平成２０年度厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

研究課題：電算機的アプローチを活用したRNase H活性を標的とするHIV-1複製阻害剤開発に関する研究（若手育成型）
課題番号：H18-エイズ-若手-003

分担研究課題：RNase H阻害剤先導化合物の酵素-化合物相互作用の電算機的解析に関する研究

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研究要旨　RNaseH阻害作用を有する低分子化合物と標的であるRNaseHドメインの結合様式に関して、既にドッキングシミュレーションにより、解析を行なっている。この知見に基づいて、新たな化合物を設計した。本年度は、設計した化合物の合成を進めた。また生化学実験でのスクリーニングによって見出された薬物と標的タンパク質分子であるHIVの逆転写酵素のRNaseHドメインとの結晶化実験を進めた。また、本研究で対象としている化合物の中心骨格と、既に認可されているインテグラーゼ阻害剤との骨格を比較して、RNaseHドメイン、逆転写酵素のPolymeraseドメイン、あるいはインテグラーゼ活性部位に対して、薬物が作用するときの共通の化合物構造の検討を行った。

A．研究目的

現在のエイズ治療において最も深刻な問題の一つは、ウイルスの薬剤耐性の獲得である。HIV感染症治療においては、主に逆転写酵素阻害薬とプロテアーゼ阻害薬を用いた多剤併用療法(HAART)が行われている。ところがHIV-1は遺伝子変異を起こしやすいため、長期間の薬剤投与により、ウイルスが薬剤耐性を獲得する現象が起こる。抗エイズ薬の開発では、薬剤耐性の問題を常に念頭に置かなくてはならない。薬剤耐性を回避するための方策の一つは、従来からの逆転写酵素阻害剤やプロテアーゼ阻害剤とは異なり、別の部位に標的を定めた抗エイズ薬の開発である。もう一方のアプローチは、薬剤耐性ウイルスにも薬効を大きく低下させない逆転写酵素阻害剤やプロテアーゼ阻害剤の開発である。本研究班では、前者のアプローチ、すなわち新しい標的部位に作用する抗エイズ薬の創出を研究目的としている。具体的には、逆転写酵素を構成する蛋白質p66に含まれるRNaseHドメインに作用して、RNaseH活性を阻害する薬物を創出することが目的である。

昨年度までに、研究代表者（感染研：駒野）により、化合物ライブラリーからのスクリーニングにより、RNaseHドメインに作用する化合物が幾つか見出された。この化合物は、RNA切断機能を阻害して酵素の働きを抑える効果がある。活性化合物と標的タンパク質であるRNaseHドメインのドッキングシミュレーションを実行して、薬物と標的タンパク質の結合に関する知見を得た。この知見を生かして、コンピューターを用いて、標的タンパク質(RNaseH)に対して結合親和性の強い複数の化合物構造を考案した。計算機により、考案

した化合物について再度、精度を上げたシミュレーション計算を実行した。さらに合成成功の可能性も考慮して、合成を行う化合物構造を選定した。本年度の分担課題として、設計した化合物について、有機合成を行った。さらに標的タンパク質分子である生化学実験でのスクリーニングによって見出された薬物と RNaseH ドメインとの結晶化実験を進めた。計算機解析と合成および生化学実験を繰り返して最適なリード化合物を見出すことが、本研究の目的である。

B. 研究方法

(B-1)計算機解析

生化学実験でのスクリーニングによって見出されたヒット化合物の構造を参考に、RNaseH に対する阻害活性を持つと期待される化合物を考案した。次に考案した化合物と RNaseH の結合配置を、ドッキングシミュレーションソフトウェアを使用して予想した。RNaseH ドメイン中の化合物の結合サイトの探索範囲を絞り込むために、逆転写酵素の RNaseH 部位にある４つの荷電性アミノ酸残基(Asp443, Glu478, Asp498, Asp549)から 20 Å以内を、結合部位として指定した。ドッキングシミュレーションは、Gold（ケンブリッジ結晶データセンター）で行った。さらに、独自に開発した薬物と標的タンパク質の結合親和性評価ソフトウェア（Orientation）を使用して、結合構造の最適化と結合エネルギーの再評価を行った。これにより考案した化合物の中から有望なものを選び出し、実際に有機合成する対象を絞り込んだ。

(B-2)有機合成

RNaseH に対する阻害活性を持つと期待される設計化合物２種類を選定して、合成経路を立案した。合成反応では、薄層クロマトグラフィーで反応生成物の有無を確認し、主にカラム精製を行い、生成物を分離した。また必要に応じて、再結晶化を行い、中間精製物の純度を高めた。合成で得られた化合物は、核磁気共鳴分光法（NMR）ならびに電子線イオン化質量分析法(EI-MASS)により、その構造を確認した。

(B-3)生化学実験

RNaseH ドメインと生化学実験でのスクリーニングによって見出されたヒット化合物の結合構造を実験で確認するために、両者の共結晶の作成を試みた。RNaseH ドメインは HIV の逆転写酵素の一部である。逆転写酵素の結晶化は既に多く達成されている。ところが結晶の X 線解析の解像度は 3 - 6Å と十分でない場合が多い。最近、アミノ酸変異の導入により、解像度を 2Å以下にすることができるとの報告があった。本実験では、この発現ベクターを提供して頂き、大腸菌にて発現を行った。発現した逆転写酵素を His-タグによるアフィニティー法ならびにゲルろ過法で精製した。

C. 研究結果

(C-1)計算機設計

生化学的スクリーニングにより活性が確認されている化合物２種類（5-nitro-furan-2-carboxylic acid adamantan-1- carbamoyl-methyl ester : NAC と 5-nitro-furan-2-carboxylic acid [[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydro-furan-2-methyl)-carbamoyl]-methyl ester : NBTC）の化学構造ならびに化合物形状を図１に示す。NAC と NBTC のともに、フラン環にカルボニル基を通してアミド結合が接続し、アミノ結合の先に疎水性官能基が結合する共通の骨格を持っている。またフラン環のカルボニル基の結合部位とは反対側に、ニトロ基が結合している。フラン環の両端にあるカルボニル基とニトロ基

およびフラン環の酸素原子が核酸を模倣しており、RNaseH ドメインの核酸結合部位に有効に吸着するものと考えられる。NAC と NBTC は、HIV の RNaseH 活性を、IC_{50} = 3-30 μM で抑制することが、主任研究者の駒野による実験で確認されている。

生化学的スクリーニングにより見出された RNaseH 活性阻害化合物である NAC と NBTC について、これら化合物が標的である RNaseH ドメインに結合した時の結合構造をドッキングシミュレーションと呼ばれる計算機解析により予測したのが図２である。ドッキングシミュレーションには市販のソフトウェアである Gold を用いた。RNaseH ドメインでは、活性部位に４つの荷電性アミノ酸残基(Asp443, Glu478, Asp498, Asp549)が存在するが、これを横切るような形で溝ができている。溝と４つの残基の交わる部分には、Mg^{2+}イオンが配位している。ヒット化合物は、丁度、この Mg^{2+}イオンに配位結合して安定化している。図２に示すように、RNaseH ドメインの活性部位の荷電性アミノ酸残基(Asp443, Glu478, Asp498, Asp549)は、いずれも NAC と NBTC のニトロ基およびフラン環と水素結合を形成している。従って、フラン環を中心とした骨格は、阻害剤として非常に重要な化学構造であると判断できる。カルボニル基に続くアミド基の酸素原子は、RNaseH ドメインの Ser553 と水素結合を形成している。従って、この部分はその先に接続する疎水基の結合向きを規定する重要な部分である。NAC に比べ NBTC は、若干、活性阻害効果が高いが、これは疎水基の先端に電荷を持つ領域があり、この部分が Lys 残基と相互作用を持つためと推察できる。

