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HIV-1 integrase inhibitors that block HIV-1 replication in infected cells. Planning synthetic derivatives from natural products*

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Abstract: Combination therapy using reverse transcriptase (RT) and protease (PR) inhibitors is currently the best clinical approach in combatting acquired immunodeficiency syndrome (AIDS), caused by infection from the human immunodeficiency virus type 1 (HIV-1). However, the emergence of resistant strains calls urgently for research on inhibitors of further viral targets such as integrase (IN), the enzyme that catalyzes the integration of the proviral DNA into the host chromosomes. Recently, we started studies on new IN inhibitors as analogs of natural products, characterized by one or two 3,4-dihydroxycinnamoyl moieties, which were proven to be IN inhibitors in vitro. Then, we designed and synthesized a number of derivatives sharing 3,4-dihydroxycinnamoyl groups, obtaining potent IN inhibitors active at submicromolar concentrations. Unfortunately, these derivatives lacked antiretroviral activity, probably owing to their high cytotoxicity. So we designed a number of 3,4,5-trihydroxycinnamoyl derivatives as less-cytotoxic IN inhibitors, which were proven to be antiretrovirals in cell-based assays. Finally, we designed and synthesized a number of aryldiketohexenoic acids, strictly related to the aryldiketo acid series recently reported by Merck Company, which were shown to be potent antiretroviral agents endowed with anti-IN activities either in 3′ processing or in strand transfer steps.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), caused by infection from the human immunodeficiency virus type 1 (HIV-1), remains a serious global health problem.

After years of hard work, a number of inhibitors of reverse transcriptase (RT) and protease (PR) were discovered and introduced in clinical practice [1,2]. Unfortunately, all the monotherapies using either RT or PR inhibitors have failed owing to the rapid emergence of HIV-resistant strains, and the long-term goal of eradicating the virus from infected cells is still unattained. However, the use of combinations of both RT and PR inhibitors has resulted in significant increases in disease-free survival [3]. This multiple attack is more effective, blocking two different steps of the virus replication cycle and

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causing a delay in the emergence of resistant strains. Therefore, it is evident that the development of new inhibitors targeted toward other viral proteins is of paramount importance.

A further viral protein as potential target for antiretroviral therapy is HIV-1 integrase (IN) [4]. Recently, anti-IN agents in combination with RT and PR inhibitors have been found to be synergistic in in vitro assays [5], and a combination therapy that uses inhibitors of all three enzymes at the same time could result in a real breakthrough in the HIV-1 therapy. Unfortunately, no inhibitor of HIV-1 IN is currently used in clinical practice, and this fact led us to make greater efforts in research in this field.

The full-length HIV-1 IN (288 amino acids) has three domains: the catalytic core, the C-terminal, and the N-terminal domains. It is thought that the catalytic core contains the active site responsible for catalysis of all the reactions of integration. Three amino acids (Asp64, Asp116, and Glu152) in the catalytic core domain are highly conserved among retrotransposon and retroviral INs. Mutation of these residues generally leads to a loss of all the catalytic activities of these proteins, and they are therefore thought to be essential components of the IN active site [6]. The catalytic core domain socked with 5-chloroindolyltetrazolylpropenone (5CITEP) (a potent inhibitor of IN reported by Shionogi Company) was resolved by D. Davies [7]. The crystal structure shows a dimeric model in which two monomers interact with each other, but actually it is not clear whether IN works in vivo as a monomer, a dimer, or a tetramer.

Multiple steps in the integration process are catalyzed by HIV-1 IN, as shown schematically in Fig. 1. The integration of HIV-1 DNA into the host chromosome is achieved by the IN performing a series of DNA cutting and joining reactions (A–C). The first step in the integration process is 3′ processing, in which the enzyme removes two nucleotides from each 3′ end of the proviral DNA, leaving recessed CA OHs at the 3′ ends. Moreover, IN cuts the human DNA at the site of integration 5 bases apart (Fig. 1A) [8].

In a second step, termed "strand transfer", the IN protein joins the previously processed 3′ ends to the 5′ ends of strands of target DNA at the site of integration (Fig. 1B). Finally, in the 5′ end, the joining IN fills in the gaps and ligates the unjoined strands (Fig. 1C).

