

Research

Rationalizing the crosslinking reaction of an α,β unsaturated carbonyl of clovamide from *Adansonia digitata* L. and the cysteine residue of HIV-1 integrase enzyme

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Abstract

In this study, clovamide was identified for the first time in *Adansonia digitata* L. fruit pulp using the UHPLC-q-TOF-MS. The inhibition potential of the naturally occurring clovamide, specifically in the *SE* and *SZ* configurations and their yet to be identified enantiomers (*RE* and *RZ*) on HIV-1 integrase (HIV-1 INT) were investigated using molecular docking studies. The results revealed that all the four stereoisomers of clovamide bind to key residues crucial for the catalytic activity of HIV-1 INT (ASP64, ASP116 and GLU152) as well as other significant residues including, LYS152 and LYS159. This indicates that clovamide has the potential to inhibit this enzyme and possibly slow down HIV-1 replication. Interestingly, the docking results showed that CYS65 was in close proximity to ASP64, allowing nearly all isomers of clovamide to interact with this residue. This suggested a potential crosslinking reaction via Michael addition between clovamide and CYS65. The consistent proximity of all ligands to CYS65 in the studied protein throughout the entire molecular dynamics simulation period also showed the potential of permanent covalent bonds formation via a Michael addition reaction. Density functional theory modelling confirmed that the α,β -unsaturated carbonyl group of clovamide and cysteine interact, forming a clovamide-integrase adduct, potentially leading to irreversible inhibition of HIV-1 INT. This study not only highlighted the potential of clovamide as an inhibitor of HIV-1 INT but also demonstrated that clovamide possesses various functional groups that can be exploited in different biological activity studies. Findings of this study suggest that clovamide and its stereoisomers could be valuable candidates for the development of new antiretroviral therapies, offering a novel approach to overcoming drug resistance through multiple inhibition mechanisms.

Keywords Clovamide · Hydroxycinnamic acid amides · HIV-1 integrase · Molecular docking · Density functional theory · Michael addition · Inhibition mechanism

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1 Introduction

Clovamide also known as (2S)-3-(3,4-Dihydroxyphenyl)-2-(1-amino) propanoic acid, *N*-caffeoyldopamine or *N*-caffeoyl-L-DOPA is a naturally occurring caffeic acid derivative that belongs to the hydroxycinnamic acid amides (HCAAs) subclass of polyphenols [1]. Clovamide exists naturally as *E* (*trans*) and *Z* (*cis*) isomers due to the presence of the double bond in the caffeoyl moiety and these isomers have been identified in some plants [2, 3]. Moreover, this molecule can exist as enantiomers (*R* and *S* configurations) due to the presence of a stereogenic centre in this compound, although the *S* enantiomer has been reported to be the one occurring naturally in plants [4, 5]. The primary source of clovamides in the human diet is chocolate and other cocoa products which are derived from some plants of the *Theobroma* genus [6, 7]. However, clovamides have been also reported in other plants such as *Vernonia fastigiata* [2, 4], *Acmella oleracea* [3] and *Trifolium pallidum* [8]. HCAAs are known to be part of the defence machinery of plants against biotic and abiotic stress [9]. Besides this important role these compounds play in plants, a HCAA clovamide has been reported to exhibit a wide range of biological activities such as, antioxidant [4, 6], anti-inflammatory [10] and neuroprotective [11] properties. Due to these diverse biological effects, clovamide and its analogues have attracted significant research interest as potential drug leads in recent years.

The search for natural products to manage notorious viruses, including HIV-1 integrase, an enzyme crucial for viral replication and the development of acquired immune deficiency syndrome, remains an active area of research [12]. Although the currently available HIV-1 INT enzyme inhibitors and other therapeutics have significantly reduced the mortality rates from HIV, there is still no cure for this disease. Most importantly, access to these expensive drugs is also limited in other parts of the world, including many African countries. Therefore, the search for novel, cheap and easy to access therapeutics continues, particularly those targeting alternative binding sites on HIV-1 INT. This is critical since the currently available inhibitors bind directly to the active site, which is susceptible to mutations leading to drug resistance [13]. Naturally occurring caffeic acid containing molecules such as chlorogenic acids (CGAs) [14] and chicoric acid [15] have been shown to bind to HIV-1 INT enzyme and sometimes this inhibition has been reported to be irreversible [16]. Nonetheless, the mode of action mechanism on HIV-1 INT enzyme of these caffeoyl containing compounds is still not fully understood. Understanding the mode of action of potential therapeutic agents is essential in drug discovery processes for more targeted and effective treatment. Recently, clovamide was virtually explored for its antiviral properties, showing potential in managing a virus-related disease COVID-19 [5]. One possible mechanism responsible for clovamide's antiviral properties is the Michael addition reaction [16]. This reaction occurs between a cysteine residue of the virus and clovamide, forming covalent bonds, wherein the α,β -unsaturated carbonyl group of clovamide is deemed as a potential Michael addition acceptor during this crosslinking reaction [17]. To the best of our knowledge the inhibitory activity of clovamide against HIV-1 INT has never been studied.

In this study, *trans*-clovamide (*SE*), identified for the first time in *Adansonia digitata* L. fruit pulp was investigated for its potential to inhibit HIV-1 INT using molecular docking studies. Three other possible stereoisomers of clovamide (*SZ*, *RE* and *RZ*) were studied, given that isomerism can have important biological implications. Molecular dynamics simulations were used to examine the stability of the ligand-protein complex and to further explore the interactions between clovamide stereoisomers and the HIV-1 INT enzyme. The mechanism of HIV-1 INT inhibition was explored through density functional theory (DFT) calculations, with particular emphasis on the binding interaction between clovamide and the free cysteine residue (CYS65) of HIV-1 INT. This knowledge will be useful in maximizing the therapeutic potential of natural products in the fight against HIV and other viral diseases.