RNaseH ドメインと RNaseH 阻害活性を持つ化合物の構造を参考に、図３ならびに図４に示すように新規の化合物構造を考案した。いずれの分子も、活性部位の溝に填るために、細長い形をしている。図３はフラン環の代わりに構造安定性の高いチオフェンを用いたものである。これは両者の性質がそれほど変わらないが、チオフェンの方が安定で合成に向いているからである。さらにニトロ基の代わりにスルフォニル基を用いた。これは標的ドメインとの結合強化という狙いがある。チオフェンの反対の端には、疎水性の官能器を配置した。２つの芳香族間が窒素原子で結合された構造があり、この部分が強い疎水性領域を形成し、しかも柔軟性のある形となっている。さらに主骨格が途中の窒素原子で分岐して、疎水性領域が直線構造より曲がった構造とした。化合物中央のペプチド結合を形成する窒素原子部分で炭素鎖が分岐することで、化合物の標的への結合向きを制御することができる。図４に示す化合物では、フラン環は図１のヒット化合物の構造をそのまま用いて、他の部分はシアノ基を除いて、図３の構造と同じとした。

ドッキングシミュレーションの結果、考案した化合物はいずれも RNaseH の活性ドメインの溝の部分に適切に結合した。Gold ソフトウェアで算出されるスコアー値は、図３の構造の方がやや良好であるが、化合物の合成のしやすさでは、図４の方が有利と思われる。

(C-2)有機合成

図３に示した化合物について、図５に示す合成経路を立案した。初めに、硫酸基をチオフェンに結合させる。次にアミノアゾベンゼンをペプチド結合で反対側に結合させる。さらにペプチド結合部位の窒素原子にシアノ基を結合させて完成させる。実際の合成では、硫酸基を結合させる反応の反応性が高く、目的の部位以外にも水酸基が結合し、目的の反応物だけを精製することが困難であった。

図４に示した化合物について、図６に示す合成経路を立案した。初めに、グリシンに保護基であるBoc基をアミド結合で付加する。次にアミノアゾベンゼンをペプチド結合で結合させる。Boc基を脱保護した後に、ニトロフランをアミド結合で結合させて完成させる。実際の合成での合成産物を、図７に示す。目的の化合物が少量ではあるが、合成することに成功した。現在、合成化合物の活性を測定している。

(C-3)生化学実験

X線結晶解析の解像度を2Å以下にすることのできる変異が導入された逆転写酵素の発現ベクター（RT69A）を、Rutgers大学のEddy Arnold教授より分与頂いた。このベクターを使用して、大腸菌（Rosetta）にて、逆転写酵素を発現した。37℃で大腸菌をストレプトマイシン入りの液体培地で培養し、O.D.値が0.9になった時点で、IPTGを投入して発現を誘導した。IPTG投入後、３時間後に遠心機により集菌した。発現した逆転写酵素には、ヒスチジンタグがついているので、一段階目の精製として、Niカラムを用いた。この後にエンテロキナーゼによる酵素反応で、ヒスチジンタグ部分を切断した。フィルターろ過後、ゲルろ過クロマトグラフィーで、二段階目の精製を行った。精製した逆転写酵素には、ポリメラーゼ活性もRNaseH活性もあることが、基質の切断反応から確認できた。現在、NACあるいはNBTCとの共結晶化を試みている。

D. 考察

RNaseH 阻害活性が期待できる化合物の特徴を以下に示す。 (1)活性中心にあるMg^{2+}イオンとキレートするような形で結合することが有効である。フラン環やチオフェンとその近傍の酸素原子が適当な間隔を空けて配置する構造は、Mg^{2+}イオンへのキレートに適しており、これまでスクリーニングで得られた化合物の多くが、この基本骨格を持っている。(2) フラン環やチオフェンにはニトロ基あるいは硫酸基を付加すると良い。これは水溶性を上げる効果と RNaseH 側のアミノ酸と水素結合を形成する役目を持っている。(3) フラン環あるいはチオフェンと反対側には、疎水相互作用をする領域が必要である。本研究では２つのベンゼン環を配置した。標的ドメインの結合部位を十分に適切に埋めるために、ジアゾ基を導入した。

設計した化合物は、既存の認可薬との共通の構造を持たせることにも配慮している。これは合成の実績がある構造であることと、副作用の出にくい構造であることを担保するためである。図８は、最近、国内でも承認されたHIVインテグラーゼ阻害剤のラルテグラビルの構造を示している。オキサジアゾールの部分はフラン環あるいはチオフェンと同等の骨格を持っている。逆転写酵素のRNaseHドメインもHIVインテグラーゼも、核酸を分解する機能がある。従って、フラン環、チオフェン、オキサジアゾールの部分はフラン環あるいはチオフェンは、有効に核酸を捕捉する働きがあるものと予想される。

E. 結論

生化学実験でのスクリーニングによって見出された RNaseH に対する阻害活性を持つ薬物の構造をもとに、新規の化合物を考案した。考案した薬物について、RNaseHとの結合構造をドッキングシミュレーションにより予測し、有望な化合物構造を２つ選び出した。それらについて、有機合成を試みたが、期間内に一方の化合物の合成には成功した。この化合物について生化学実験により活性の評価を行っている。今後、合成に成功した化

合物を足掛かりとして、幾つかの化合物の合成を実施し、より有望な RNaseH 阻害活性化合物を探索する予定である。また、設計合成した化合物は HIV インテグラーゼ阻害剤とも類似した構造を持っている。これは阻害作用機構が、承認済みの阻害剤と共通であることを意味しており、HIV の酵素阻害剤として効果が期待できる。

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G. 知的所有権の出願・取得状況（予定を含む）

実績無し。

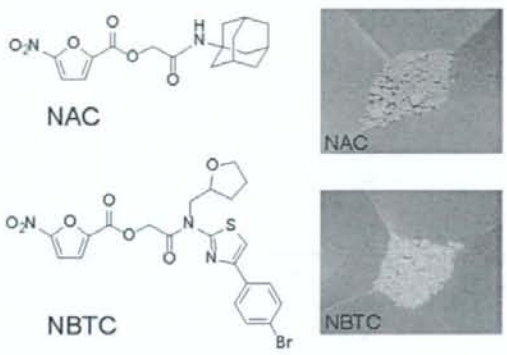

NAC
NBTC
Br
NAC
NBTC

図1.

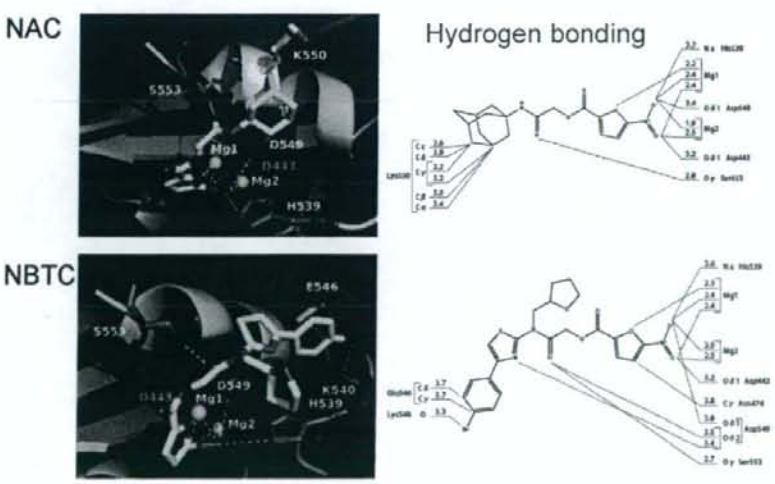

NAC
S553
K550
Mg1
D549
Mg2
H539
NBTC
S553
E546
D549
Mg1
Mg2
K540
H539
Hydrogen bonding

図2.

