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1-[2-(2-Benzoyl- and 2-benzylphenoxy)ethyl]uracils as potent anti-HIV-1 agents

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Abstract

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are key components in highly active antiretroviral therapy for treating HIV-1. Herein we present the synthesis for a series of N1-alkylated uracil derivatives bearing oxo-(2-benzyl- and 2-benzylphenoxy)alkyl substituents as novel NNRTIs. These compounds displayed anti-HIV activity similar to that of nevirapine and several of them exhibited activity against the K103N/Y181C RT mutant HIV-1 strain. Further evaluation revealed that the inhibitors were active against most nevirapine-resistant mono- and di-substituted RTs with the exception of the V106A RT. Thus, the candidate compounds can be regarded as potential lead compounds against the wild-type virus and drug-resistant forms.

Keywords: HIV-1, Non-nucleoside reverse transcriptase inhibitors, Reverse transcriptase, Uracil, Benzophenone

1. Introduction

A third decade of pandemic AIDS is underway, despite extensive efforts from scientists across the globe. In the USA alone, almost 1.6 million patients require antiretroviral therapy, while in many developing countries this number is an order of magnitude greater.1 To date 25 antiretroviral drugs have been approved for the developing countries this number is an order of magnitude greater.2 These compounds inhibit HIV replication, however their use is often hampered by toxic side-effects over long periods of therapy, as well as a loss of activity when faced with emerging drug-resistant virus strains. As a result, the search for new therapeutics with minimal side-effects and the ability to retain activity against drug-resistant virus strains is regarded as a critical goal for medicinal chemists.

These anti-HIV drugs comprise six different classes of compounds: eight nucleoside (or nucleotide) reverse transcriptase (RT) inhibitors (NRTIs), four non-nucleoside RT inhibitors (NNRTIs), ten protease inhibitors (PIs), one integrase inhibitor (INI), one fusion inhibitor (FI) and one CCR5 inhibitor.2 The first four classes of inhibitors target three different viral enzymes: HIV–RT, HIV protease and HIV integrase, respectively. NRTI and NNRTI are primary components of HAART.

One area of ongoing research in our laboratories has focused on the design and synthesis of NNRTIs. These compounds are highly specific, non-competitive inhibitors of HIV-1 RT, the key enzyme involved in HIV replication. Since 1996, five NNRTIs (shown in Fig. 1) have been approved by the FDA for clinical use: nevirapine (Viramune, Boehringer Ingelheim), delavirdine (Rescriptor, Pharmacia & Upjohn), and efavirenz (Sustiva, DuPont) are classified as first generation NNRTIs, whereas etravirine (Intellence, Tibotec) and rilpivirine (TMC278, Tibotec) are considered as second-generation NNRTIs, since they retain activity against a variety of drug-resistant virus strains.3 NNRTIs have been reported to bind to a non-nucleoside inhibitor binding pocket in HIV-1 RT (NNIBP), which is located in the ‘palm’ domain of the p66 subunit at a distance of approximately 10 Å from the enzyme catalytic site.4,5 The pocket is primarily hydrophobic and key amino acid residues involved in inhibitor binding include aromatic (Y181, Y188, F227, W229 and Y232), hydrophobic (P59, L100, V106, V179, F227, L234 and P236) and hydrophilic (K101, K103, S105, D132 and E224) residues in the p66 RT subunit, as well as three additional amino acids (I135, E138 and T139) in the p51 subunit.4,5 One major limitation of many NNRTIs however, is the rapid emergence of HIV-1 variants resistant to these drugs due to one or more point mutations in the RT binding site.
reported. Inhibitors of this structural type were found to be highly active against both wild-type HIV-1 and clinically significant drug-resistant forms.\textsuperscript{21-23} This class of compounds is based on the benzophenone fragment bridged by a short linker to an ortho-methyl-aniline residue. SAR studies have further revealed that the substituents in the two meta-positions of the benzoyl moiety as well as the benzophenone fragment play crucial roles in retaining antiviral activity against mutant strains.

Thus, by combining these leads, ‘chimeric’ molecules containing the uracil residue such as that found in scaffold 1, as well as the benzophenone fragment found in scaffold 2, may not only inhibit HIV-1 RT, but also exhibit strong antiviral activity towards both wild-type HIV-1 and drug-resistant virus strains (Fig. 2). Herein we report the synthesis of N\textsuperscript{1}-alkylated uracil derivatives and the initial biological studies describing their antiviral activity.

\section{2. Results and discussion}

\subsection{2.1. Chemistry}

Synthesis of the target compounds was carried out by condensation of equimolar quantities 2,4-bis(trimethylsilyloxy)pyrimidine or 5-methylpyrimidine 3 with 1-bromo-\(\omega\)-(2-benzylphenoxy)-4 or 1-bromo-\(\omega\)-(2-benzylphenoxy)alkanes 5. Starting bromides 4 and 5 were prepared according to previously described methods.\textsuperscript{24} This synthetic approach has a key advantage: the reaction results in the N\textsuperscript{1}-substituted uracils as the only product. Formation of the N\textsuperscript{3}-substituted products was not observed, likely due to the steric hindrance of the adjacent trimethylsilyl groups blocking the approach to the N\textsuperscript{3}-nitrogen atom.