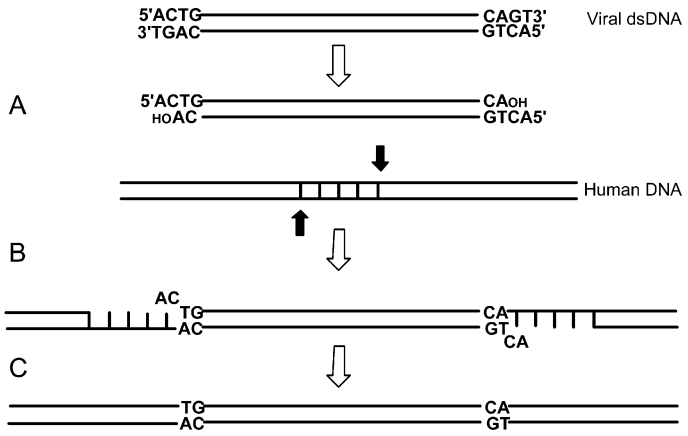


Fig. 1 Schematic steps for HIV-1 integration.

DIHYDROXYCINNAMOYL DERIVATIVES

In the last 10 years, many different classes of compounds have been reported to inhibit the HIV-1 IN. They belong to different classes, namely DNA binders, peptides, oligonucleotides, nucleotides, and polyhydroxylated aromatics [9].

The most important one is the polyhydroxylated aromatic class. A number of these compounds are natural products such as aurintricarboxylic acid (ATA) [10], caffeic acid phenethyl ester (CAPE) [11], tyrphostin [12], and quercetin [13], which inhibited the IN enzyme at micromolar concentrations (Fig. 2).

Unfortunately, the majority of the cited compounds proved inactive in cell-based assays. Notable exceptions are curcumin [14], chicoric acid [15], and 3,5-dicaffeoylquinic acid [16], which, in addition to be very potent and specific IN inhibitors in enzyme assays, blocked the HIV-1 multiplication in acutely infected cells at noncytotoxic concentrations (Fig. 3).

A few years ago, we started our studies in this field and focused our attention on the chemical features shown by these compounds. We noted that the majority of natural products endowed with anti-IN activity were characterized by one or two 3,4-dihydroxycinnamoyl moieties sometimes incorporated in a ring structure such as in quercetin. Then we started the design and synthesis of new IN inhibitors, hopefully active in HIV-1 infected cells. We designed a series of cinnamoyl derivatives as geometrically and conformationally constrained structures characterized by a *syn* disposition of the carbonyl group with respect to the vinylic double bond [17]. The rationale of freezing this moiety in a *syn* arrangement relied on the observation that some flexible *syn* derivatives, such as curcumin, display anti-HIV-1 activity in cell-based assays, while flavones such as quercetin, having this fragment fixed in *anti*, are inactive against HIV-1 infected cells (Fig. 4).

So we hoped that stabilization in a *syn* arrangement could give novel IN inhibitors endowed with anti-HIV-1 activity and synthesized various polyhydroxylated derivatives **1**–**7** containing one or two cinnamoyl moieties (Fig. 5).

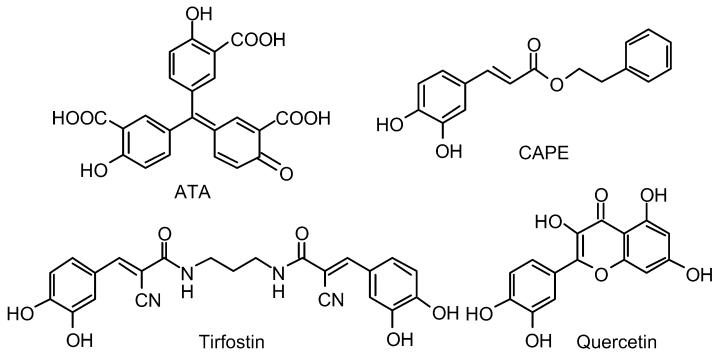


Fig. 2 Natural polyhydroxylated compounds active as IN inhibitors.

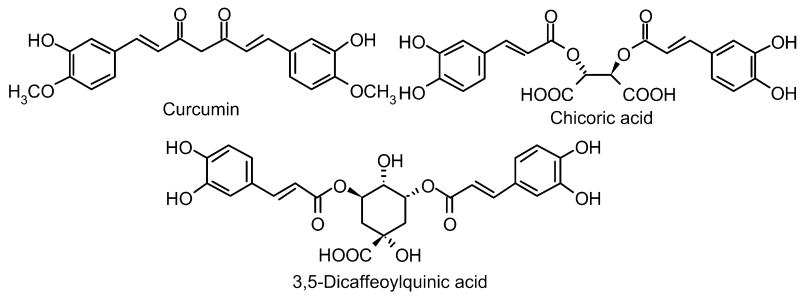


Fig. 3 Natural products that inhibit either IN enzyme or HIV-1 replication in cell-based assays.