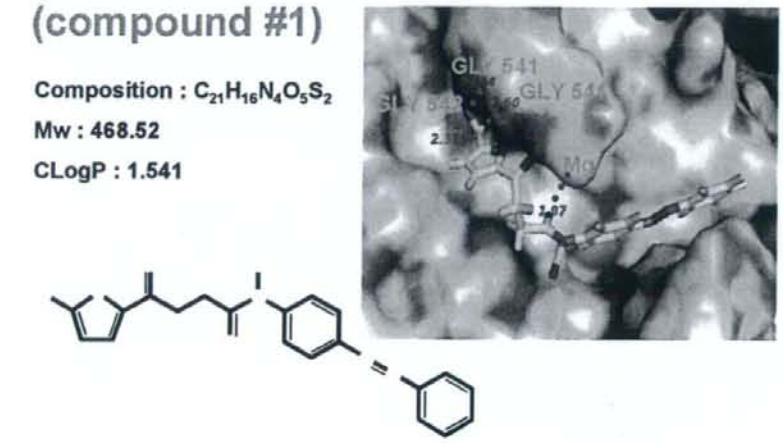


図３ *都合により一部意図的に図を消去するなど修正を施しています。

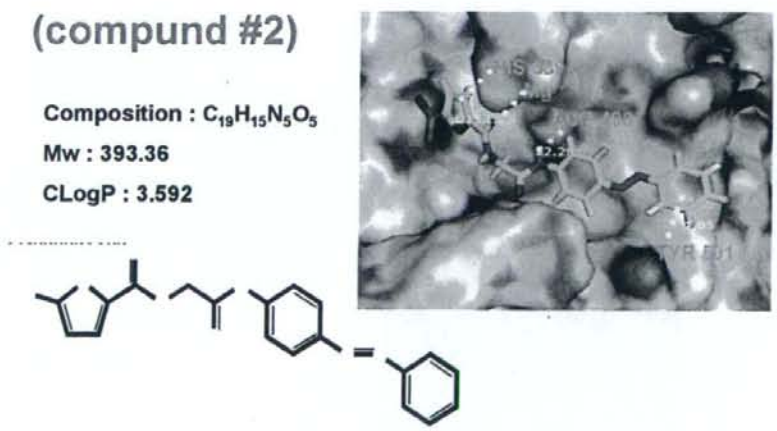
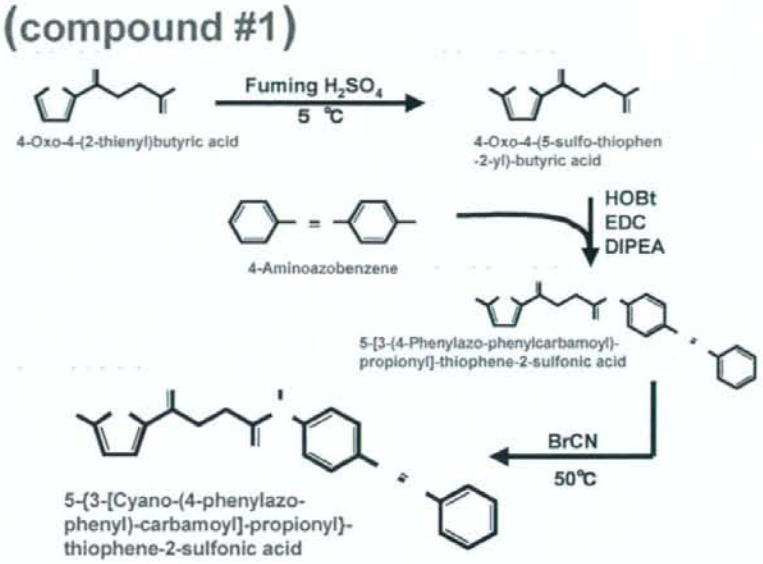


図４ *都合により一部意図的に図を消去するなど修正を施しています。



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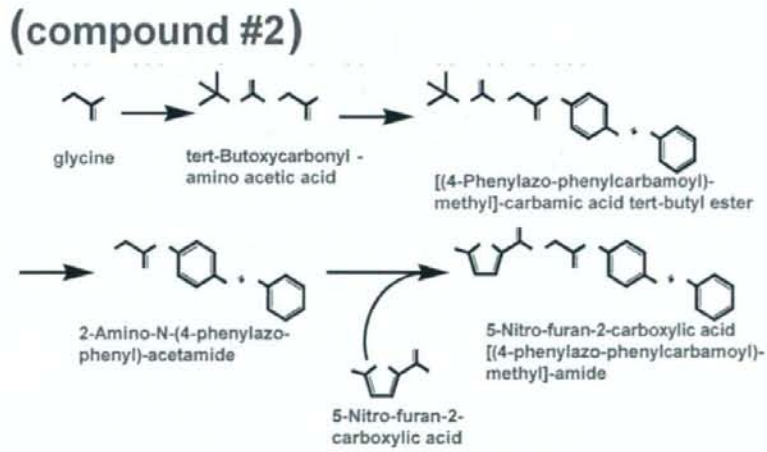

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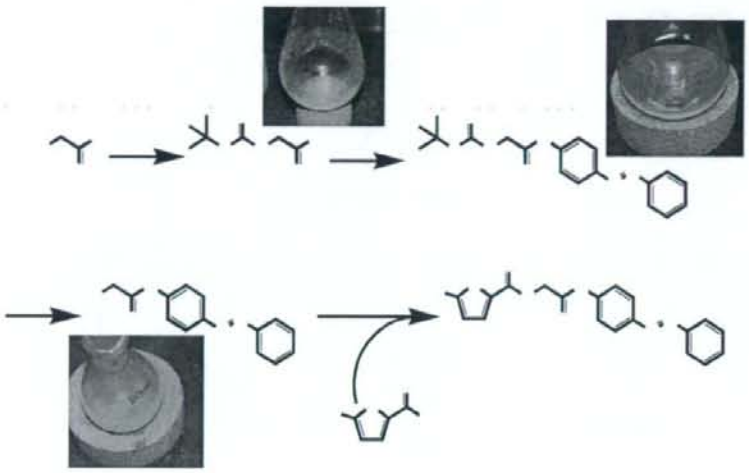

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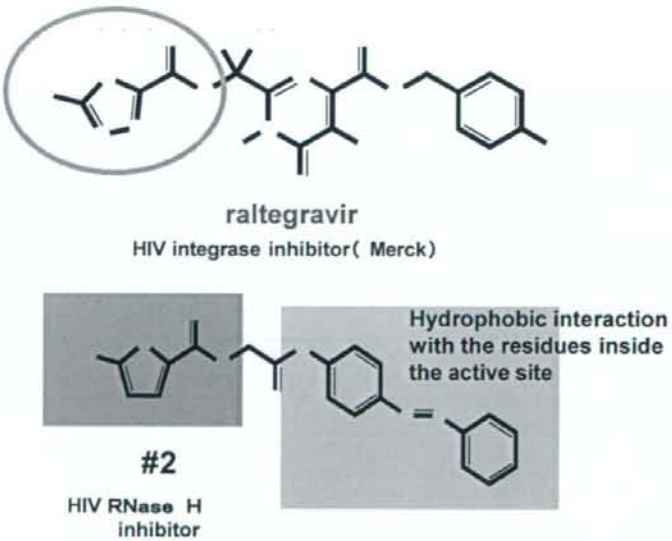