2 Material and methods

2.1 Sample preparation and metabolite extraction

Adansonia digitata fruits from plants growing in the wild were collected from Manenzhe, Limpopo, South Africa were air dried until they were completely dry. Samples were prepared and extracted according to a previous study by Moyo et al. [18]. The obtained pulp was ground and kept at room temperature until ready for extraction. The fruit pulp samples (2 g) were extracted overnight by shaking on a digital rotisserie tube rotator (Dlab Scientific, Beijing, China) at 70 rpm in 20 mL of 80% methanol. A benchtop fixed angle centrifuge (Thermofischer, Johannesburg, South

Africa) was used to centrifuge the crude extracts at 2739 *g* for 20 min. The samples were filtered through 0.22 μm nylon filters into HPLC vials and kept at 4°C until analysis on the UHPLC-q-TOF-MS.

2.2 Chromatographic separation

The *A. digitata* sample extracts were analyzed using a liquid chromatography-quadrupole time-of-flight tandem mass spectrometer (LC-MS-9030 q-TOF, Shimadzu Corporation, Kyoto, Japan) according to a method established in a previous study carried out by Moyo et al. [19]. Secondary metabolites were separated using a Shim-pack Velox C₁₈ column (100 × 2.1 mm, 2.7 μm) (Shimadzu Corporation, Kyoto, Japan) maintained at 55°C. A volume of 5 μL was injected into the instrument and the compounds in the fruit pulp extracts were separated using a binary mobile phase gradient elution at a flow rate of 0.3 mL min⁻¹. The mobile phases A and B contained 0.1% (v/v) formic acid in ultrahigh purity water and methanol, respectively. From 0 to 3 min, the mobile phase composition was 5% mobile B and it was kept constant until 8 min. The composition of mobile B was increased to 40% at 8 min and then to 95% between 23 and 25 min. At 27 min, the gradient was altered to 5% mobile phase B and was held at this composition until 30 min. A q-TOF high resolution mass spectrometer with an electrospray interface (ESI) operating in negative ionisation mode was employed for mass spectral analysis. The parameters of the mass spectrometer were set as follows: interface voltage of 4.0 kV, interface temperature of 300°C, nebulization and dry gas flow of 3 L min⁻¹, heat block temperature of 400°C, DL temperature of 280°C, detector voltage of 1.8 kV, and flight tube temperature of 42°C. To monitor high mass accuracy, sodium iodide was utilized as a calibration solution. MS¹ and MS² were generated concurrently (using data-dependent acquisition) for all ions with *m/z* values ranging from 100 to 1000 Da and an intensity threshold greater than 5000. Argon was utilized as a collision gas in the MS² runs, with a collision energy of 30 eV.

2.3 Molecular networking and metabolite annotation

A classical molecular network was generated on the GNPS ecosystem (accessed on 16 Jun 2024). Raw data from the Shimadzu LCMS-9030 q-TOF was converted to an open-source format (.mzML) before it was uploaded to the online workflow. The precursor and MS² fragment ion mass tolerances were set at 0.02 Da. For spectral similarity, the cosine score was set to 0.6 with a minimum of 4 matched fragments. The generated molecular network was visualized on the cytoscape software [20]. The tentative identification of clovamide was confirmed by its molecular formula generated from the accurate mass based on MS/MS data of this compound. Additionally, this putative annotation was compared to literature findings.

2.4 Molecular docking studies

Clovamide stereoisomers and the catalytic domain of HIV-1 INT (PDB code: 1QS4) were prepared in YASARA Structure [21] (version 20.7.4) and molecular docking was performed using Autodock Vina [22]. Structural preparation and docking were carried out as described in previous studies by Makola et al. [14] and Nobela et al. [15], thus briefly, 500 docking runs were performed for each compound using the Autodock Vina local search algorithm with default settings. Afterwards the docking poses obtained were clustered using a root mean square deviation (RMSD) of 5 Å cut-off. Finally, interaction analysis of the clusters was carried out using YASARA and Python scripts. Each docking run produced 400 binding configurations that were then clustered using a RMSD cut-off less than 5 Å. An X-ray crystal structure of the inhibitor 5CITEP, 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone, bound to the catalytic domain of HIV-1 INT (PDB: 1QS4) was used as receptor and reference ligand [23]. The crystal structure (bound to its inhibitor, 1QS4) was overlaid with the most favorable docking poses to confirm that binding occurred at the expected site.

2.5 Molecular dynamics

The protein alone together with four protein ligand complexes were parameterized using the Amber ff19SB force-field [24] for the protein and the GAFF2 force-field for the ligands. The system was solvated and neutralized with chloride anions. The coordinate files and parameter files were converted to gromacs format using Acpype [25]. Gromacs was used for all molecular dynamics simulations [26]. The initial equilibration was for 10 ps at 300 K. Production dynamics was for 100 ns. All systems were analyzed looking at RMSD for the protein and ligand, and root mean square fluctuation (RMSF)

and principal component analysis (PCA) for the protein. The distance of the β -carbon of the ligand (of the α,β -unsaturated system) to CYS65 was also monitored through the simulation.