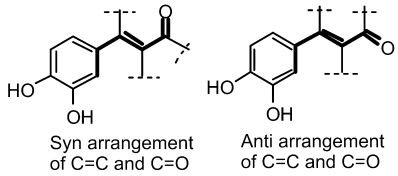


Fig. 4 *Syn* and *anti* dispositions of C=C and C=O groups in 3,4-dihydroxycinnamoyl derivatives.

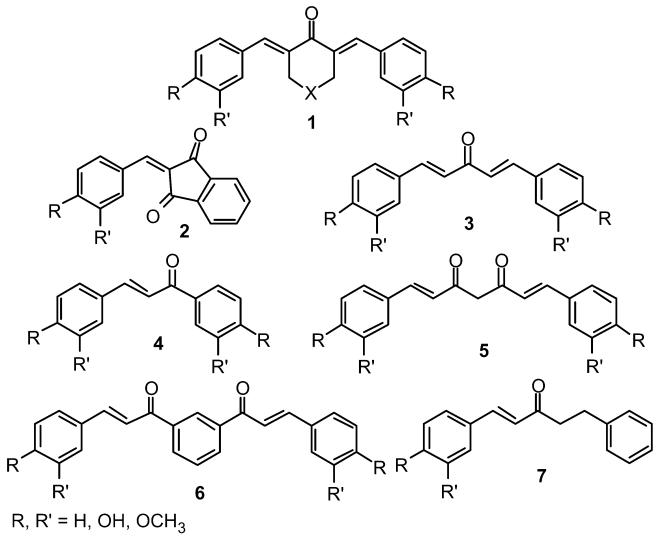
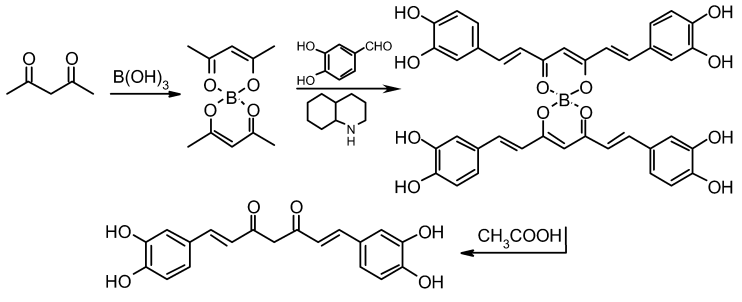


Fig. 5 Cinnamoyl-based derivatives investigated as IN inhibitors.

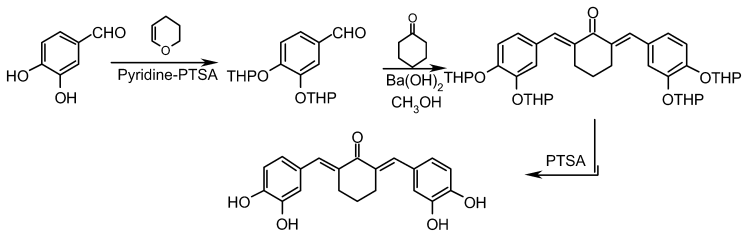
The curcumin-like derivatives were synthesized starting from acetylacetone, which was treated with boric acid to block the ketoenolic form, preventing the condensation on the methylene group.



Scheme 1 Synthesis of curcumin-like derivatives.

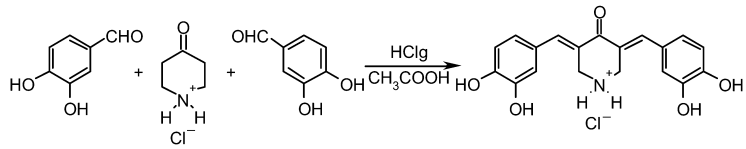
The intermediate that formed was reacted with the appropriate benzaldehyde leading to the protected complex, which was broken with acetic acid to obtain the curcumin-like derivatives (Scheme 1).

The cyclovalone analogs were achieved starting from 3,4-dihydroxybenzaldehyde. Firstly, hydroxyls were protected with tetrahydropyranyl (THP) groups, and the condensation of the appropriate protected aldehyde with cyclohexanone was performed in the presence of barium hydroxide. Finally, the THP groups were easily removed by treatment with *para*-toluenesulfonic acid (PTSA) (Scheme 2).