It is known that condensation of trimethylsilyl pyrimidines with highly reactive alkylating agents occurs in a facile manner in aprotic solvents under mild conditions and with high yields.\textsuperscript{25,26} In contrast, treatment of 2,4-bis(trimethyl-silyloxy)pyrimidines with alkylating agents of lower reactivity requires elevated temperatures. Condensation of trimethylsilyl uracil derivatives with allylbromides\textsuperscript{27,28} or benzylhalides\textsuperscript{28} successfully proceeds in 1,2-dichloroethane in the presence of a catalytic amount of iodine. Heating 2,4-bis(trimethyl-silyloxy)pyrimidines with an excess of alkyl halide leads to 1-substituted uracils with good yields.\textsuperscript{29,30} Previously we have reported a method of synthesis of 1-[2-(phenoxy)ethyl]-uracil derivatives which was based on condensation of trimethylsilyl uracil derivatives with 1-bromo-2-(phenoxy)ethanes.\textsuperscript{31}

Analogous to this route, we have now synthesized a number of 1-[2-(2-benzophenyl)oxy]ethyl] and 1-[\(\omega\)-(2-benzylphenoxy)ethyl]uracil derivatives 6-24. The synthesis of 1-[2-(2-benzophenyl)oxy]ethyl]uracil 6 was accomplished by heating trimethylsilyl derivative 3 and bromide 4 or 5 at 160–170°C for 2 h without a solvent. The target compounds were obtained in 62–83% yields, and formation of the N\textsuperscript{1}- and N\textsuperscript{3}-disubstituted byproducts was not observed.

The presence of a substituent at position 6 of the pyrimidine can affect regioselectivity of 6-R-uracil alkylation.\textsuperscript{32} For example, condensation of 2,4-bis(trimethylsilyloxy)-6-methylpyrimidine with 4-5 molar excess of 1,4-dibromobutane leads to a complex mixture of N\textsuperscript{1}- and N\textsuperscript{3}-mono- as well as to N\textsuperscript{1},N\textsuperscript{3}-disubstituted uracils, the ratio of which depends upon the reaction temperature.\textsuperscript{33} As a result, an alternative approach for synthesis of 1-[2-(3,5-dimethylbenzoyl)-4-methylpyrimidinyl]ethyl]-6-methyluracil 25 was needed. Thus treatment of 6-methyluracil (5 molar excess) with bromide 5 in the presence of KCl in DMF (Scheme 1) afforded 25 in a 64% yield following separation from the N\textsuperscript{1},N\textsuperscript{3}-disubstituted product.

To study the influence of the bridging carbonyl group on the antiviral properties of the compounds, the reduced analogue of compound 17 was pursued. As shown in Scheme 2, treatment of 17 with NaBH\textsubscript{4} in the presence of NaOH resulted in the hydroxyl derivative 26 (93%).
or two substituents at the R4 position(s). As such, compound C-5 (pyrimidine ring whereas introduction of the same substituent at 17 pound. The most potent anti-HIV agents were found to be uracils 24 whereas their 2-(2-benzoylphenoxy)ethyl counterparts 6–10 oxy)ethyl uracils (SAR) of the compounds, it became evident that 2-(2-benzylphen- against HIV-1. In examining the structure–activity relationship however most compounds exhibited notable antiviral activity ble 1). All compounds were found to be inactive against HIV-2, (ROD), or the double mutant (K103N + Y181C RT) virus strain (Ta-

2.2. Antiviral activity

The anti-HIV properties of the uracil derivatives were studied in human T-lymphocyte CEM (compounds 6–10) or MT-4 (compounds 11–26) cell cultures infected with HIV-1[IIIB] or HIV-2 (ROD), or the double mutant (K103N + Y181C RT) virus strain (Table 1). All compounds were found to be inactive against HIV-2, however most compounds exhibited notable antiviral activity against HIV-1. In examining the structure–activity relationship (SAR) of the compounds, it became evident that 2-(2-benzylphen-

Scheme 1. Reagents and conditions: (i) 160–170 °C, 2 h; (ii) K2CO3, DMF, 70 °C, 8 h.

Scheme 2. Reagents and conditions: (i) NaBH4, NaOH, EtOH (aq.), 50 °C, 8 h.

Concentrations. Most compounds were found to be nontoxic in MT-4/CEM cell cultures. Benzophenone 16, which bears a methyl group at the R3-position, showed the highest toxicity albeit at a minor level (CC50:179 μM).

The studied compounds were also investigated as potential inhibitors of replication against a large panel of other DNA and RNA viruses. However, none of the compounds displayed notable activity against HIV-2 or any DNA or RNA viruses other than HIV-1 (HSV-1, HSV-2, Vaccinia virus, Para-influenza-3 virus, Reovirus-1, Sindbis virus, Vesicular stomatitis virus, Respiratory syncy-

2.3. Enzymatic study

Next, the inhibitory properties of the uracil derivatives against HIV-1 RT enzyme were studied in standard DNA- and RNA-dependent DNA-polymerase assays using activated DNA and wild-type p66/p66 homodimer RT or poly(rC)-oligo(dG) template–primer and p66/p51 heterodimer recombinant enzyme. Noteworthy that these enzyme preparations had similar activity: when activated DNA and wild-type HIV-1 RT enzyme were studied in standard DNA- and RNA-depen-

An increase in antiviral activity was obtained by inserting one or two substituents at the R3 position(s). As such, compound 17 bearing two methyl groups exhibited more pronounced activity than the analogous 15 possessing only one methyl group or 13, which has no methyl groups. Moreover, substitution of these methyl groups by various halogens had no significant impact on the antiviral activity as exemplified in 17 versus 19-21 and 18 versus 22. A similar level of anti-HIV activity was exhibited by the compound 25 bearing a methyl group in the C-6-position of the pyrimidine ring whereas introduction of the same substituent at C-5 (24) slightly decreased the antiviral properties of the compound. The most potent anti-HIV agents were found to be uracils 17, 20, 22 and 25 which inhibited viral replication at nanomolar concentra-

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A preliminary investigation of their mechanism of action was performed with compound 8 in p66/p66/activated DNA assay. It was shown that this compound acted in a non-competitive manner with regards to the dNTP substrate (Fig. S1). In addition, the uracil derivative 8 retained its efficacy when the enzymatic reactions were carried out in the presence of heparin, which was added
simultaneously with the inhibitor and dNTPs after a short pre-incubation period of the RT with activated DNA (Fig. S2). Since heparin prevents reinitiation of the polymerization reaction, this indicates that the inhibitor only impacts the elongation process of the RT reaction and has no effect on binding of the enzyme to nucleic acids.