2.6 Density functional theoretical study

All calculations were performed using the density functional theory in the framework of the M062-2X functional. The hybrid meta-GGA functional is known for producing meaningful estimates of the geometries, barrier heights and thermodynamic properties related to molecules containing main group elements [27–29]. The standard 6-31 + + g (d,p) basis set, which include both polarization and diffusion basis functions has been selected for the investigation as it is a compromise between accuracy of results and computational cost for the system under investigation. Frequency calculations were performed using the same functional and basis set in order to characterize the type of stationary points found as well as to obtain the thermodynamic properties. Reaction transition states were obtained using the synchronous transit-guided quasi-Newton method. To confirm if the obtained transition state connects the reactant species and product species, the intrinsic reaction path analysis approach [30] was utilized. All calculations were performed using the Gaussian software program [31].

3 Results and discussion

3.1 Clovamide characterization using the UHPLC-q-TOF-MS and molecular networking

Over the years, there has been tremendous growth in research on natural products that can manage the increasing number of diseases that pose a threat to public health, including HIV. *A. digitata* has been reported to possess many ethnomedical uses [32, 33], however, the richness of its phytochemistry is still not well studied. In this study *trans*-clovamide at m/z 358.092 $[M-H]^-$ (Fig. 1B) was identified for the first time in *A. digitata* extracts using the UHPLC-q-TOF-MS and classical molecular networking approach. This compound was characterized by a precursor ion $[M-H]^-$ at m/z 358.092 and corresponding fragment ions at m/z 178 and 135 (Figure S1), characteristic of the loss of the deaminated 3,4-dihydroxyphenylalanine fragment and the presence of a vinyl-catecholic moiety, respectively [2, 7, 34]. Moreover, clustering of this compound with other clovamide-like compounds through molecular networking as shown on Fig. 1B further reinforced the correct identification of this compound. A key advantage of molecular clustering in molecular networking, where compounds with similar gas-phase chemistries are grouped together, is that it significantly simplifies the identification of new compounds in complex samples, including plant extracts [35–37]. This approach enhances the detection of compounds that might otherwise be overlooked through manual annotation, particularly in samples containing a diverse range of chemical entities as illustrated on Fig. 1A. A clovamide-like compound, coumaroyl-*N*-tyrosine has been

Fig. 1 A hydroxycinnamic acid amides cluster observed in *Adansonia digitata* using molecular networking generated from the UHPLC-q-TOF-MS data (**A**) full molecular network showing the whole metabolic profile of *Adansonia digitata* (**B**) a molecular family of clovamide-like compounds contained in this plant grouped according to their shared gas phase chemistries. Clovamide (m/z 358.092) is indicated by the enlarged node in yellow

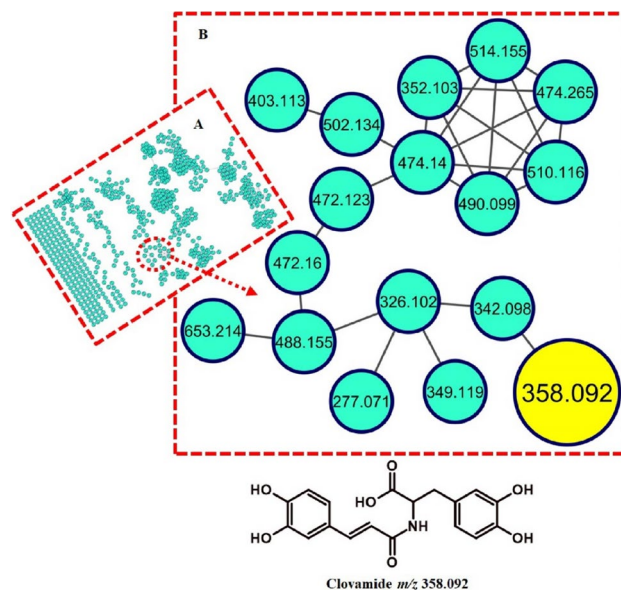
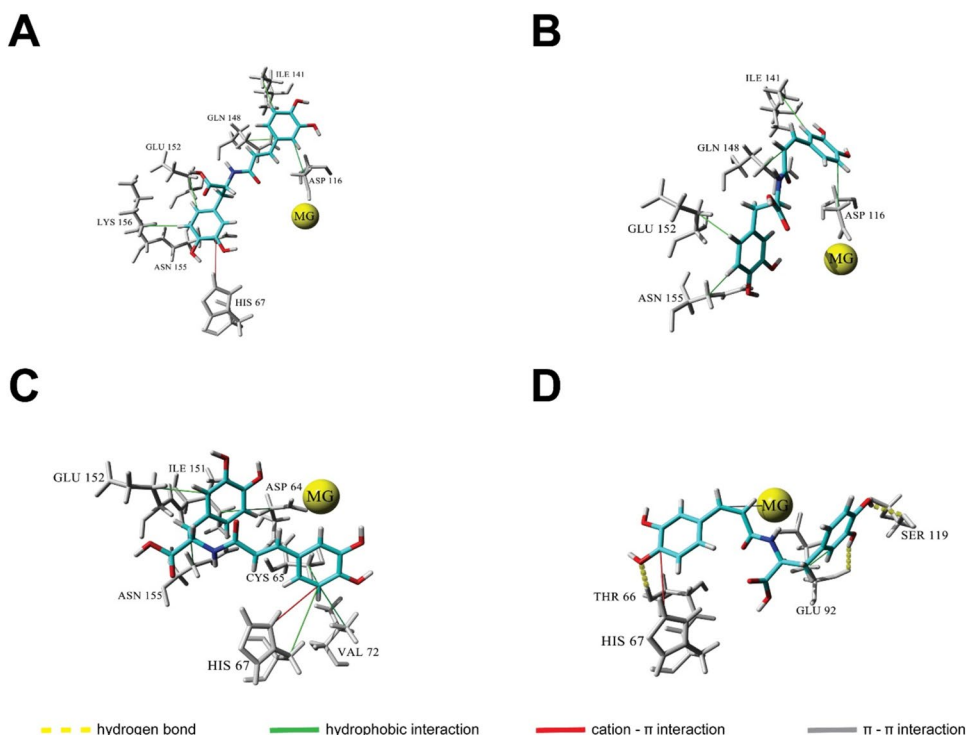