図８　*都合により一部意図的に図を消去するなど修正を施しています。

III. 平成２０年度　業績一覧

研究成果の刊行に関する一覧表

駒野　淳

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Fuji H, Urano E, Futahashi Y, Hamatake M, Tatsumi J, Hoshino T, Morikawa Y, Yamamoto N, Komano J.	Derivatives of 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester inhibit RNase H activity associated with HIV-1 reverse transcriptase	J Med Chem		(in press)	2009
Hamatake M, Aoki T, Futahashi Y, Urano E, Yamamoto N, Komano J.	Ligand-independent higher-order multimerization of CXCR4, a G-protein-coupled chemokine receptor involved in the targeted metastasis.	Cancer Sci.		(in press)	2009
Urano E, Kariya Y, Futahashi Y, Ichikawa R, Hamatake M, Fukazawa H, Morikawa Y, Yoshida T, Koyanagi Y, Yamamoto N, Komano J.	Identification of the P-TEFb complex-interacting domain of Brd4 as an inhibitor of HIV-1 replication by functional cDNA library screening in MT-4 cells.	FEBS Let.	Dec 10 582 (29)	4053-8	2008
Urano E, Aoki T, Futahashi Y, Murakami T, Morikawa Y, Yamamoto N, Komano J.	Substitution of the myristoylation signal of human immunodeficiency vius type 1 Pr55Gag with the phospholipase C delta 1 pleckstrin homology domain results in infectious pseudovirion production.	J Gen Virol.	Dec; 89 (Pt12)	3144-9	2008
Urano E, Shimizu S, Futahashi Y, Hamatake M, Morikawa Y, Takahashi N, Fukazawa H, Yamamoto N, Komano J.	Cyclin K/CPR4 inhibits primate lentiviral replication by inactivating Tat/positive transcription elongation factor b-dependent long terminal repeat transcription.	AIDS.	May 31;22(9)	1081-3	2008
Yoshida T, Kawano Y, Sato K, Ando Y, Aoki J, Miura Y, Komano J, Tanaka Y, Koyanagi Y.	A CD63 mutant inhibits T-cell tropic human immunodeficiency virus type 1 entry by disrupting CXCR4 trafficking to the plasma membrane.	Traffic.	Apr;9(4)	540-58	2008

星野　忠次

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Fuji H, Urano E, Futahashi Y, Hamatake M, Tatsumi J, Hoshino T, Morikawa Y, Yamamoto N, Komano J.	Derivatives of 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester inhibit RNase H activity associated with HIV-1 reverse transcriptase	J Med Chem		(in press)	2009
Fuji H., Suzuki M., Neya S., Hoshino T.	Development of Software Program Predicting the Binding Site and the Binding Mode of Ligands Against a Target Protein.	e-J. Surf. Sci. Nanotech.	6	241-245	2008
Katagiri D, Fuji H, Neya S, Hoshino T.	Ab initio Protein Structure Prediction with Force Field Parameters Derived from Water Phase Quantum Chemical Calculation.	J Comput Chem.	29	1930-44	2008
Hoshino T, Iwamoto K, Ode H, Ohdomari I.	Accurate evaluation method of molecular binding affinity from fluctuation frequency, Jpn.	Jpn J. Appl. Phys.	47	3719-25	2008

IV. 平成２０年度　刊行物別刷（抜粋）

Derivatives of 5-Nitro-furan-2-carboxylic Acid Carbamoylmethyl Ester Inhibit RNase H Activity Associated with HIV-1 Reverse Transcriptase

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The RNase H activity associated with human immunodeficiency virus type 1 (HIV-1) is an attractive target for an antiretroviral drug development. We screened 20000 small-molecular-weight compounds for RNase H inhibitors and identified a novel RNase H-inhibiting structure characterized by a 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester (NACME) moiety. Two NACME derivatives, 5-nitro-furan-2-carboxylic acid adamantan-1-carbamoylmethyl ester (compound **1**) and 5-nitro-furan-2-carboxylic acid [[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydro-furan-2-ylmethyl)-carbamoyl]-methyl ester (compound **2**), effectively blocked HIV-1 and MLV RT-associated RNase H activities with IC_{50}s of 3−30 μM but had little effect on bacterial RNase H activity in vitro. Additionally, 20−25 μM compound **2** effectively inhibited HIV-1 replication. An in silico docking simulation indicated that the conserved His539 residue, and two metal ions in the RNase H catalytic center are involved in RNase H inhibition by NACME derivatives. Taken together, these data suggest that NACME derivatives may be potent lead compounds for development of a novel class of antiretroviral drugs.

Introduction

Highly Active Anti-Retroviral Therapy, a combination of antiretroviral drugs, has become the standard treatment for HIV-infected individuals. However, the emergence of drug-resistant viruses is problematic because there are few alternative treatment regimens. HIV-1 has three known enzymes, protease, reverse transcriptase (RT), and integrase (IN). The RT, a heterodimer of p51 and p66 subunits, has three enzymatic functions: RNA- and DNA-dependent DNA polymerase and RNase H activity. Inhibitors of the protease, RT-associated polymerase, and IN activities are now in clinical use; however, a suitable inhibitor of the RT-associated RNase H activity has not been found. Thus, developing a highly specific inhibitor against HIV-1 RT-associated RNase H activity could provide another option for treatment of HIV-1-infected individuals.

RNase H activity is a ribonuclease activity that recognizes RNA hybridized to DNA. The RT-associated RNase H activity resides in the carboxy-terminal region of p66 and is essential for synthesis of double-stranded DNA from the HIV-1 single-stranded RNA genome. The RNase H activity is also involved in HIV-1 drug resistance to RT inhibitors as well as generating a diversity of viruses in vivo by homologous recombination.[1−7] Thus, RT-associated RNase H activity is another attractive target for development of a novel class of antiretroviral drugs.[8] Dual inhibitors that target RNase H and IN or RT-associated polymerase activities have been reported because these enzymes possess structural similarities.[9−11] Such inhibitors should be more effective than inhibitors that merely target RNase H activity.

The difficulty in developing an RNase H inhibitor for the treatment of HIV-1 infection lies in the specificity and toxicity of the drug. Although several RNase H inhibitors with different structures have been reported,[9,12−20] the 50% inhibitory concentration (IC_{50}) of many previous derivatives are on the order of micromolar concentration and they often lack sufficient specificity for the HIV-1 RT-associated RNase H activity. Most problematically, they often display cytotoxicity to mammalian cells. Many RNase H inhibitors are assumed to bind to the catalytic center and interact with Mg^{2+} ions. The toxicity of such inhibitors may be due to inhibition of other cellular proteins that require divalent metal cations to function.

More chemical compounds need to be tested in order to find a novel RNase H inhibitor with a chemical structure that can be used to design a potent and specific HIV-1 specific RNase H inhibitor. For a structure−function based approach to drug design, the chemical−enzyme interactions are examined by in silico docking simulations and the models critically assessed by comparing them to data obtained from experiments. This approach is possible because the crystallized protein structures of the HIV-1 RT and RNase H proteins from diverse organisms have been solved. Indeed, structure-based drug design has been successful in identifying several of the drugs currently available for HIV-1 treatment including protease inhibitors. Molecular docking simulations can reveal the molecular details of the interaction between HIV-1 RT and RNase H inhibitors.