The inhibition constants ($K_I$) reflecting the affinity of each compound for RT were assessed using a Dixon plot. Comparison of the results revealed that the compounds displayed similar inhibitory activity in activated DNA (p66/p66) and poly(rC)-oligo(dG) (p66/p51) assays. The correlation ($r$) between the $K_I$ values of the test compounds against HIV-1 RT in these assays was 0.979 (Fig. S3). Moreover, the correlation ($r$) between the EC50’s for HIV-1 replication in cell culture and the $K_I$’s for HIV-1 RT inhibition using p66/p66 and p66/p51 were 0.911 and 0.952, respectively (Fig. S4).

In examining the SAR of the compounds, the results show that, in general, as also noticed in the HIV-1-infected cell culture system, 2-(2-benzoylphenoxy)ethyl uracils 11–22 exhibit greater inhibition of HIV–RT than their corresponding 2-(2-benzylphenoxy)ethyl counterparts (compounds 6–10). The differences between the benzophenones and the diphenylmethane series were especially pronounced when comparing the efficacy of compounds 11 and 6. In contrast, the carbonyl group had almost no influence on the activity of uracil derivatives bearing methyl groups at the R3 and R4 positions (16 vs 8).

Nonetheless, considering that the reduced benzohydroxy analogue 26 failed to inhibit HIV–RT, the benzophenone carbonyl fragment appears to be crucial for retention of activity and must be interacting with the enzyme in some fashion.

### Table 1

| Compound | R1 | R2 | R3 | R4 | CC50 (µM) | CEM or MT-4 HIV-1 (III B) wild type | HIV-1 (III B) K103N + Y181C |
|----------|----|----|----|----|----------|----------------------------------|-----------------------------|
| 6        | H  | H  | 4-Me | H  | 48c      | 3.3c                             | 3.3c                        |
| 7        | H  | H  | 4-Me | 3-Me | 53c      | 0.22c                            | 0.22c                       |
| 8        | H  | H  | 4-Me | 3,5-Me2 | 66c      | 0.13c                            | 0.13c                       |
| 9        | H  | H  | 4-Cl | 3,5-Me2 | >200c | 0.22c | >909 |
| 10       | H  | H  | 4-Br | 3,5-Me2 | >220c | 0.22c | >1000 |
| 11       | H  | H  | Me   | H   | >357     | 0.31 | >1136 |
| 12       | H  | H  | F    | H   | >353     | 0.51 | >1136 |
| 13       | H  | H  | Cl   | H   | >337     | 0.089 | >1136 |
| 14       | H  | H  | Br   | H   | >301     | 0.065 | >1136 |
| 15       | H  | H  | Cl   | 3-Me | >325     | 0.052 | >1136 |
| 16       | H  | H  | Me   | 3,5-Me2 | 179     | 0.058 | >1136 |
| 17       | H  | H  | Cl   | 3,5-Me2 | >313    | 0.016 | >1136 |
| 18       | H  | H  | Br   | 3,5-Me2 | >282   | 0.023 | >1136 |
| 19       | H  | H  | Cl   | 3,5-F2 | >307   | 0.027 | >1136 |
| 20       | H  | H  | Cl   | 3,5-Cl2 | >284  | 0.018 | >1136 |
| 21       | H  | H  | Cl   | 3,5-Br2 | >236   | 0.032 | >1136 |
| 22       | H  | H  | Br   | 3,5-Cl2 | >258 | 0.017 | >1136 |
| 23       | H  | H  | Cl   | 3,5-Me2 | >290c | 5.8c | >1136 |
| 24       | Me | H  | Cl   | 3,5-Me2 | >303   | 0.044 | >1136 |
| 25       | H  | Me | Cl   | 3,5-Me2 | >303   | 0.020 | >1136 |
| 26       | H  | H  | Cl   | 3,5-Me2 | >200c | >200c | >200c |
| Nevirapine | -- | -- | -- | -- | >50 | >242 |
| Efavirenz | -- | -- | -- | -- | >50 | >242 |

# SI = selectivity index; CC50/EC50.
# RI = resistance index; EC50 (resistant virus)/EC50 (wild-type virus).
# a Data obtained in CEM cell cultures.

### Table 2

Inhibitory activity of the compounds against wild-type HIV-1 RT in DNA-dependent and RNA-dependent polymerase activity assays