Table 1 Docking clusters for clovamide stereoisomers ranked according to binding energy

Cluster Rank	Binding Energy (kcal.mol ⁻¹)			
	<i>RE</i> -m06-b	<i>RZ</i> -m06-b	<i>SE</i> -m06-b	<i>SZ</i> -m06-b
1	-7.837	-7.792	-7.563	-7.595
2	-7.584	-7.592	-7.213	-7.539
3	-7.234	-7.587	-7.187	-7.405
4	-7.213	-7.23	-7.006	-7.404
5	-7.161	-7.107	-6.993	-7.366
6	-7.151	-7.04	-6.929	-7.116
7	-6.958	-6.887	-6.879	-6.856
8	-6.846	-6.748	-6.597	-6.695
9	–	-6.736	-6.526	–
10	–	-6.672	–	–

Fig. 2 Interactions of the clovamide stereoisomers with residues in the HIV-1 integrase catalytic domain in the representative docking poses for the top-ranked clusters. The *RE* (**A**), *RZ* (**B**), *SE* (**C**) and *SZ* (**D**) stereoisomers are indicated as sticks and coloured according to their constituent atoms (carbon as cyan, oxygen as red, hydrogen as grey and nitrogen as blue). The Mg²⁺ is included as reference. Hydrogen bonds (yellow dashed lines), hydrophobic (green solid lines), cation- π (red solid lines) and π - π (grey solid lines) interactions are indicated. Molecular visualization was performed using YASARA and POV-Ray



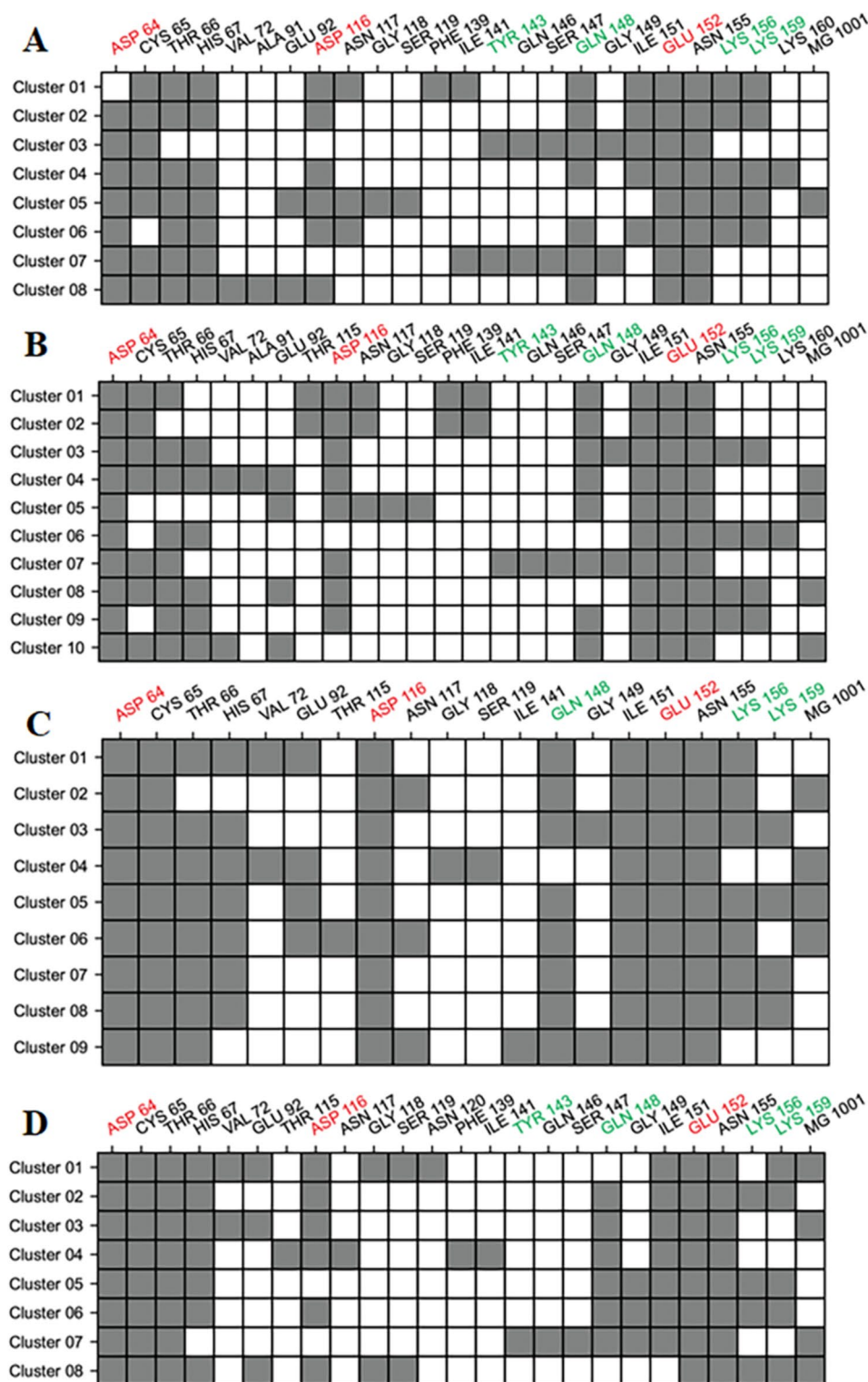
identified in *A. digitata* [33], suggesting that this plant could be an additional source of these specialized compounds, complementing the already known sources. *A. digitata* is already consumed in many countries where it is native due to its nutritional and ethnomedicinal uses [38, 39]. Therefore, identifying important compounds like clovamide and its analogues could further enhance the value of food products derived from these plants, particularly the fruit pulp.