In this study, we screened 20000 small molecular weight compounds to find chemicals that suppress the HIV-1 RT-associated RNase H activity. We found that derivatives of 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester inhibit retroviral RNase H activity in vitro. One of the derivatives was

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⊥ Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NACME, 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester; compound 1, 5-nitro-furan-2-carboxylic acid adamantan-1-carbamoylmethyl ester; compound 2, 5-nitro-furan-2-carboxylic acid [[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydro-furan-2-ylmethyl)-carbamoyl]-methyl ester.

10.1021/jm801071m CCC: $40.75

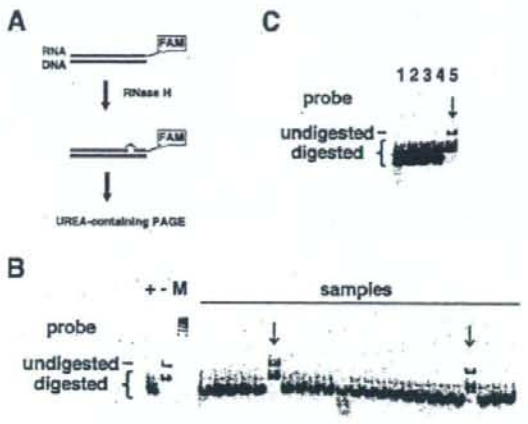

Figure 1. Screening for RNase H inhibitors. (A) Schematic diagram of the screening method. (B) An example from the first screen. Groups of 5 chemicals were pooled at 100 μM each for testing. Some pools exhibited RNase H inhibitory activity (arrows). (C) An example from the second screen. The five chemicals from a positive pool were tested individually at 100 μM each. One of the chemicals was responsible for the RNase H inhibition (arrow).

able to limit HIV-1 replication in tissue culture. The docking simulations were consistent with the experimental data and indicate some critical drug−enzyme interactions. The in silico docking simulation may help us to design a highly specific inhibitor against HIV-1 RT-associated RNase H activity.

Results

To find a novel chemical that inhibits HIV-1 RT-associated RNase H activity, we screened a chemical library containing 20000 compounds representing key structural features of three million chemicals. We used a purified RT encoded by a clade B HIV-1_{LAI} and a polyacrylamide gel electrophoresis (PAGE)-based assay for the first and second rounds of screening (Figure 1A). A fluorochrome-labeled synthetic oligoribonucleotide was annealed to a complementary oligodeoxyribonucleodide, and the hybridized DNA/RNA probe was incubated with HIV-1 RT. The RT-associated RNase H activity introduces nicks into the RNA strand that yields low molecular weight bands in UREA-containing denaturing PAGE, which are visualized by fluorescence scanning. The p51 preparation did not yield detectable low-molecular-weight bands, indicating that the RT preparation was not contaminated with RNase activities of bacterial origin (data not shown). Initially, five chemicals were pooled for screening purposes, and the screen was performed with each chemical at a concentration of 100 μM. Some of the pooled chemicals protected the RNA/DNA probe from cleavage by the RT-associated RNase H (Figure 1B). Pooled chemicals that showed ~50% protection efficiency were chosen for the second round of screening (43/20000 chemicals, 0.22%). Individual chemicals were tested at a concentration of 100 μM (Figure 1C). We found 17 chemicals (0.085%) that possessed detectable RNase H inhibitory activities. Interestingly, six of these compounds (6/17 chemicals, 35.3%) included a common structure characterized by a 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester (NACME, Figure 2A).

To clarify the structure−activity relationship, we re-examined 91 chemicals from the chemical library bearing NACME homologues and tested their RNase H inhibitory activity using a real-time monitoring assay described previously.[21] Among these 91 chemicals, 32 compounds displayed detectable RNase H inhibition (32/91 chemicals, 35.2%). Derivatives carrying various substituents at the end of the carbamoylmethyl ester group (R1) retained RNase H inhibitory activity (32/48 chemicals, 66.7%; Figure 2A,B), but substitution of the 5-nitro-furan (R2) with various structures resulted in a loss of RNase H inhibition. These data indicate a critical role for the 5-nitro-furan group in RNase H inhibition (0/43 chemicals, 0.0%; Figure 2C). NACME derivatives displayed RNase H inhibitory activity when R1 was substituted by a relatively long and bulky group (Figure 2A). Conversely, compounds with relatively small volume substituents in the R1 position showed no inhibitory activity (Figure 2B). A steric effect could explain why some derivatives with large substituents that did not show RNase H inhibitory activity (Figure 2A,B). We tested a series of additional NACME derivatives not included in the original chemical library and found that five compounds inhibited RNase H (5/20 chemicals, 20%). The significant increase in the percentage of RNase H inhibitors in this "biased" population compared to the original library (20.0% vs 0.085%, 235.3-fold) suggests that the NACME motif is a novel RNase H inhibitory structure. We focused on two representative derivatives for detailed analysis: 5-nitro-furan-2-carboxylic acid adamantan-1-carbamoylmethyl ester (compound **1**, hereafter) and 5-nitro-furan-2-carboxylic acid [[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydro-furan-2-yl-methyl)-carbamoyl]-methyl ester (compound **2**, hereafter; Figure 2A).

The IC_{50}s of compounds **1** and **2** for the HIV-1 RT-associated RNase H were defined as the concentration of the chemical yielding half the substrate cleavage rate of the control (DMSO) and were measured using the real time monitoring assay[21] (Figure 3A). The estimated IC_{50}s for compounds **1** and **2** for clade B HIV-1_{LAI}-derived RT-associated RNase H activity were 29.6 ± 9.2 μM ($n = 6$) and 26.7 ± 13.5 ($n = 5$), respectively (Figure 3B and Table 1). Similar IC_{50}s were obtained in assays performed independently by Beutler et al. (data not shown) with a previously described protocol.[22] RTs from different HIV-1 strains were also tested. The IC_{50}s of compounds **1** and **2** for the HIV-1 clade C (93IN101) RT were 26.5 ± 15.0 μM ($n = 5$) and 32.2 ± 7.5 μM ($n = 3$) and for the CRF01 A_E (93JP-NH1) RT were 3.8 ± 1.5 μM ($n = 3$) and 2.6 ± 1.8 μM ($n = 5$), respectively (data not shown, Figure 3B, and Table 1). The IC_{50}s of compounds **1** and **2** for a murine leukemia virus (MLV) RT were 8.4 ± 9.4 μM ($n = 6$) and 8.6 ± 8.9 μM ($n = 7$), respectively (Figure 3B and Table 1). Neither compound inhibited RNase H from *Escherichia coli* (data not shown). Of note, inhibitory activity against human RNase H1 was observed for compound **2** (IC_{50} ~50 μM) but not compound **1** (Beutler et al., data not shown). The enzymatic activities of the HIV-1 IN and the RT RNA-dependent DNA polymerase were not detectably affected by these compounds (data not shown, Pommier et al., data not shown; Table 1). These data suggest that compounds **1** and **2** selectively inhibit RNase H.