Scheme 2 Synthesis of cyclovalone derivatives.

Nitrogen derivatives were obtained by condensing 3,4-dihydroxybenzaldehyde with 4-piperidone in acetic acid under a stream of gaseous hydrogen chloride (Scheme 3).



Scheme 3 Synthesis of 4-piperidone derivatives.

All the compounds described were tested in enzyme assays against IN and RT to prove their selectivity against the integration step. Moreover, assays on 3′ processing, strand transfer, and disintegration steps were performed. Finally, we performed cell-based assays either against HIV-1 MT-4 acutely infected cells, or in mock-infected cells to state their cytotoxycity [17].

In general, our polyhydroxylated derivatives **1**–**7** were found to be very potent IN inhibitors and the values of 3′ processing paralleled with strand transfer ones.

The most potent group of inhibitors was the cyclohexanone derivatives with IC_{50} ranging from 0.2 to 0.9 μM, no matter whether the ring was cut (**RDS 1028**, IC_{50} = 0.6 μM) or the methylene was replaced by isosteric O, S, or NH (**RDS 1158**, IC_{50} = 0.9 μM; **RDS 1190**, IC_{50} = 0.2 μM; **RDS 1211**, IC_{50} = 0.2 μM; **RDS 1195**, IC_{50} = 0.2 μM) (Fig. 6).

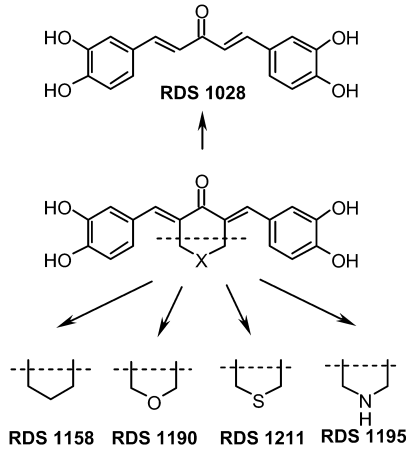


Fig. 6 Cyclovalone analogs as IN inhibitors.

Some conclusions could be drawn on the structure–activity relationships within this series of IN inhibitors:

(i) The catechol group plays a vital role in IN enzyme inhibition. In fact, in curcumin series, the derivative **RDS 1222**, in which there are two phenolic hydroxyl groups instead of methoxyl groups, showed IC_{50} = 0.7 μM, while curcumin was about 40 times less potent (IC_{50} = 30 μM) (Fig. 7); moreover, the dimethoxy, monohydroxy, and unsubstituted derivatives (**RDS 1212**, **RDS 1217**, **RDS 1220**, respectively) were totally inactive.

(ii) The activity of the bis dihydroxycinnamoyl derivatives was relatively unrelated to both the distance between the two cinnamoyl units and the nature of the linker. In fact, the compound with a partial superimposition of the cinnamoyl moieties on the carbonyl group (**RDS 1028**, IC_{50} = 0.6 μM) was nearly as active as the corresponding cyclic form (**RDS 1158**, IC_{50} = 0.9 μM). Moreover, similar activities were shown by **RDS 1222** (IC_{50} = 0.7 μM) and **RDS 1155** (IC_{50} = 0.8 μM), in which the cinnamoyl groups were spaced by a methylene or a phenyl linker, respectively (Fig. 8).

Molecular modeling studies on compounds **1**–**7** (Fig. 5) were performed by Buolamwini and Assefa [18], which used comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) methods to explore the binding mode of the above polyhydroxylated derivatives at the active site of IN. They docked **1**–**7** in the IN core and found two clusters, the horizontal one and the vertical one. Interestingly, the cited model could explain why molecules with very different lengths (i.e., **1** and **6**) showed similar activities. In fact, the extension of the molecule goes out to the IN core with no negative interactions with enzyme, while the binding with the enzyme was ensured by the dihydroxycinnamoyl moiety with hydrogen bonds in the integration site.

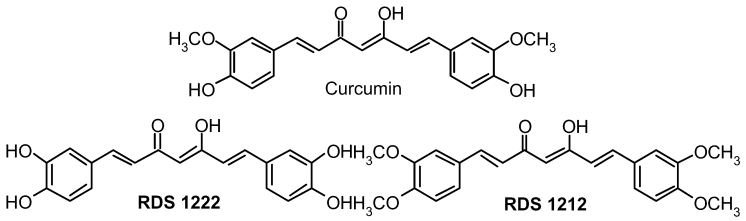


Fig. 7 Curcumin analogs as IN inhibitors.