3.2 Molecular docking studies

Molecular docking experiments to probe the potential binding capabilities of clovamide (*SE*) identified in *A. digitata* and its stereoisomers (*SZ*, *RE* and *RZ*) to HIV-1 integrase were performed according to a previous study conducted by Nobela et al. [15] with L-chicoric acid. Previously it was found that Autodock Vina was able to redock a known inhibitor 5CITEP, 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl)-propenone, with a score of -6.218 kcal.mol⁻¹ and a RMSD cut-off of 5 Å, however, the X-ray crystal structure position was only found in the second-best docking cluster [15]. In the current study, all clovamide-HIV-1 INT docking poses produced binding scores greater than that of the known inhibitor 5CITEP, the top clusters of all the clovamide stereoisomers produced scores greater than -7.00 kcal.mol⁻¹ (Table 1, Fig. 2

and Figure S2). Moreover, all the stereoisomers of clovamide in different clusters exhibited similar binding energies indicating that these compounds are most likely binding to HIV-1 INT in a similar fashion. The clovamide stereoisomers could cover a larger area of the HIV-1 INT binding site because of their larger size compared to 5CITEP, however all poses covered the position of 5CITEP (Figure S2 and S3). Additionally, all clusters contacted most of the important catalytic integrase residues (D64, D116 and E152) [40] and, selectively, other important residues in the integrase active site (Y143, Q148, K156 and K159) [41, 42] as shown on Fig. 3A-D through various interactions including cation - π , hydrogen bonds,

Fig. 3 Contacts of the clovamide stereoisomers with residues in the HIV-1 integrase catalytic domain in the representative docking poses for the top-ranked clusters. The contacts of the *RE* (A), *RZ* (B), *SE* (C) and *SZ* (D) stereoisomers are indicated. Important catalytic residues are highlighted in red and other important integrase residues are indicated in green



hydrophobic and π - π interactions (Fig S4-S7). K156 and K159 are important residues as they are near GLU152 and have been reported to disrupt viral DNA integration [43]. The results for the top cluster (cluster 1) as shown on Fig. 3B-D show that clovamide stereoisomers *RE*, *SE* and *SZ* interacted with all the residues that form part of the catalytic triad of HIV-1 INT mainly through hydrophobic interactions (Fig S6). This suggests that all these stereoisomers can inhibit HIV-1 integrase, even though naturally occurring clovamide exists primarily in the *SE* and *SZ* configurations. Thus, these compounds have potential as drug leads for managing the HIV infection. It is noteworthy that the *SZ* configuration can also occur naturally, as excessive exposure of plants to UV light may induce its formation [2]. However, in this study, it was not identified in *A. digitata* and understandably so because the fruit pulp of this plant is covered within a hard shell, making it hard for light to penetrate through. Previous research has indicated that the caffeoyl moiety in L-chicoric acid [15] and 3,5-dicaffeoylquinic acid [14] is the main pharmacophore responsible for inhibiting HIV-1 INT through molecular docking studies, as some interactions with catalytically important residues occur via the caffeoyl moiety. Additionally, Zhu et al. [16] reported that the catechol groups of dicaffeoylquinic acids were responsible for the integrase inhibition. It is interesting that in the current study, CYS65 was in close proximity to ASP64 as shown on Fig. 2C and Fig S8 for clovamide in the *SE* configuration which forms part of the catalytic triad of this enzyme. This suggests that clovamide has a high likelihood of also interacting with CYS65. CYS65 has been reported to be inside the HIV-1 INT active site [43, 44], therefore exploring interactions of potential HIV-1 INT inhibitors with this novel residue could provide new insights into the development of novel antiretroviral therapies. Dicaffeoylquinic acids have been reported to bind irreversibly to the HIV-1 INT presumably through the formation of a covalent adduct between these compounds and the integrase enzyme, making them potent inhibitors of this enzyme [16]. However, the mechanism leading to the formation of this covalent bond has not yet been identified. Building on recently published work conducted by Ye et al. [5], it has been shown that clovamide can interact with the cysteine residue of the main protease enzyme of the coronavirus, resulting in crosslinking (covalent bonding) through a Michael addition reaction. Therefore, based on the close proximity of CYS65 to ASP64, it is suspected in the current study that the sulfhydryl group of cysteine in HIV-1 integrase disrupts the enzyme's activity by facilitating a nucleophilic Michael addition reaction with the α,β -unsaturated carbonyl of clovamide, generating a clovamide-integrase adduct [5]. The molecular inhibition mechanism of HIV-1 INT by clovamide was further investigated using density functional theory calculations.