We examined whether these compounds were able to limit HIV-1 replication using two assay systems. The 50% cytotoxicity concentration was >25 μM for T cell lines and NP2CD4-CXCR4 cells, and 25−50 μM for primary $CD4^+$ T cells (data not shown). First, we infected NP2CD4CXCR4 cells with HIV-1_{NL4-3} in the presence of compound **2**. Four days postinfection, the culture supernatant was recovered and the concentration of viral p24 antigen was measured by ELISA. The p24 levels decreased in an compound 2 dose-dependent manner, and the

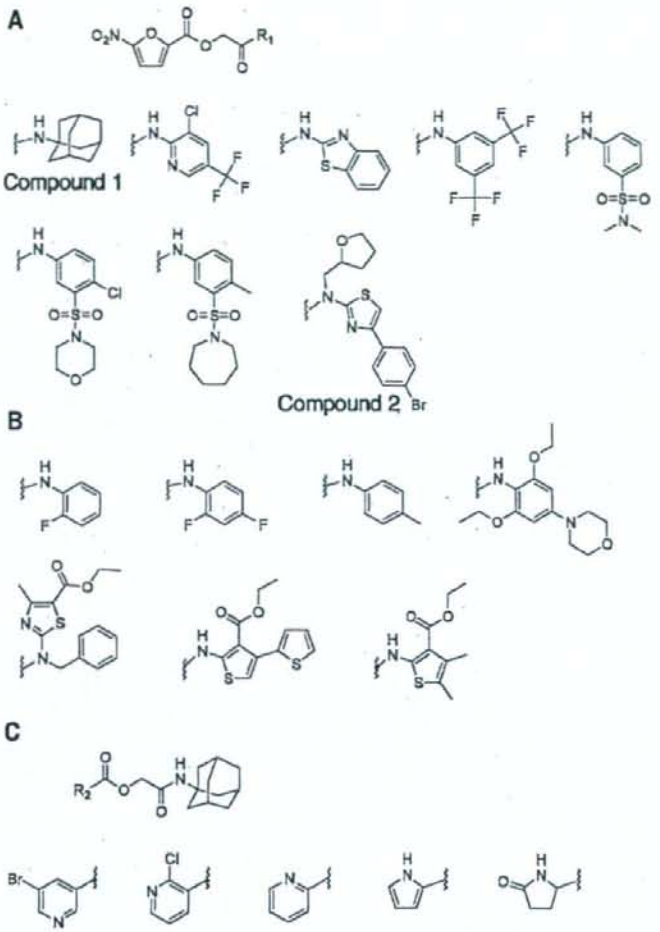

Figure 2. Structure−activity relationships of NACME homologues. (A) NACME derivatives with RNase H inhibitory activity carrying various substituents at the R1 position. The structure at the top is the NACME core structure. The structures below are the R1 substituents. (B) NACME derivatives with no RNase H inhibitory activity carrying various substituents at the R1 position. The structures show the R1 substituents. (C) NACME derivatives carrying various chemical structures at the R2 position. None of these chemicals displayed RNase H inhibitory activity.

IC_{50} was estimated to be ~22.7 μM (Figure 4A). Finally, we infected primary $CD4^+$ T cells with HIV-1_{NL4-3} and monitored the viral replication kinetics in the presence of 25 μM compounds **1** or **2**. Compared to control, HIV-1_{NL4-3} replication was slowed to some extent by compound **1**, but a greater attenuation of viral replication was observed in the presence of compound **2** (Figure 4B).

We conducted an in silico docking simulation using the GOLD software and the RT structure of PDB code: 1RTD[23] to examine the molecular mechanism of RNase H inhibition. Overall docking results for compounds **1** and **2** were similar (Figure 5A−C). The best docking pose for compound **1** showed that the nitrofuran group chelates the two Mg^{2+} ions and orients toward His539 to form a possible hydrogen bond (Figure 5B). Additionally, the carbonyl oxygen atom in the amide group of compound **1** interacted with the side chain of Ser553. There were hydrophobic contacts between the adamantan group of compound **1** and the side chain of Lys550. For compound **2**, the best docking pose showed that, in addition to the above interactions, a nitrogen atom in the thiazole group interacted with Asp549 by hydrogen bonding (Figure 5C). A hydrophobic contact between the phenyl group of compound **2** and the side chain of Glu546 was also observed (Figure 5C). In contrast, most of the inactive compounds did not chelate the two Mg^{2+} ions simultaneously, and His539 was not involved in the chemical−enzyme interaction (Figure 5D). The possible interactions of compounds **1** and **2** with the RNase H domain of HIV-1 RT are summarized in Figure 5E. Similar findings were obtained by additional simulation using the Glide (Schrodinger Inc.) docking simulation software (data not shown). These results suggest that the interactions with both of the Mg^{2+} ions and His539 are essential to the inhibition of HIV-1 RNase H activity by NACME derivatives. We measured the ChemScores of compounds **1** and **2** in binding to HIV-1 RT and compared the scores with those of the *E. coli* and human RNase H using X-ray structures of *E. coli* (PDB code: 1RDD[24]) and human RNase H (RNase H1, PDB code: 2QKK[25]). For compound **2**, the ChemScores were 31.27, 18.02, and 26.03 against HIV-1, *E. coli*, and human RNase H, respectively. For compound **1**, the ChemScores were 30.79, 17.09, and 28.10, respectively. In

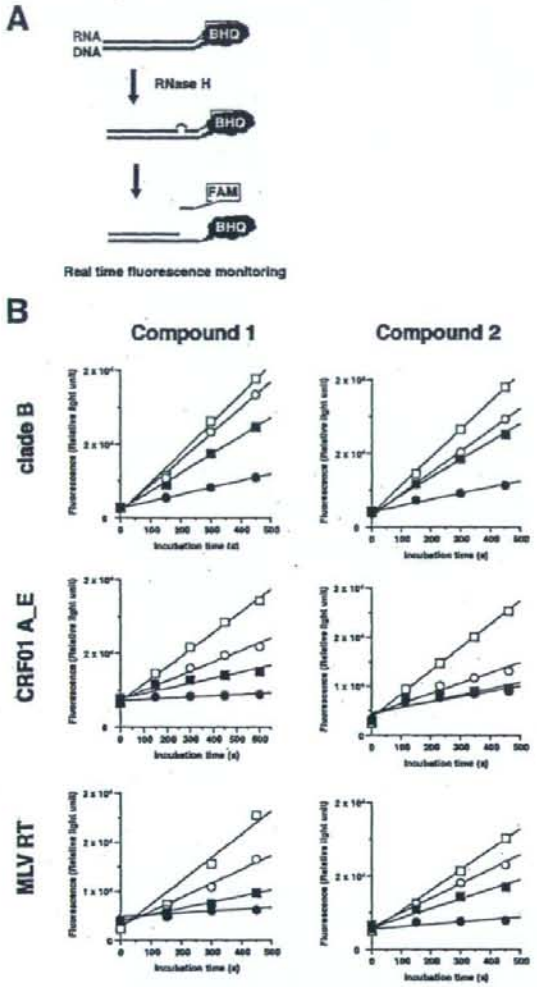

Figure 3. Measuring the IC_{50}s of compounds **1** and **2** against various RNase H activities. (A) Schematic presentation of the experimental system. The black hole quencher (BHQ) blocks the fluorescence emission from FAM when placed close to FAM. (B) Real-time monitoring of RNase H activity in the presence of increasing concentrations of compounds **1** (left) and **2** (right). HIV-1 RT derived from clade B and CRF01 A_E, and MLV RT were tested (top to bottom, respectively). Drug concentrations were 50 (solid circle), 10 (solid square), 2 (open circle), and 0 μM (DMSO, open square), respectively.