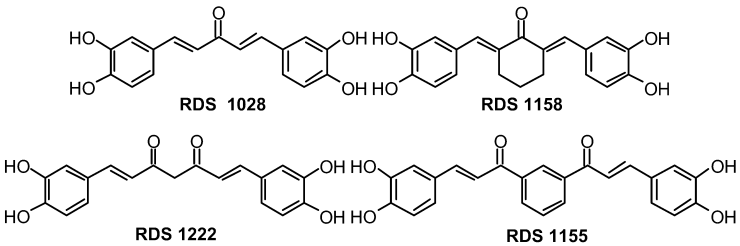


Fig. 8 Potent IN inhibitors with different linkers between the two cinnamoyl moieties.

TRIHYDROXYCINNAMOYL DERIVATIVES

After all, the dihydroxycinnamoyl moiety could be considered an excellent pharmacophore for obtaining potent IN inhibitors. Unfortunately, polyhydroxylated compounds **1**–**7**, although being very potent anti-IN agents, were totally inactive against virus replication cycle in cell-based assays. We hypothesized that this inactivity at concentrations lower than cytotoxic ones was due to the facile in vivo oxidation of the catechol moiety to quinone species, endowed with high toxicity for cell machinery. In fact,

quinones, which are very good electrophile reagents, are easily attacked by nucleophilic centers of the proteins, which remain irreversibly alkylated (Fig. 9) [19].

Therefore, bearing in mind that disruption of catechol moiety of 3,4-dihydroxycinnamoyl pharmacophore leads to inactive anti-IN compounds and that at the same time the presence of 3,4-dihydroxyaryl group leads to highly cytotoxic derivatives, we designed new IN inhibitors characterized by a 3,4,5-trihydroxycinnamoyl group. We chose this moiety for the following reasons: (i) a third substituent on the aryl moiety could reduce the overall process of alkylation of the cellular proteins; (ii) a further hydroxyl is added in a part of the pharmacophore highly involved in hydrogen bonds with the biological target (Fig. 10).

In that way, we achieved a breakthrough and, for the first time, obtained IN inhibitors endowed with anti-HIV-1 activity in cell-based assays. Figure 11 shows a few examples of 3,4,5-trihydroxycinnamoyl derivatives endowed with antiviral activity showing EC_{50} ranging from 20 to 34 μM [20].

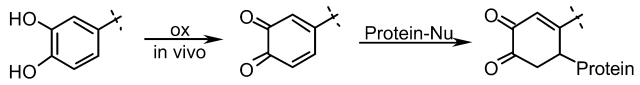


Fig. 9 Presumed mechanism of toxicity of catechol derivatives.

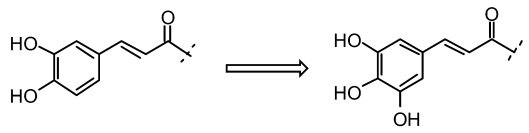


Fig. 10 Design of new IN inhibitors endowed with lower cytotoxicity.

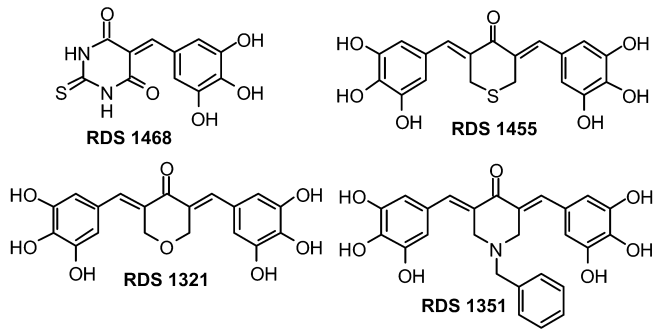


Fig. 11 Examples of new IN inhibitors endowed with anti-HIV-1 activity in cell-based assays.

CARBOXYLIC DERIVATIVES

A comparison of the structural features of compounds synthesized by our group with chicoric acid and the recently reported styrylquinolines led us to introduce a carboxylic acid function in the skeleton of trihydroxycinnamoyl derivatives. In fact, in addition to structural similarities, the carboxylic acid function could give additional interactions with Lys-156 and Lys-159 being in the catalytic site of IN. Interestingly, this introduction led to increased potency in the series of trihydroxycinnamoyl derivatives. As a matter of fact, carboxylic derivative **RDS 1541** (EC_{50} = 2 μM) was 10 times more active than **RDS 1455** in cell-based assays (Fig. 12) [20].