For the sake of the discussion, the dihydroxyphenyl closest to the stereogenic center of clovamide (from the L-DOPA moiety) will be referred to as DHP1 and the other dihydroxyphenyl from the caffeoyl moiety will be referred to as DHP2. The *RE* and *RZ* stereoisomers produced the best-scoring positions (Table 1 and Fig. 2A, B, Supplementary Fig. 2A-B and Supplementary Fig. 3A-B), although they are yet to be identified in nature. In both cases, one of the dihydroxyphenyl rings were positioned in a planar position within a hydrophobic pocket formed by D116, I141, N148 (Fig. 2A, B). This also orientated the other dihydroxyphenyl ring between the α 4-helix and the β 1-sheet, and in contact with the catalytically important integrase residues mentioned above. Additionally, for both stereoisomers, the DHP2 dihydroxyphenyl ring was positioned in the hydrophobic pocket. In fact, the first two clusters for the *RZ* stereoisomer show the positions of the two dihydroxyphenyl rings reversed, with DHP1 in the hydrophobic pocket. However, due to the geometric configuration of the molecule, DHP1 is bound perpendicularly to the plane of the enzyme surface (Fig. 2B). This position however shifts the DHP2 away from the backbone of CYS65 and preventing the formation of the hydrogen bond observed in the top cluster (Figure S5B).

With both the *SE* and *SZ* stereoisomers, the top clusters had one of the dihydroxyphenyl rings bound in the vicinity of the bound Mg^{2+} (Table 1 and Fig. 2C, D). The activity of integrase requires Mg^{2+} . In a study conducted by Esposito and Craigie [42], it was reported that viral DNA 3' processing is optimal when Mg^{2+} is used as a metal cofactor. However, the other dihydroxyphenyl ring was bound in a similar position as the *R*-stereoisomers, i.e. between the catalytically important residues of the integrase. Both the *S*-stereoisomers yielded clusters that had one of the dihydroxyphenyl ring bound in the hydrophobic pocket similarly to the *R*-stereoisomers, however due to configuration of the stereoisomers, this tends to shift the binding orientation of the other dihydroxyphenyl ring, which seemed to yield lower binding scores. Regardless of the contrasting binding poses and scores, all the investigated isomers were found to interact with the CYS65 residue. This led to the hypothesis that a crosslinking reaction between clovamide and this cysteine residue can occur, as previously demonstrated with a different enzyme [5].

3.3 Molecular dynamics

During production dynamics the *apo*-protein and the *RZ* complex were the first to equilibrate (as per RMSD), while the other complexes equilibrated later during simulation. The protein residue RMSF was relatively similar for all

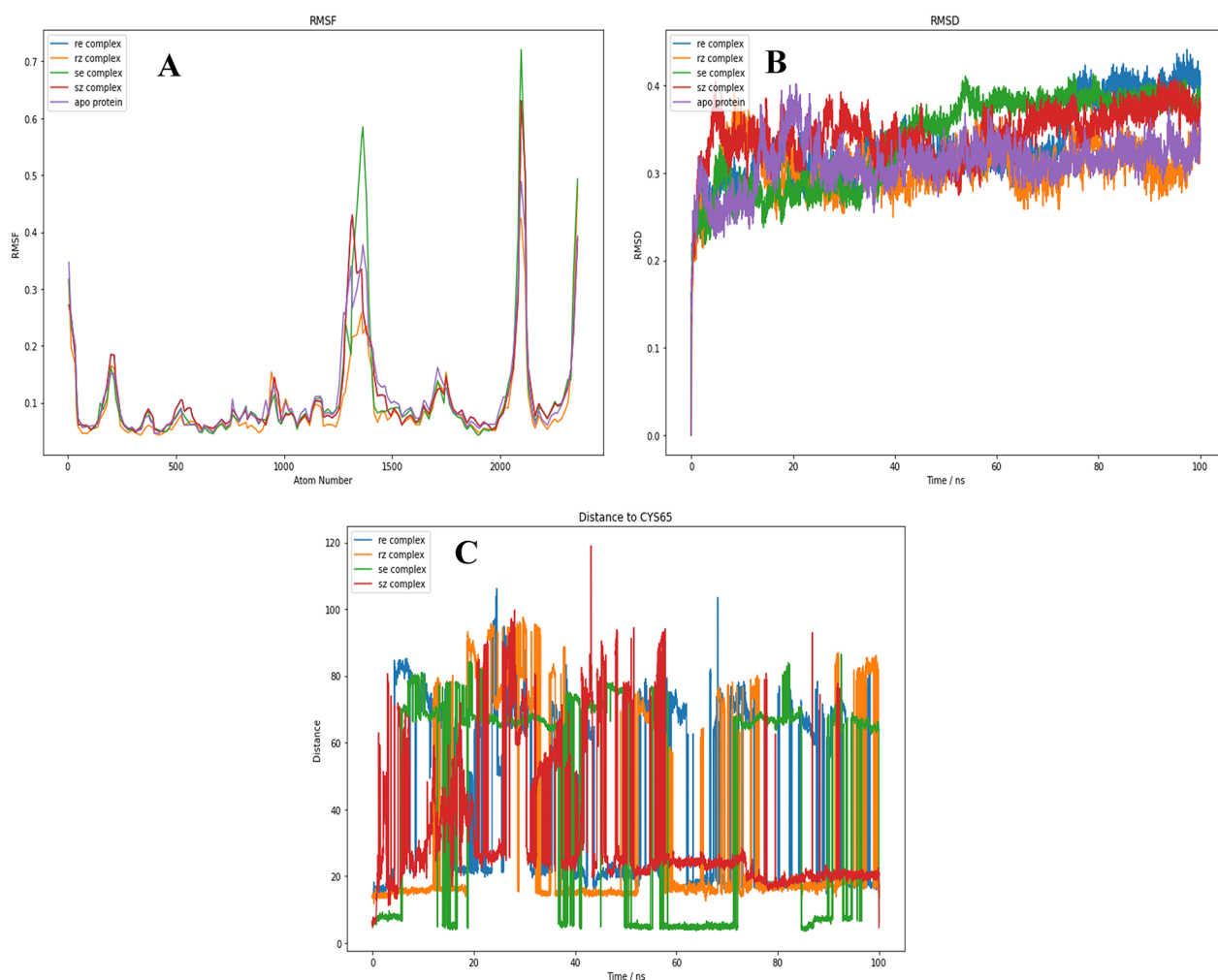
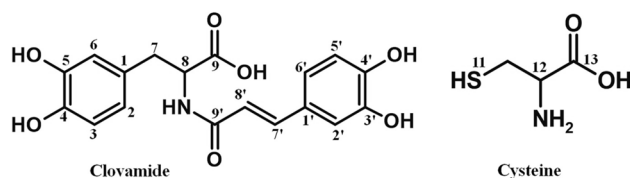