Table 1. Summary of Compounds **1** and **2** IC_{50}s

enzyme	compd **1**	compd **2**
HIV-1 RT		
clade B, μM	29.6 ± 9.2 (n = 6)	26.7 ± 13.5 (n = 5)
clade C, μM	26.5 ± 15.0 (n = 5)	32.2 ± 7.5 (n = 3)
CRF01 A_E, μM	3.8 ± 1.5 (n = 3)	2.6 ± 1.8 (n = 5)
MLV RT, μM	8.4 ± 9.4 (n = 6)	8.6 ± 8.9 (n = 7)
E. coli RNase H	no inhibition	no inhibition
human RNase H1, μM	no inhibition	~50
HIV-1 IN	no inhibition	no inhibition

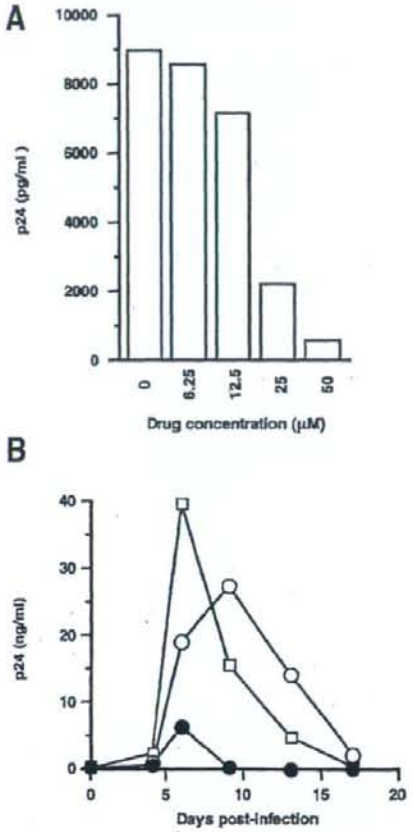

Figure 4. Inhibition of HIV-1 replication by compounds **1** and **2**. (A) Inhibition of HIV-1 replication was measured using the NP2CD4CXCR4/HIV-1_{NL4-3} system. The estimated IC_{50} was ~22.7 μM. (B) Effect of compound **1** (open circle) and compound **2** (filled circle) on HIV-1_{NL4-3} replication in PBMC. DMSO was used for the negative control (square).

agreement with the in vitro experiments, the ChemScores against *E. coli* RNase H were the lowest for both inhibitors. Inhibition of human RNase H by compound **2** can be explained by the docking structure, which indicated that compound **2** interacted with the highly conserved His264 (which corresponds to the HIV-1 His539) and two metal ions simultaneously in the human RNase H active site. In contrast, there is no interaction between compound **1** and His264 in the human RNase H. Similarly, there was no interaction between either inhibitor and the highly conserved His124 (which corresponds to the HIV-1 His539) of the *E. coli* RNase H. These data justify the reliability of docking simulation results and suggest a critical role of the conserved His residues in regulating RNase H function.

Discussion

This is the first report characterizing the NACME moiety as a functional group with the ability to inhibit HIV-1 RT-associated RNase H activity. The representative NACME derivatives compounds **1** and **2** were able to inhibit RTs derived from various HIV-1 clades with varying potencies. However, compounds **1** and **2** did not inhibit IN or RT polymerase activities, indicating that NACME derivatives are selective RNase H inhibitors.

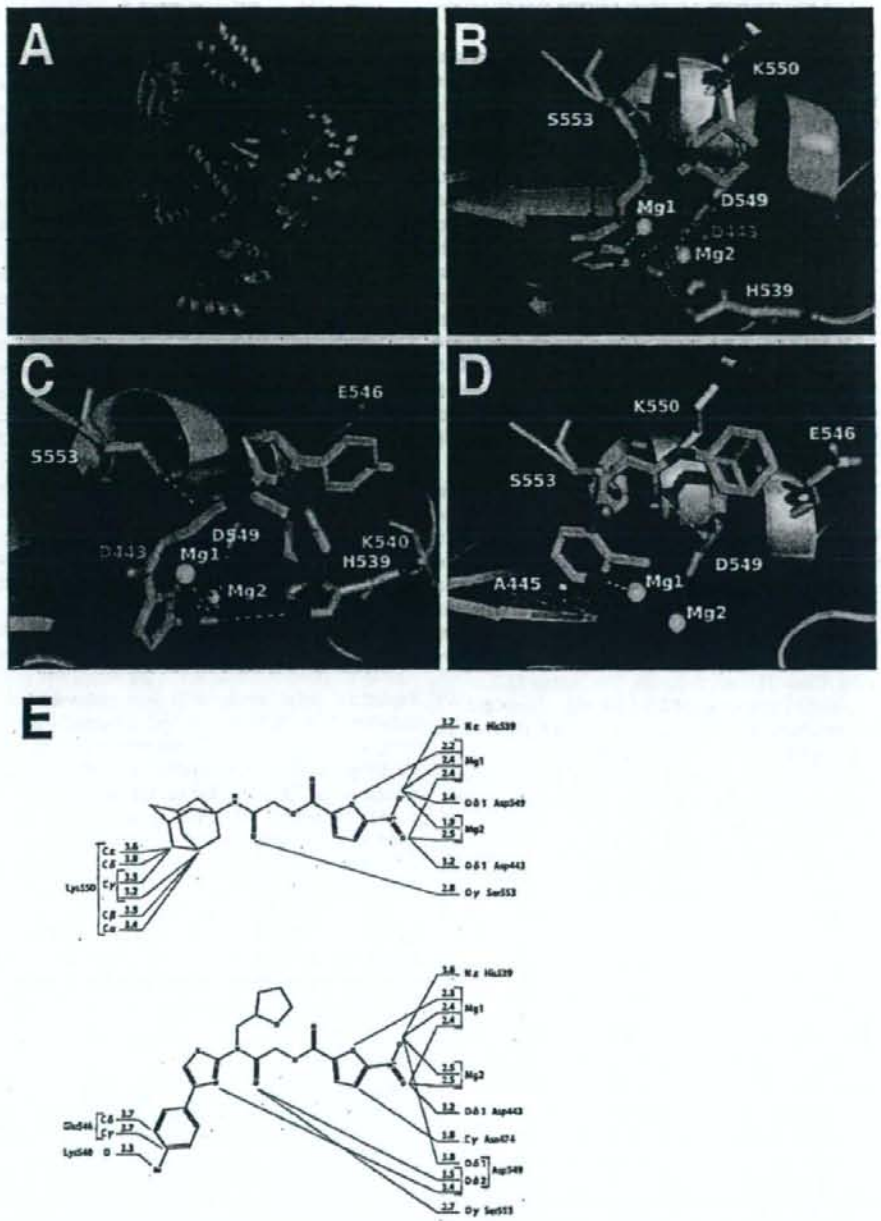

Figure 5. In silico simulation of compounds **1** or **2** docking to the RNase H domain of HIV-1 RT. (A) Overview of HIV-1 RT docked with compound **1**. HIV-1 RT is depicted as a ribbon model, with p66 in green and p51 in orange. Compound **1** is shown using a space filling model with carbon in cyan, oxygen in red, and nitrogen in blue. (B) Structure of compound **1** bound to the RNase H domain. (C) Structure of compound **2** bound to the RNase H domain. (D) Structure of a noninhibitor bound to the RNase H domain. NACME derivatives are presented as stick model with carbon in cyan, oxygen in red, nitrogen in blue, sulfur in yellow, bromine in brown, and chlorine in green. HIV-1 RNase H is depicted in a ribbon mode in green. Magnesium ions are depicted as green sheres. Amino acid residues forming hydrophobic contacts with NACME derivatives are depicted by yellow stick models. Amino acid residues forming hydrogen bonds with compounds **1** or **2** are depicted by magenta stick models, and hydrogen bonds are in dashed yellow lines. Figures were generated by PyMOL. (E) Atoms of compound **1** (top) and compound **2** (bottom) that may interact with amino acids and Mg^{2+} ion in HIV-1 RT are shown with contact distances. All residues in contact with the compounds are from the RNase H domain of HIV-1 RT.