| Compound | $K_I$ (µM) | Activated DNA (p66/p66) | Poly(rC)-oligo(dG) (p66/p51) |
|----------|------------|------------------------|-----------------------------|
| 6        | 76         | 18                     | Poly(rC)-oligo(dG) (p66/p51) |
| 7        | 36         | 6.3                    | Poly(rC)-oligo(dG) (p66/p51) |
| 8        | 5.9        | 2.7                    | Poly(rC)-oligo(dG) (p66/p51) |
| 9        | 5.0        | 2.1                    | Poly(rC)-oligo(dG) (p66/p51) |
| 10       | 11         | 3.1                    | Poly(rC)-oligo(dG) (p66/p51) |
| 11       | 5.6        | 1.7                    | Poly(rC)-oligo(dG) (p66/p51) |
| 12       | 2.9        | 2.6                    | Poly(rC)-oligo(dG) (p66/p51) |
| 13       | 0.98       | 0.63                   | Poly(rC)-oligo(dG) (p66/p51) |
| 14       | 2.0        | 0.45                   | Poly(rC)-oligo(dG) (p66/p51) |
| 15       | 0.49       | 0.21                   | Poly(rC)-oligo(dG) (p66/p51) |
| 16       | 2.4        | 2.0                    | Poly(rC)-oligo(dG) (p66/p51) |
| 17       | 2.4        | 0.52                   | Poly(rC)-oligo(dG) (p66/p51) |
| 18       | 0.77       | 1.7                    | Poly(rC)-oligo(dG) (p66/p51) |
| 19       | 0.77       | 0.36                   | Poly(rC)-oligo(dG) (p66/p51) |
| 20       | 0.68       | 0.82                   | Poly(rC)-oligo(dG) (p66/p51) |
| 21       | 2.3        | 0.47                   | Poly(rC)-oligo(dG) (p66/p51) |
| 22       | 0.69       | 0.16                   | Poly(rC)-oligo(dG) (p66/p51) |
| 23       | >50        | >242                   | Poly(rC)-oligo(dG) (p66/p51) |
| 24       | 1.2        | 1.1                    | Poly(rC)-oligo(dG) (p66/p51) |
| 25       | 0.73       | 1.7                    | Poly(rC)-oligo(dG) (p66/p51) |
| 26       | >50        | >249                   | Poly(rC)-oligo(dG) (p66/p51) |

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Comparison of the antiviral activity data for the compounds bearing various substituents at R¹ led to surprising results. On the one hand, substitution of the methyl group of 2- (2-(3,5-dimethyl)benzyl)phenoxy)ethyl uracil with a chlorine or bromine had almost no effect on the potency of the compounds (8 vs 9 and 10). However the same substitution on the benzophenone-containing compounds led to a marked enhancement of activity. The inhibitors bearing chlorine or bromine in R¹ position inhibited RT in lower concentrations in comparison to their counterparts with methyl or fluorine substituents (13 and 14 vs 11 and 12, or 17 and 18 vs 16). It is notable that these results are in agreement with the anti-HIV activity observed for the uracil derivatives.

Interestingly, several of the compounds (14, 17, and 18) showed decreased inhibitory activity in one of the enzymatic assays. Since there were no similarities between structure and the type of assay, this discrepancy could be attributed to the poor solubility observed for some of the halogenated compounds in aqueous media, in particular, in the enzymatic reaction conditions required for the assays.

Moving forward with the SAR evaluation, the role of the R¹ substituent was less clear. On the one hand, for compounds lacking the carbonyl group (6–10), introduction of one or two methyl substituents significantly enhanced the inhibitory activity against RT (8 vs 7 vs 6). On the other hand, in the benzophenone series, the corresponding 3,5-dimethyl or 3,5-dichloro-containing inhibitors displayed activity similar to that of their unsubstituted counterparts (16 vs 11, 17 and 20 vs 13). These observations were in disagreement with the activity of the compounds in infected cell culture. A correlation was found only in the pairs of the benzophenones 13 and 15 (all assays), 18 and 14 (activated DNA assay), and 22 and 14 (poly(rC)-oligo(dG) assay). In all other cases, the discrepancies again could be attributed to two effects: the greater affinity of the substituted benzophenones for the enzyme, and/or the lower solubility of the compounds bearing the halogen substituents.

Next, in order to more fully understand the activity of the benzophenones against drug-resistant HIV strains as well as to verify the reasons for the poor levels of activity of the compounds against the K103N/Y181C RT double mutant virus, a panel of RT's bearing one or two of the most clinically significant mutations (L100I, K103N, V106I, Y181C, Y188L, G190A, and K103N/Y181C) confering resistance of the virus to NNRTIs was constructed. Based on the results of the previously highlighted antiviral studies, two structurally-related benzophenones were selected for further study. In that regard, compounds 17 and 20 both exhibited the highest activity against the wild-type virus but exhibited quite different levels of activity towards the K103N/Y181C double mutant HIV-1 strain (Table 3).

Both benzophenones 17 and 20 retained activity against most HIV-RT forms with the exception of V106A (Table 3). Although the absolute Kᵢ values were closer to that of nevirapine, the resistance susceptibility profile of compounds 17 and 20 towards most HIV–RT mutant forms more closely resembled the profile of efavirenz. For example, nevirapine and efavirenz exhibited a 15-fold and 10-fold lower activity, respectively, towards the L100I RT mutant compared to the wild-type enzyme, whereas benzophenones 17 and 20 retained almost full activity, exhibiting only a very minor degree of resistance (1.4-fold and 2.5-fold, respectively). Similarly, against K103N, a common drug-resistant HIV–RT form, the inhibitory activity of nevirapine was -400-fold lower than that towards wtRT, whereas activity of efavirenz or the studied benzophenones 17 and 20 was only 24–, 13.8 and 8-fold lower, respectively.

Both compounds proved to be inactive against the V106A RT, while efavirenz retained activity. In contrast to nevirapine, the benzophenones retained partial activity against all other forms of RT, and in some cases the decrease was lower than that of efavirenz. For the Y181C RT mutant, compounds 17 and 20 inhibited the polymerase 3.3- and 2-fold less effectively, as compared to a decrease of 395- and 2.6-fold for nevirapine and efavirenz, respectively. In the case of the Y188L mutant RT, the activity of benzophenone 17 was 4.3-fold lower than for the wild-type enzyme, while compound 20 exhibited 12-fold lower activity. Nevirapine and efavirenz were >250 and 12-fold less active, respectively. Interestingly, benzophenone 17 displayed even higher inhibitory activity toward the mutant G190A RT form, while 20 exhibited somewhat lower activity (2.3-fold), more closely in line with efavirenz (2.7-fold decrease) but in sharp contrast to nevirapine (423-fold decrease). Lastly, benzophenones 17 and 20, as well as efavirenz, exhibited a 23.8-, 14.9- and 19.3-fold lower efficacy towards the K103N/Y181C double mutant RT, as compared to a >250-fold lower inhibitory activity for nevirapine.