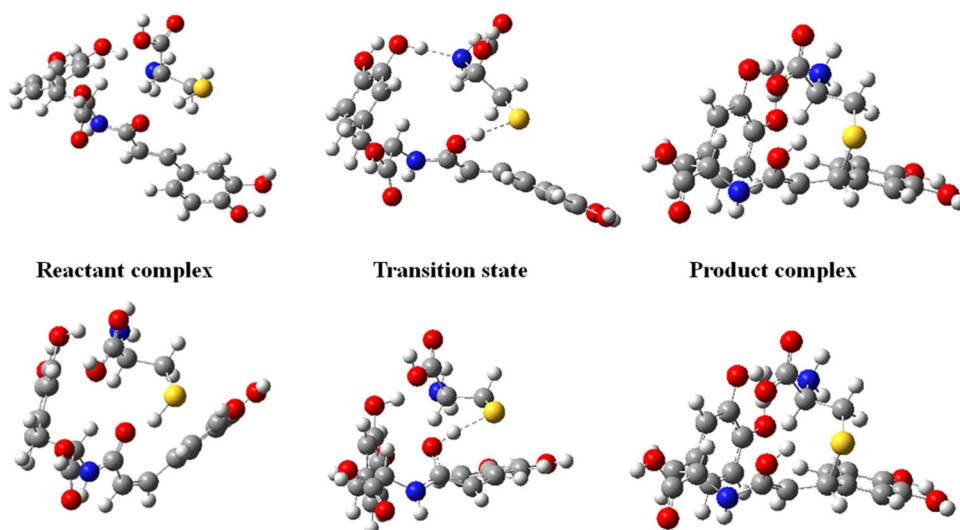
Fig. 4 (A) RMSF, (B) RMSD and (C) ligand distance (to CYS65) plots during 100 ns of molecular dynamics simulation

Fig. 5 Schematic representation of the clovamide molecule and the cysteine molecule along with the atom numbering utilised in the discussion



five simulations. It was noticed, however, that the respective ligands underwent exchange at the site during the simulation and as such the distance (from the β position of the α,β -unsaturated system to the sulfur group of CYS65) was monitored for all ligands during dynamics. In particular, the *SE* ligand was at a large percentage of the simulation as a close proximity to CYS65 suggesting that the Michael addition of sulfur to ligand would be facile particularly in this case (Fig. 4). Following formation of this bond (which this simulation cannot render) the ligands will permanently inhibit the enzyme. Most importantly, although it has been noted that not all stereoisomers occur naturally, the results of this study, using PCA, reveal that each ligand displayed distinct motion throughout the 100 ns simulation. This suggests that each isomer follows a unique yet consistent trajectory towards forming a covalent bond with the proteins (Figure S9).

Fig. 6 M06-2X/6-311 + +G(d,p) optimise reactant complex (RC), transition state (TS) and product complex (PC) for the reactions of *E* clovamide with cysteine (first row) and *Z* clovamide with cysteine (—●—)



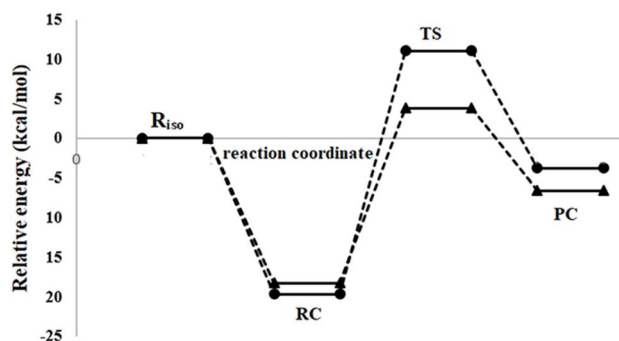
3.4 Rationalising the interaction of clovamide and cysteine through DFT modelling

In this study the HIV-1 INT inhibition mechanism was investigated using density functional theory calculations. Only the *SE* and *SZ* configurations of clovamide were investigated through DFT in this study, as these are the configurations that can naturally occur. The structures of clovamide and cysteine and the numbering used in this section are shown in Fig. 5. The optimised geometries of the reactant species, transition states and product complex species are shown in Fig. 6. The clovamide molecule reacts with the cysteine molecule by forming an initial reactant complex (RC) species in which the two molecules interact by the formation of two intermolecular hydrogen bonds. In the first hydrogen bond (N12...H-5O5), the amino group of the cysteine molecule acts as hydrogen bond acceptor while the *meta* phenolic OH of the catechol unit acts as the hydrogen atom donor. In the second hydrogen bond (O9'...H11-S11), the *keto* group in the clovamide molecule acts as hydrogen bond acceptor while the sulfide group is the hydrogen bond donor, suggestive of the fact that this reaction proceeds through a Michael addition reaction mechanism. The carboxylic group of the cysteine molecule appears to have a minimal role in the stabilisation of the RC structure. Clovamide is a versatile natural product that exhibit different inhibition mechanisms due to the various functional groups and stereochemistry it possesses. While Ye et al. [5] showed that the inhibition mechanism of the coronavirus by clovamide was through a thioesterification mechanism occurring on the carboxyl group of this compound, Jin et al. [17] showed that inhibition of the same virus proceeded through the Michael addition reaction.