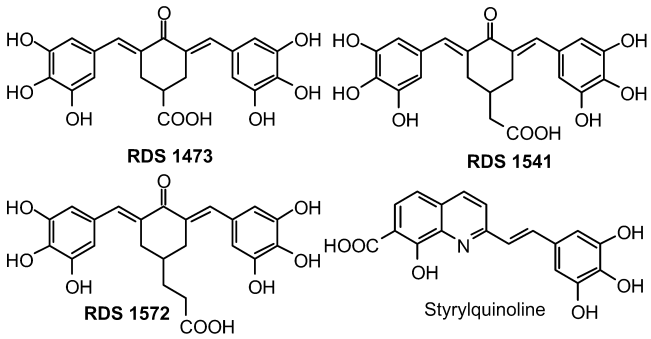


Fig. 12 Comparison of the structural features of the newly synthesized carboxy derivatives with a styrylquinoline derivative.

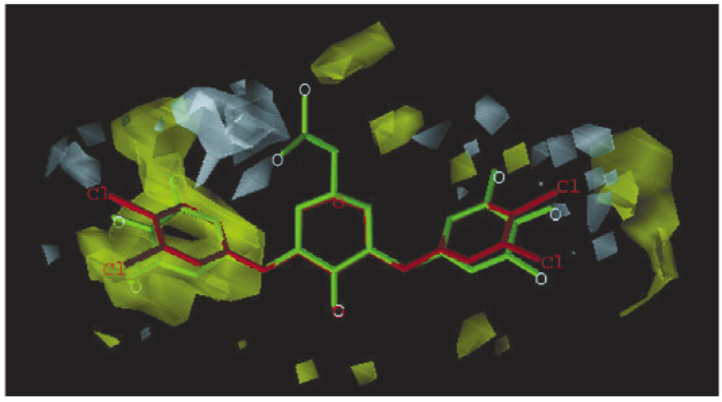


Fig. 13 GRID/GOLPE partial least square (PLS) coefficients contour maps for the 3D-QSAR model of cinnamoyl-derivatives. Highly active derivative **RDS 1541** (green) and inactive dichloroderivative **RDS 1310** (red) are displayed.

On the contrary, the substitution of the carboxylic function with esters, nitriles, or alcohol groups, led to inactive derivatives, proving the unique role played by the carboxylic group in obtaining potent IN inhibitors in cell-based assays.

Recently, we performed 3D quantitative structure–activity relationship (QSAR) studies on either di- or trihydroxycinnamoyl series with a GRID/generating optimal partial least squares estimations (GOLPE) approach. The main results are shown in Fig. 13, which shows the most potent derivative **RDS 1541** (green), the inactive dichloroderivative **RDS 1310** (red), and the steric and electrostatic contour maps. The blue polyhedra represent zones with positive interactions with positively charged groups, while yellow polyhedra represent zones with low steric tolerance. Blue polyhedra are all around the hydroxyls and the carboxylic group, which can give hydrogen bonds, while yellow polyhedra, farther out than the blue ones, point out the low tolerance for even not so bulky groups such as methoxy or chlorine ones.

ARYLDIKETOHEXENOIC ACIDS

In the last two years, a new class of potent IN inhibitors was reported, namely, aryldiketo acids. The first inhibitor L-731,988, which was a pyrrole derivative, was synthesized by the Merck Company [21], while a compound containing an indole ring, named 5CITEP, was reported later by Shionogi Pharmaceuticals [22].

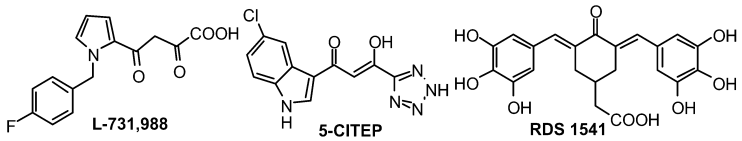


Fig. 14 Lead compound **RDS 1541** and aryldiketoacids.