Thus, the data obtained are highly encouraging and appear to point to a resistance susceptibility profile for the synthesized benzophenones that is closer to efavirenz than to nevirapine. Moreover, the resistance index for the compounds against the Y181C, Y188L, and K103N/Y181C mutant RT's was comparable to that of efavirenz, and even lower for the L100I, K103N and G190A mutants, although for the V106A mutant, efavirenz was more potent than 17 and 20. While these data do not directly support the results of the antiviral activity studies, which revealed different resistance susceptibility profiles for the structurally-related benzophenones 17 and 20, it is possible that this difference is due to differences in solubility for some of the compounds in aqueous media, rather than to a different mode of interaction with mutant HIV–RTs.

In that regard, it should be noted that 10% DMOS was used in the HIV-1 RT poly(rC)-oligo(dG) experiment, whereas cell cultures only contained 0.1% DMOS at most. To test this possibility, we determined solubility of several compounds in 10% DMOS solution (as was used in the RT activity assay) or in 0.1% (as was used in the cell culture experiments) by UV spectroscopy. It was found that the maximum concentration of the compounds bearing halogens (including 17 and 20) could not exceed 2–3 μM in 10% DMOS solution (Supplementary data, Table S2). Assuming that the resistance index of the compounds 17–19 against K103N + Y181C HIV-1 strain is 50–200 as was shown for benzophenones 20, 24, and 25, they should display antiviral activity against this strain at micromolar concentrations, that is, in the same range as they tend to precipitate. If this is indeed the case, the next generation of benzophenones should be designed with enhanced solubility characteristics, which may lead to obtaining more effective antiretroviral agents active against a variety of drug-resistant viral strains.

### 3. Conclusions

Herein we have described the synthesis, SAR, and biological evaluation of a series of potential NNRTI anti-HIV agents. Several of the uracil derivatives exhibited potent antiviral activity in infected cell
culture and effectively inhibited recombinant HIV–RT. Notably, the compounds did not exhibit toxicity in cell culture, and thus have high selectivity indices. Two of the uracil derivatives effectively inhibited several mutant HIV-1 RTs and also partially retained antiviral activity against several drug-resistant HIV strains. Despite these encouraging results, the poor solubility of some of the compounds hindered true assessment of their potential biological activity. Although the activity levels of the compounds towards wild-type virus and HIV-1 RT were similar to that of nevirapine, they more closely resemble the activity profile of efavirenz by retaining their inhibitory activity towards mutant RT forms resistant to nevirapine. Given the promising and potent antiviral activity exhibited by these compounds, it is clear that the benzophenone-linked uracil scaffold is an excellent lead for the development of additional compounds. As a result, current efforts are underway to improve the solubility of some of the more insoluble compounds in order to design more effective anti-HIV drugs. Those results will be reported elsewhere as they become available.

4. Materials and methods

4.1. General

Activated DNA was purchased from GE Healthcare (Little Chalfont, UK) Oligonucleotides were obtained from Lytech (Moscow, Russia); [6-32P]dATP (5000 Ci/mmole) and [8-3H]dGTP were from Izotop (Moscow, Russia), and Moravek Biochemicals, Brea, CA), respectively. Ni-NTA-agaroate resin and Rosseta(DE3) Escherichia coli strain were from Novagen (Madison, WI). All other reagents of highest grade were obtained from Sigma (St. Louis, MI), and Fermentas (Vilnus, Lithuania). All chemicals were obtained from commercial sources and used without further purification unless otherwise noted. Anhydrous DMF, isopropanol, and ethylene glycol were purchased from Sigma–Aldrich Co. Anhydrous acetone, CH3Cl2, 1,2-dichloroethane, and ethyl acetate were obtained by distillation over P2O5.

Melting points were measured on Mel-Temp 3.0 Pro apparatus (Laboratory Devices Inc.) and uncorrected. All 1H and 13C NMR spectra were registered on a Varian Mercury 300B and AMXIII-400 Bruker, operated at 300 and 400 MHz for 1H; 75 and 100 MHz for 13C, respectively (1H and 13C NMR) homogeneous materials. The purity of the compounds was verified using HPLC and all compounds were verified to be >98% pure with the only minor impurity being uracil.

2.1.1. 1-[2-(2-Benzyl-4-methylphenoxy)ethyl]uracil (6).

Yield: 80%, mp: 191–192.5

2H, ArCH2), 4.03 (t, 2H, J = 6 Hz, N–CH2), 4.14 (t, 2H, J = 6 Hz, O–CH2), 5.48 (dd, 1H, J = 8 Hz, H-5), 6.84–6.98 (m, 3H, aromatic H), 7.12–7.25 (m, 5H, aromatic H), 7.60 (s, 1H, J = 8 Hz, H-6), 11.31 (s, 1H, NH); 13C NMR (DMSO-d6): 31.81, 32.9, 45.3, 63.5, 98.8, 110.1, 124.2, 126.7, 127.2, 127.8, 129.1, 139.2, 144.5, 149.4, 151.9, 162.2; HRMS calcld for C20H20N2O3, [M+H]+ 337.1547; found (FAB) m/z ([M+H]+): 337.1549.

2.1.2. 1-[2-(3-Methylbenzyl)-4-methylphenoxy]ethyl]uracil (7).