It is intriguing that HIV-1 replication was restricted by compound **2** because most RNase H inhibitors are unable to inhibit HIV-1 replication in tissue culture. RDS1643 is one of the diketo RNase H inhibitors and has been shown to inhibit HIV-1 replication in vivo at micromolar concentrations.[12] Although we detected the anti-HIV-1 activity of compound **2**,

the cytotoxicity still remained a concern because the selectivity window was small. compound **2** and RDS1643 still need to be improved to lower both IC_{50} and the cytotoxicity. Compound **1** did not show an anti-HIV-1 replication activity in tissue culture even though the IC_{50}s of compounds **1** and **2** were similar in vitro. NACME is composed of many polar chemical groups; thus, NACME derivatives may have difficulty crossing a lipid bilayer. Cell membrane permeability may be conferred by the hydrophobic groups compound **2**, so that the intracellular concentration of compound **2** can reach higher levels than compound **1**. Alternatively, compound **1** may be less stable in vivo than compound **2** or cellular enzymes may attack the ester group to degrade compound **1**, whereas compound **2** may be protected sterically. As the docking model indicates, compound **2** has more contacts in the protein−drug complex than compound **1**, suggesting that the binding of compound **2** to HIV-1 RNase H may be more selective and stable than that of compound **1** in vivo. Therefore, further modifications should be considered to lower the IC_{50} of NACME derivatives to inhibit HIV-1 replication and achieve specificity.

The feasibility of the drug−protein docking structure was supported by the following evidence: two independent simulation softwares programs (GOLD and Glide) gave similar drug−protein interaction models, the predicted binding affinities from the simulations were largely consistent with the experimental data as demonstrated by the inverse correlation between docking scores and IC_{50}s, and the involvement of conserved His residues and metal ions in the predicted drug−enzyme interactions at the RNase H catalytic center. The human RNase H1 was weakly inhibited by compound **2** but not with compound **1**, where the ChemScore of compound **1** was slightly higher than that of compound **2**. This could be explained by compound **1**'s hydrophobic interaction with Met212 and Phe213 of human RNase H1 that compound **2** lacks, which may result in lowering the compound **1**'s ability to inhibit human RNase H1 activity. It is also possible that the modest ChemScore difference between compounds **1** and **2** may be within the cross validation error.[26] Compounds **1** and **2** are predicted to interact with both Asp443 and Asp549, chelators of magnesium ion. On the other hand, the inactive NACME compounds depicted in Figure 5D are predicted to interact only with D549 but not with D443. Thus, we propose a hypothesis that the interaction of NACME compounds to both D443 and D549 is important for their abilities to inhibit RNase H activity. The metal ions are essential for RNase H function[27] and His539, highly conserved residue, plays an important role in binding the scissile phosphate of the RNA.[28] Results from previous studies have shown that mutation of His539 in RT leads to a significant reduction in RNase H activity.[29] The three-dimensional structure of the RNase H domain is similar among HIV-1, *E. coli*, and human enzymes. In the catalytic center, several conserved amino acids can be identified including the His residue: His539 of HIV-1 RT, His124 of *E. coli* RNase H, and His264 of Human RNase H1. The presence of predicted contacts between compounds **1** or **2** and His539 correlated well with their ability to inhibit RNase H activity. On the basis of the structural models, we hypothesize that compounds **1** and **2** block RNase H activity by interfering with the role of His539 and the metal ions rather than through the physical interference with nucleic acid−enzyme binding because RT has sufficient molecular surface available for nucleic acid binding.

Compounds **1** and **2** showed lower IC_{50}s against RNase H from the HIV-1 clade CRF01 A_E compared to those against HIV-1 clade B and C, even when using enzymes with comparable purities and RT polymerase activities. The amino acids at or close to the RNase H active site are identical among the clade B, C, and CRF01 A_E enzymes. The subtle amino acid changes present at noncatalytic site regions could affect enzyme function. For HIV-1 RT, mutations in the connection or RNase H domains, remote from the polymerase catalytic site, were shown to affect the polymerase function.[30−32] The nonnucleoside RT inhibitor efavirenz positively affects the RNase H activity even though the binding site is distinct from the RNase H catalytic site.[33] Thus, we hypothesize that the structure of the RNase H domain of clade A_E RT is affected by noncatalytic site amino acids that differ from the clade B or C RT in such a way that compounds **1** and **2** can access and/or bind to the RNase H catalytic site more efficiently (Figure S1, Supporting Information). It is also possible that the altered RNase H structure may allow NACME derivatives to bind more tightly by generating additional drug−protein interactions.

Cytotoxicity was not detected for compound **1** but was detected for compound **2** at concentrations of 30 μM or more depending upon the cell line. Despite the differences in primary structure between HIV-1 RNase H and human RNase H, their three-dimensional structures are remarkably similar. The specificity to HIV-1 RNase H needs to be improved based on in silico design, to decrease the binding affinity between NACME derivatives and human RNase H, and thereby decrease the cytotoxicity of these inhibitors.

To improve the efficacy of NACME derivatives, we propose to attach additional chemical moieties at the carbamoyl ester side to fill pockets connected to the active site of the enzyme. Replacing polar atoms with less polar atoms should be considered to increase the hydrophobicity and allows the compound to cross the plasma membrane. A cell permeant chemical could be taken orally. Also, the ester group in the middle of the NACME motif should be modified to protect from potential attack by cellular esterases. The nitrofuran group may yield a toxic metabolite in vivo because a structurally similar carcinogen semicarbazide is metabolically generated from the banned drug nitrofurazone.[34] These points should be considered in modifying the structure of the NACME core. Interestingly, a mutation in His539 decreases the polymerase activity by 50%.[1] Given that the NACME derivatives appear to interfere with His539, we may be able to generate a dual inhibitor targeting both RNase H and polymerase functions of HIV-1 RT.

Methods

Enzymes. A DNA fragment encoding the HIV-1 RT was amplified from molecular clones with the following primers: for p66, 5′- TGGTTGTACTTTAGGATCCCCAATTAGTCC-3′, and 5′- TATTCCATCGGATCCCTACTATAGCTCTTTCCTGATTC-3′; for p51, 5′-TGGTTGTACTTTAGGATCCCCAATTAGTCC-3′, and 5′-AGCCCCATCGGATCCCTACTAGAAAGTCTCTGC-3′. The PCR products were digested with *Bam*HI and cloned into the *Bam*HI site of pQE-9 (QIAGEN GmbH, Hilden, Germany) to generate plasmids expressing p66 or p51 RT with a hexahistidine tag at the amino terminus. The *E. coli* strain BL21(DE3)pLysS (Promega, Madison, WI) was transformed with the plasmids, and protein expression was induced by treatment with 1 mM isopropyl β-D-thiogalactoside for 3 h. The basic procedures for purification of the hexahistidine-tagged p66 and p51 monomers of HIV-1 RT have been described.[35,36] For heterodimer purification, bacteria expressing p66 or p51 were mixed prior to purification. The AKTA prime step gradient system (GE Healthcare Bio-Sciences, Piscataway, NJ) was used with HiTrap HP columns (GE Healthcare) according to the manufacturer's protocol. The yields of purified proteins were estimated using a Protein assay kit (Bio-Rad, Hercules, CA.) with bovine serum albumin as a standard. All