Figure 14 shows **RDS 1541** (EC_{50} = 2 μM; IC_{50} = 0.2 μM for 3′ processing and IC_{50} = 0.3 μM for strand transfer), L-731,988 (EC_{50} = 1 μM; IC_{50} = 6 μM for 3′ processing and IC_{50} = 0.17 μM for strand transfer) and 5CITEP (IC_{50} = 35 μM for 3′ processing and IC_{50} = 2.1 μM for strand transfer). Interestingly, our lead compound is a potent inhibitor of HIV-1 replication cycle in acutely infected cells at concentrations similar to that of L-731,988, while 5CITEP lacked antiretroviral activity. Moreover, the aryldiketo acids are selective inhibitors of the strand transfer step, whereas **RDS 1541** blocks 3′ processing and strand transfer at similar concentrations.

A structural analysis of the polyhydroxylated compounds and the aryldiketo derivatives, led us to observe that the above described IN inhibitors are characterized by one or more following features: (i) a cinnamoyl moiety, typical for a number of natural products and for synthetic derivatives, including the series synthesized by our group; (ii) a 1,3-diketo group, present in aryldiketo acid series; (iii) a carboxylic acid function, found in natural products (i.e., chicoric acid), in diketo acid series (i.e., L-731,988 and 5CITEP), and in our derivatives (i.e, **RDS 1473**, **RDS 1541** and **RDS 1572**); (iv) an aromatic portion (i.e., 1-benzylpyrrole, indole, or trihydroxyphenyl group) (Fig. 15).

Bearing in mind these structural features, we designed new IN inhibitors belonging to the diketohexenoic acid class. These molecules show all the above structural features. In fact, there is a Ψ-cinnamoyl moiety in which the phenyl group is replaced by the isosteric pyrrole ring, there are 1,3-diketo group and carboxylic acid functions, and, finally, 1-benzyl pyrrole portion is included. Finally, the newly designed derivatives could be considered as vinylogs of Merck derivatives, obtained with the insertion of the vinyl group between 1-benzylpyrrole and the 2,4-diketobutanoic moieties [23].

The synthesis of the diketohexenoic acids is illustrated in Scheme 4. Firstly, 1*H*-pyrrole-2-carboxaldehyde was alkylated with the appropriate benzyl bromide in alkaline medium (K_2CO_3) obtaining the *N*-alkylated derivatives that underwent condensation with acetone in 5N sodium hydroxide at room temperature. Then, the butenones that formed were condensed with diethyl oxalate in the pres-

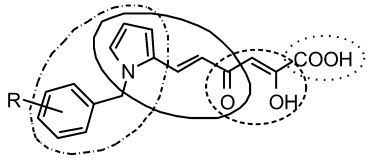
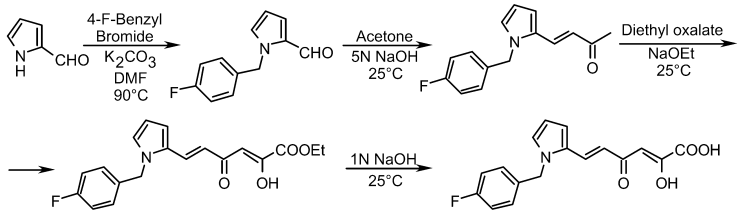


Fig. 15 Chemical features of newly synthesized aryldiketohexenoic acids.



Scheme 4 Synthesis of aryldiketohexenoic acids.

ence of sodium ethoxide to obtain the diketoesters that were easily hydrolyzed in 1N sodium hydroxide to afford the required diketohexenoic acids (Scheme 4).

The results of the screening carried out in either enzymatic or cell-based assays for the newly synthesized aryldiketohexenoic acids are reported in Table 1. Compound **RDS 1699** was the most potent derivative against replication of HIV-1 infected cells in cell-based assays, showing EC_{50} = 1.5 μM comparable to that of L-731,988.

Table 1 Cytotoxicity and antiviral activities in enzyme (IN) and cell-based assays of aryldiketohexenoic acids.

Compound	R	CC_{50}[a] (μM)	EC_{50}[b] (μM)	S.I.[c]	IC_{50} (μM)[d]	
					3′ Processing	Strand Transfer
RDS 1699	H	61	1.5	41	7.9	7.0
RDS 1714	Cl	95	>95		nd[e]	nd
RDS 1644	F	80	11	7.3	nd	nd
RDS 1693	Me	41	>41		nd	nd
RDS 1716	OMe	78	>78		nd	nd
RDS 1704	NO_2	33	>33		nd	nd
L-731,988			1.0		6.0	0.05

[a]Cytotoxicity: compound dose required to reduce the viability of the mock infected cell by 50 % as determined by the MTT method.
[b]Compound concentration required to reduce the exponential growth of MT-4/KB cells by 50 %.
[c]Selectivity index: CC_{50}/EC_{50} ratio.
[d]Compound concentration required to reduce rIN processing strand transfer of 3′ end-labeled 40mer substrate by 50 %.
[e]nd: not determined.