Yield: 80%, mp: 127–128, Rf 0.35 (elution with ethyl acetate); 1H NMR (DMSO-d6): 3.17 (s, 3H, CH3), 2.95 (s, 3H, CH3), 3.79 (s, 2H, ArCH2), 4.06 (t, 2H, J = 4.9 Hz, N–CH2), 4.13 (t, 2H, J = 5 Hz, O–CH2), 5.46 (dd, 1H, J = 7.2 and 2.2 Hz, H-5), 6.55–6.86 (m, 2H, aromatic H), 6.90 (m, 1H, aromatic H), 6.93–6.96 (m, 3H, aromatic H), 7.09–7.12 (m, 1H, aromatic H), 7.58 (d, 1H, J = 7.8 Hz, H-6), 11.29 (s, 1H, NH); 13C NMR (DMSO-d6): 20.2, 21.1, 47.3, 65.4, 100.7, 111.7, 125.7, 126.5, 127.7, 128.1, 129.1, 129.2, 129.5, 131.0, 137.3, 140.9, 146.1, 151.1, 153.5, 163.7; HRMS calcld for C20H22N2O3, [M+H]+ 351.1630; found (FAB) m/z ([M+H]+): 351.1698.

2.1.3. 1-[2-(3,5-Dimethylbenzyl)-4-methylphenoxy]ethyl]uracil (8).

Yield: 74%, mp: 156–158 °C, Rf 0.58 (elution with ethyl acetate); 1H NMR (DMSO-d6): 3.20 (s, 3H, CH3), 2.12 (s, 6H, CH3), 3.68 (s, 2H, PhCH2), 4.02 (t, 2H, J = 6 Hz, N–CH2), 4.07 (t, 2H, J = 6 Hz, O–CH2), 5.38 (d, 1H, J = 8 Hz, H-5), 6.67 (s, 2H, H-2, H-6), 6.70 (1H, H-4), 6.78 (3H, m, H-3′, H-5′, H-6′), 7.52 (d, 1H, J = 8 Hz, H-6), 11.28 (s, 1H, NH); 13C NMR (DMSO-d6): 18.4, 19.1, 32.8, 45.5, 63.6, 98.8, 110.0, 124.4, 124.6, 125.3, 125.5, 126.0, 126.7, 127.6, 128.9, 129.2, 135.3, 138.9, 144.2, 149.2, 151.6, 161.9; HRMS calcld for C21H23N2O3, [M+H]+ 365.1839; found (FAB) m/z ([M+H]+): 365.1854.

2.1.4. 1-[2-(4-Chloro-2-(3,5-dimethylbenzyl)-4-methylphenoxy)ethyl]uracil (9).

Yield: 80%, mp: 191–192.5 °C, Rf 0.59 (elution with ethyl acetate); 1H NMR (DMSO-d6): 3.14 (s, 3H, CH3), 3.71 (s, 2H, ArCH2), 4.03 (t, 2H, J = 4 Hz, N–CH2), 4.19 (t, 2H, J = 5 Hz, O–CH2), 5.46 (dd, 1H, J = 7.9 and 2.2 Hz, H-5), 6.75 (s, 2H, H-2, H-6), 6.79 (s, 1H, H-4), 6.95–6.97 (m, 1H, H-6), 7.20 (d, 1H, J = 2.4 Hz, H-3), 7.32 (dd, 1H, J = 8.8 and 2.5 Hz, H-5), 7.60 (d, 1H, J = 7.8 Hz, H-6), 11.30 (s, 1H, NH); 13C NMR (DMSO-d6): 19.0, 32.5, 45.3, 63.8, 98.9, 111.6, 122.5, 124.4, 124.6, 125.0, 125.7, 127.7, 127.9, 129.9, 135.5, 138.0, 144.4, 149.2, 152.5,
161.9: HRMS calcd for C$_{21}$H$_{21}$BrN$_2$O$_3$: [M+H]$^+$ 429.0804; found (FAB) m/z ([M+H]$^+$): 429.0804 ($^1$HBr). 31.0765 ($^1$Cl). 194.0: HRMS calcd for C$_{19}$H$_{15}$FN$_2$O$_4$: [M+H]$^+$: 429.0804 (79Br), 431.0796 ($^3$Br).

4.2.1.12. 1-[2-(2-Benzoyl-4-chlorophenoxy)ethyl]uracil (13). Yield: 76%, mp: 236–238°C, R$_f$ 0.39 (elution with ethyl acetate); 1H NMR (DMSO-d$_6$): $\delta$ 4.20 (t, 2H, $J = 4.5$ Hz, O–CH$_2$), 4.10 (t, 2H, $J = 7.5$ Hz, H-5), 6.66 (d, 1H, $J = 7.5$ Hz, H-5), 7.16–7.22 (m, 2H, aromatic H), 7.30–7.36 (m, 1H, aromatic H). 7.43–7.51 (m, 2H, aromatic H), 7.53–7.58 (m, 1H, aromatic H), 7.57–7.66 (m, 2H, aromatic H). 11.12 (s, 1H, NH); 13C NMR (DMSO-d$_6$): $\delta$ 46.9, 66.1, 100.3, 114.6, 114.6, 115.2, 115.4, 117.9, 118.1, 128.6, 129.1, 129.7, 129.7, 133.7, 136.2, 145.2, 150.9, 151.5, 151.5, 157.4, 163.4, 194.0; HRMS calcd for C$_{19}$H$_{15}$BrN$_2$O$_4$: [M+H]$^+$: 431.0793; found (FAB) m/z ([M+H]$^+$): 431.0792 ($^1$HBr), 373.0776 ($^3$Cl).

