings from this study further provide insights into the atomic binding interaction mechanistic preferences of the preclinical BACE1 inhibitor AM-6494 relative to CNP-520. It also provides a basis to explain AM-6494 high inhibitory potency and structural changes to BACE1 binding compared to CNP-520, which may assist in the future therapeutic design of BACE1 inhibitors.

Although there is no approved BACE1 inhibitor for AD treatment, BACE1 inhibitor design and development status is promising. Sequel to the following two separate computational studies, we discovered that the probability of identifying potent candidate(s) is high. Previous research has employed *in silico* methods in identifying compounds with better activity/affinity than the failed and clinical trial BACE1 inhibitors. As a result of this, we suggest that if any of these compounds are further analyzed, they might be good anti-AD agents, and more studies in this direction should be supported. Furthermore, For an

efficient and effective BACE1 inhibitor design at the theoretical level, researchers should exacerbate screening and docking with enhanced modeling techniques such as MD and QM/MM simulations or *in vitro* assay to provide details on the binding mechanism inhibitor.

Following the failures of some of the discontinued BACE1 inhibitors at the human clinical trials, we reviewed BACE1 biological and genetic properties, its therapeutic option, and exosites binding activities. We suggested that detailed elucidation of BACE1 biological roles could potentially better understand the associated diseases' mechanisms toward its therapeutic inhibition in AD. Therefore, we proposed further research on the identified peptidomimetics and non-peptide BACE1 allosteric inhibitors and antibodies. Such investigations could guide improved anti-AD drug discovery and development. In addition, we recommend further laboratory and computational investigations on BACE1 allosteric sites and exosites determination. The outcome would potentially stimulate a consensus on the secondary space available for binding on the enzyme. Therefore, a comprehensive theoretical perspective would likely unveil the interatomic and molecular properties of allosteric BACE1 inhibition. Such detailed knowledge would assist preclinical and subsequent clinical trial advancement on BACE1 targeting.

Although multi-directed ligand design for BACE1 and other AD targets are available, no BACE1 MTDL has made it to preclinical trials as the approach comes with complications, time, and resource-intensive. However, there is a need to push BACE1 MTDLs to preclinical and clinical trials to establish this approach. Researchers have explored single targeting compounds with specificity and selectivity for the BACE1 active site. We noticed little attention on targeting allosteric regions or exosites. We also reviewed allosteric site inhibition and exosite antibody for BACE1. The outcome revealed and substantiated that inhibiting BACE1 in other regions and not just the catalytic active domain is feasible and tenable. We at this moment suggest further exploration of the exosite both experimentally and theoretically as this might produce compounds that might be successful at the human clinical trials.

Finally, the implementation of computational techniques in the designing of BACE1 inhibitors has been quite interesting. Nevertheless, the designing of potent BACE1 inhibitors through the computational application of the QM method such as the density functional theory (DFT) method has been scarcely employed. We recommend the further and extensive application of the QM technique in developing and designing potent BACE1 inhibitors. We also recommend that governmental and non-governmental organizations help financially with computational resources to assist and enable more quality research on BACE1 inhibitor design. We further recommend drug repurposing giving the role it has played in the fight against the covid-19 virus, and it can as well be replicated in the search for approved BACE1 inhibitor

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