Fluoro derivative **RDS 1644** was about 10 times less potent than **RDS 1699**, whereas all remaining derivatives substituted in the 4-position of phenyl group with chlorine, methyl, methoxy, or nitro groups were inactive. **RDS 1699** was proved to be an IN inhibitor in enzyme assays. Surprisingly, even if structurally very similar to each other, **RDS 1699** and L-731,988 act in a different way against IN enzyme. In fact, the Merck derivative is a selective inhibitor of strand transfer step, while **RDS 1699** blocks either ST or 3′ processing at similar concentrations.

We performed a molecular modeling approach to try to explain the difference in the biological activity of **RDS 1699**, 5CITEP, and L-731,988. We docked these inhibitors in the IN core, obtaining the following results: (i) a common ligand receptor interaction pattern could be noted with the diketo function and the carboxylic group (or the bioisosteric tetrazole ring) being involved in similar hydrogen bonding with the receptor; (ii) the 4-F-benzyl chain of L-731,988 and the indole ring of 5CITEP point outside the receptor cavity, whereas the benzyl group of **RDS 1699** is totally buried in the enzyme pocket, probably explaining the difference in biological effects between the cited derivatives; (iii) the 4-position of the benzyl group of **RDS 1699** is hidden in the IN pocket and the 4-substituted derivatives could give negative steric interactions with the enzyme: only the little fluorine atom can fit into that narrow pocket (Fig. 16).

These preliminary results on aryldiketohexenoic acids press us to further studies on IN inhibitors belonging to the cited series. The synthesis and the biological assays on 6-(1-arylmethyl-1*H*-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids mono- and disubstituted on the phenyl ring are in progress.

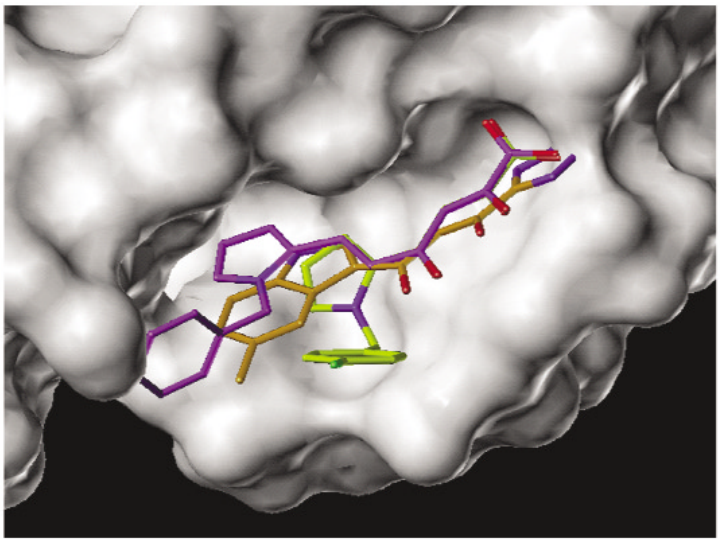


Fig. 16 RDS 1699 (purple) and L-731,988 (yellow) superimposed on 5CITEP (orange) in the IN core (white).

CONCLUSIONS

In the last five years, we designed a number of IN inhibitors with the aim of obtaining compounds active either in enzymatic or in cell-based assays. The first class of inhibitors synthesized by our group was characterized by the presence of one or two 3,4-dihydroxycinnamoyl moieties. A number of the last compounds were potent anti-IN agents, but were lacking in anti-HIV-1 activity in cell-based assays. So we designed a series of 3,4,5-trihydroxycinnamoyl derivatives as less-cytotoxic anti-IN agents and for the first time obtained potent IN inhibitors that blocked the HIV-1 replication cycle in infected cells. The potency of the above derivatives was enhanced by the introduction of a carboxylic acid function in the skeleton of the lead compounds. Finally, we designed and synthesized a series of 6-(1-arylmethyl-1*H*-pyrrol-2-yl)-2,4-dioxo-5-hexenoic acids, strictly related to the potent anti-IN agents endowed with antiretroviral activity recently reported by Merck Company, namely aryldiketo acids. The cited aryldiketohexenoic acids blocked HIV-1 replication in infected cells at micromolar concentrations, in addition to inhibiting either 3′ processing or strand transfer steps in enzyme assays.

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