4.2.1.11. 1-[2-(2,3-Dimethoxybenzoyl)phenoxy]ethyl]uracil (16). Yield: 66%, mp: 215.5–217°C, R$_f$ 0.53 (elution with ethyl acetate); 1H NMR (DMSO-d$_6$): $\delta$ 2.22 (s, 3H, CH$_3$), 3.68 (t, 2H, $J = 4.5$ Hz, O–CH$_2$), 5.07 (d, 1H, $J = 8$ Hz, H-5), 6.75 (d, 1H, $J = 7.5$ Hz, H-6), 7.23–7.28 (m, 4H, aromatic H), 7.61–7.67 (m, 4H, aromatic H), 8.51 (1H, NH); 13C NMR (DMSO-d$_6$): $\delta$ 19.8, 47.0, 65.8, 100.3, 112.8, 128.5, 129.1, 130.1, 132.3, 133.3, 137.0, 145.3, 150.6, 153.3, 163.4, 195.6; HRMS calcd for C$_{19}$H$_{15}$BrN$_2$O$_4$: [M+H]$^+$: 431.0793; found (FAB) m/z ([M+H]$^+$): 379.1652; found (FAB) m/z ([M+H]+): 379.1661.

4.2.1.10. 1-[2-(4-Chloro-2-(3-methylbenzoyl)phenoxy)ethyl]uracil (15). Yield: 79%, mp: 191–192°C, R$_f$ 0.37 (elution with ethyl acetate); 1H NMR (DMSO-d$_6$): $\delta$ 2.28 (s, 3H, CH$_3$), 3.71 (t, 2H, $J = 4.5$ Hz, N–CH$_2$), 4.10 (t, 2H, $J = 4.5$ Hz, O–CH$_2$), 5.04 (dd, 1H, $J = 8$ and 2 Hz, H-5), 6.69 (d, 1H, $J = 7.5$ Hz, H-5), 7.19 (d, 1H, $J = 9$ Hz, H-6), 7.29–7.44 (m, 5H, H-3, H-2, H-4, H-6), 7.51 (dd, 1H, $J = 8$ and 2 Hz, H-6), 11.10 (s, 1H, NH); 13C NMR (DMSO-d$_6$): $\delta$ 20.7, 46.9, 66.1, 100.3, 114.8, 125.0, 126.5, 128.1, 130.3, 131.3, 134.5, 136.3, 138.1, 145.2, 150.6, 154.0, 163.4, 193.9; HRMS calcd for C$_{19}$H$_{15}$BrN$_2$O$_4$: [M+H]$^+$: 385.0949; found (FAB) m/z ([M+H]+): 385.1319 ($^3$Cl), 387.1297 ($^3$Cl).
4.2.1.16. 1-[2-[4-Chloro-2-(3,5-dibromobenzoyl)phenoxy]ethyl] uracil (21). Yield: 75%, mp: 261–262°C, Rf 0.47 (elution with ethyl acetate). 1H NMR-spectrum (DMSO-d6): δ 3.75 (t, 2H, J = 5 Hz, N–CH2–), 4.13 (t, 2H, J = 5 Hz, O–CH2–), 5.08 (d, 1H, J = 8 Hz, H-5), 6.87 (d, 1H, J = 8 Hz, H-5′), 7.21 (d, 1H, J = 8 Hz, H-6′), 7.41 (d, 1H, J = 1.2 Hz, H-4′), 7.57 (d, 1H, J = 8 Hz, H-6), 6.69 (s, 2H, H-2′, H-6′). 8.03 (d, 1H, J = 1.5 Hz, H-4′), 11.10 (s, 1H, NH); 13C NMR (DMSO-d6): δ 47.0, 66.2, 102.0, 114.9, 122.9, 125.1, 128.4, 128.7, 130.6, 130.8, 132.3, 134.2, 150.5, 150.6, 154.2, 163.2, 191.2; HRMS calcd for C19H13Br2CINO4 [M+H]+: 526.9003; found (FAB) m/z ([M+H]+): 526.9011 (2 × [M]+Br), 528.8991 ([M]+Br2), 530.8974 (2[M]+Br, 35Cl), 413.1265 [35Cl], 415.1250 (37Cl).

4.2.1.17. 1-[2-[4-Bromo-3,5-dichlorobenzoyl]phenoxy]ethyl] uracil (22). Yield: 70%, mp: 258–259.5°C, Rf 0.47 (elution with ethyl acetate); 1H NMR (DMSO-d6): δ 3.81 (t, 2H, J = 5.1 Hz, N–CH2–), 4.19 (t, 2H, J = 5.1 Hz, O–CH2–), 5.16 (dd, 1H, J = 8 and 2.1 Hz, H-5), 6.91 (d, 1H, J = 8 Hz, H-5′), 7.16 (d, 1H, J = 8 Hz, H-6′), 7.56–7.59 (m, 3H, H-3′, H-2′, H-4′), 7.74 (dd, 1H, J = 8.9 and 2.7 Hz, H-6), 7.84–7.85 (m, 1H, H-1′), 11.14 (s, 1H, NH); 13C NMR (DMSO-d6): δ 46.9, 66.1, 100.2, 112.6, 115.3, 127.4, 128.9, 131.4, 132.9, 134.6, 135.1, 139.3, 145.2, 150.5, 154.6, 163.1, 191.3; HRMS calcd for C20H12Br2CINO4 [M+H]+: 482.9509; found (FAB) m/z ([M+H]+): 482.9511 (2 × [M]+Br), 484.9486 (alternative isotope), 486.9469 (alternative isotope).