The transition state (TS) complex was characterised by the presence of a longer C7'-C8' bond than in the individual clovamide molecule, longer than the normal $C_{sp^2} = C_{sp^2}$ double bond. However, C8' - C9' bond is shorter than a standard $C_{sp^2} - C_{sp^2}$ single bond, finally, the C9' - O9' is longer than the standard $C_{sp^2} - O_{sp^2}$ bond length in the individual clovamide. All these factors indicate the delocalisation of electronic distribution throughout the C7' - C8' - C9' - O9' region in the TS structure with respect to the individual clovamide molecule or RC structure. TS was also characterised by the transfer of the sulfide hydrogen to O9' and a shortening of the C7' - S11 distance with respect to the distances identified in the reactant complex structure. The product species is described by the formation of the C7' - S11 covalent bond, C8' = C9' double bond and the C9' - O9' single bond which is a Michael addition reaction product and this is summarized on Figure S10. It is important to note that the description of the transition state here is intended to illustrate the sequential steps leading to the product (Figure S10). However, it is strongly believed that this reaction follows a concerted mechanism, where the transfer of hydrogen from the cysteine to the carbonyl system and the subsequent attachment of S⁻ to the α,β -unsaturated carbonyl of clovamide occur simultaneously. This mechanism of inhibition is novel by targeting a different aspect of the enzyme's function and holds significant promise for the development of HIV drugs. Unlike traditional inhibition methods that target the enzyme's active site only, this approach provides an additional inhibition avenue that can potentially overcome the challenge of drug resistance.

The energy of the product species, as obtained using the M06-2X/6-311 + +G(d,p) calculation method, is lower than the sum of the energy of the reactant species, indicating that the product species is stable with respect to the isolated

Fig. 7 M06-2X/6-311 + + G(d, p) energy profile (energy vs reaction coordinate) for the reactions of clovamide with cysteine (—▲—) and Z clovamide with cysteine (—●—)



reactant species as depicted on Fig. 7. However, the reactant complex is more stable than the product complex, indicating that the reaction could proceed through the formation of the reactant complex. B3LYP/6-311 + + G(d, p) results give a high barrier height than M06-2X/6-311 + + G(d, p), which indicates that B3LYP functional tends to overestimate barrier height with respect to meta-GGA functional. The transition state involving the *E* isomer has lower barrier height than the *Z* isomer, which indicates that the *E* isomer is kinetically faster than the *Z* isomer on reaction with cysteine. The product species have higher free energy than the sum of the energy of the reactant species, suggesting that overall, the reaction is endogenic in nature. Future studies should investigate the experimental parameters that drive the Michael addition reaction, such as temperature and pH, to fully elucidate this inhibition mechanism. It is important to note that all calculations were performed in vacuo, whereas the actual reaction is expected to occur in solution (water). Recent studies using CGA as a model have demonstrated that pH is a crucial factor in the deprotonation of the sulfhydryl group of cysteine, which facilitates an effective attack on the α , β -unsaturated carbonyl and results in CYS-CGA crosslinking [45].

4 Conclusion

Natural products hold significant potential as drug leads for managing complex diseases, including HIV. The discovery of clovamide and its analogues in *A. digitata* underscores the plant's potential as a valuable source of therapeutic agents. Additionally, the use of molecular modelling tools offered valuable insights into the inhibitory mechanism of clovamide on HIV-1 INT. This study demonstrated that a Michael addition reaction between cysteine and clovamide might be an innovative strategy for inhibiting HIV-1 INT. The molecular dynamics simulations performed in this study have shown that the ligands and CYS-65 of the investigated proteins remain in proximity throughout the simulation period (100 ns), suggesting a high likelihood of the proposed reaction leading to the formation of permanent covalent linkages. Unlike traditional inhibition mechanisms that target the enzyme's catalytic site, this approach offers a novel mechanism that could effectively address the issue of drug resistance. This study further highlighted that caffeoyl derivatives, including clovamide, could serve as potent drug leads for HIV management due to the presence of the α , β -unsaturated carbonyl group in these compounds. The potential for clovamide to form various isomers is significant because it could reduce the likelihood of the HIV-1 INT enzyme mutating to all these isomers. Therefore, future design strategies for HIV-1 INT inhibitors should consider developing molecules with multiple isomeric forms, including geometric isomers (*E* and *Z*) and stereoisomers (*R* and *S*).

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Data availability The datasets used and analysed during this study will be made available from the corresponding author upon a reasonable request.

Declarations

Ethics approval and consent to participate No harm was caused to the plant and there is no requirement of a permit to collect fruits from wild growing plants which are not in demarcated nature reserves. Local guidelines were followed for the collection of *A. digitata* fruits.

Competing interests The authors declare no competing interests.

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