4.2.1.18. 1-[2-[4-Chloro-2-(3,5-dimethylbenzoyl)phenoxy]pro- pyl]uracil (23). Yield: 62%, mp: 200–201°C, Rf 0.70 (elution with ethyl acetate); 1H NMR (DMSO-d6): δ 1.70 (m, 2H, J = 6.7 Hz, CH2), 3.31 (t, 2H, J = 6.7 Hz, CH2), 3.95 (2H, J = 6.1 Hz, CH2), 5.46 (dd, 1H, J = 7.8 and 1.9 Hz, H-5), 7.14 (dd, 1H, J = 7.8 and 1.2 Hz, H-5′), 7.19 (d, 1H, J = 8.9 Hz, H-6), 7.28 (s, 1H, H-4′), 3.2 (s, 1H, H-2′, H-6′), 7.19 (d, 1H, J = 8.7 Hz, H-5′), 7.38 (d, 1H, J = 2.6 Hz, H-3′), 7.56 (dd, 1H, J = 8.9 and 2.8 Hz, H-6), 11.16 (s, 1H, NH); 13C NMR (DMSO-d6): δ 20.6, 27.7, 44.5, 65.5, 100.9, 114.9, 124.6, 126.7, 128.2, 130.4, 131.5, 134.8, 137.3, 137.9, 145.0, 150.61546, 163.6, 194.5; HRMS calcd for C22H21Cl2CINO4 [M+H]+: 413.1263; found (FAB) m/z ([M+H]+): 413.1269 ([M]+Br), 415.1248 (35Cl).

4.2.1.19. 1-[2-[4-Chloro-2-(3,5-dimethylbenzoyl)phenoxy]ethyl] thymine (24). Yield: 69%, mp: 244–245°C, Rf 0.54 (elution with ethyl acetate); 1H NMR (DMSO-d6): δ 1.53 (d, 3H, J = 1.1 Hz, CH3), 2.29 (s, 6H, CH3), 3.75 (t, 2H, J = 5.1 Hz, N–CH2–), 4.18 (t, 2H, J = 5 Hz, O–CH2–), 6.80–6.81 (m, 1H, H-5′), 7.25–7.28 (m, 4H, H-3′, H-2′, H-4′, H-6′), 7.32 (d, 1H, J = 2.7 Hz, H-6), 7.55 (dd, 1H, J = 8.9 and 2.7 Hz, H-6), 11.08 (s, 1H, NH); 13C NMR (DMSO-d6): δ 11.6, 20.6, 46.8, 66.2, 107.8, 115.0, 124.9, 126.8, 128.0, 130.5, 131.1, 135.2, 136.2, 137.8, 141.3, 150.6, 154.0, 163.9, 193.7; HRMS calcd for C22H21NO4 [M+H]+: 390.1190; found (FAB) m/z ([M]+H+): 390.1190 (35Cl), 402.1168 (37Cl).

5. Biological evaluation

5.1. Antiviral assays

The methodology of the anti-HIV assays was as follows: human CEM (3 × 10^5 cells/cm^2) cells were infected with 100 CCID50 of HIV(IIIB) or HIV-2(ROD)/ml and seeded in 200 μl wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, HIV-induced CEM giant cell formation was examined microscopically.

The methodology of the anti-HIV assays in MT-4 cell cultures was as follows: virus stocks were titrated in MT-4 cells and expressed as the 50% cell culture infective dose (CCID50). MT-4 cells were suspended in culture medium at 1 × 10^5 cells/ml and infected with HIV at a multiplicity of infection of 0.02. Immediately after viral infection, 100 μl of the cell suspension was placed in each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. The test compounds were dissolved in 100% DMSO at 50 mM or higher. After 4 days of incubation at 37°C, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The selection and characterization of mutant virus strains have been performed previously.

5.2. Reverse transcriptase plasmids, RT expression and purification

Plasmid encoding the HIV-1 reverse transcriptase (RT) was obtained as previously reported. Plasmids encoding RT in which one or two amino acid residues were substituted (L100K, K103N, V106A, Y181C, Y188L, G190A and K103N/Y181C) were constructed by amplifying the gene in two fragments using the oligonucleotides listed in Table S1. ‘Head’ and ‘Tail’ PCR-products were mixed
together in the absence of oligos and 30 additional amplification cycles were performed in order to fulfill the length-genes. The resulting products were digested with Xhol and Xbal restriction endonucleases and ligated into pBRP-HR or pET-21d-2c vectors.37 RT genes containing mutations K103N and G190A were inserted into a pBRP-HR vector, other mutant genes were subcloned into pET-21d-2c. The wild-type and mutant RTs were expressed and purified in E. coli Rosetta (DE3) strain as described earlier for HCV RNA polymerase.37

5.3. RT enzyme assay

The RT assays using activated DNA were performed as follows: the standard reaction mixture (20 μl) contained 0.75 μg of activated DNA, 0.05 μg p66/p66 RT, 3 μM dATP, 30 μM of dCTP, dGTP and dTTP, 1 μCi [α-32P]dATP in a Tris–HCl buffer (50 mM, pH 8.1) containing also 10 mM MgCl2, and 0.2 M KCl. The RT assays using poly(rC)-oligo(dG) as the template–primer complex were performed as follows: the reaction mixture (40 μl) contained 0.02–0.1 mM poly(rC)-oligo(dG12–18), 0.02–0.1 M dATP, 30 μM MgCl2, 25 mM NaCl, and 5% glycerol. The test compounds were dissolved in DMSO and added to both assays to the 10% final DMSO concentration. The reaction mixtures were incubated for 30 minutes at 37 °C, and applied onto Whatman 3MM filters. After drying on air the filters were washed twice with 10% trichloracetic acid, then air-dried and counted in a liquid scintillation counter. Radioactivity was measured by the Cherenkov method.38 Alternatively, 1 ml ice-cold 5% TCA in 0.02 M Na4P2O7 was added as described earlier for HCV RNA polymerase.37

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Supplementary data

Supplementary data (1H, 13C NMR and solubility data, as well as Dixon plots for the compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.025.