Synthesis and antiviral activity of some 5'-N-phthaloyl-3'-azido-2',3'-dideoxythymidine analogues

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Introduction

3'-Azido-2',3'-dideoxythymidine (AZT, zidovudine) was the first nucleoside drug approved for anti-HIV treatment. Although AZT has proven effective in suppressing HIV infection, it has several limitations; these include dose-limiting toxicities and the emergence of drug-resistant virus strains (De Clercq, 2002). Several attempts have been made to deliver the 5'-monophosphate derivatives of nucleoside analogues in intact cells directly by lipophilic phosphate triester prodrugs (for an overview, see Meier, 1998). However, 5'-O-esters of 3'-azido-2',3'-dideoxythymidine have also been synthesized as prodrugs of AZT (Young et al., 2000). In these compounds, the conjugating moiety is linked to the deoxyribose ring via 5'-O-carboxylate ester groups. Such AZT ester prodrugs need to be converted to free nucleosides before they can be phosphorylated to AZT monophosphate (AZT-MP) by thymidine kinase. AZT-MP is phosphorylated to its diphosphate derivatives by thymidylate kinase and NDP kinase, respectively. The triphosphate of AZT is then incorporated in the growing viral DNA chain by the HIV-encoded reverse transcriptase, upon which it causes premature DNA chain termination.

Whereas most prodrugs of AZT have been prepared mainly by ester derivatization at the 5'-O-position, there are not many available 5'-N-derivatives to act as prodrugs. However, we have found that adamantyl ester imides containing amino acid residues as N-substituents exhibit strong antibacterial activity (Orzeszko et al., 2000, 2002). Thalidomides with N-phenyl and N-benzylphthalimide skeletons, as well as simple halogenated phthalimides, show bi-directional TNF-α production-regulating activity (Hashimoto, 1998; Shibata et al., 1998; Miyachi et al., 1997). Naphthalimides such as amonafide and mitonafide, exhibit substantial anticancer activities against various animal tumours (Samanta et al., 2001).

Recently, it has been found that phthalimide-derived nucleoside analogues can bind CG base pairs via specific hydrogen bonds (Langler & Weisz, 2001). As mentioned, the majority of bio-transformations of nucleoside agents occur in the phosphorylation process. For 5'-O-esters this reaction could be easily achieved after hydrolytic removal of the ester group. However, N-C bonds in N-substituted imides are much more chemically and biologically stable than ester linkages. Because phthalimide moieties are present in a variety of biologically active compounds, it would be interesting to study the biological properties of compounds containing an imide skeleton in their structures. In particular, from a pharmaceutical point of view, it would be interesting to explore the biological activity for nucleoside analogues in which the 5'-position has been substituted with imide groups. For the above reasons, in the present report we have decided to synthesize a series of novel 5'-N-phthaloylderivatives of AZT and investigate them on their potential value as antiviral agents.
Materials and methods

Chemistry

Melting points were taken in open capillary tubes on a Gallenkamp 5 melting point apparatus and were uncorrected. The structures of products were confirmed by elemental analysis, FTIR and 1H NMR spectroscopy. The NMR spectra were measured on a Varian Gemini 200 MHz spectrometer in CDCls solutions. FTIR spectra were recorded on a Perkin Elmer 2000 apparatus using the KBr pellet method. Analyses indicated by symbols were within ±0.4% of the theoretical values.

All chemicals used were analytical-grade commercial products (Aldrich) and were used without any further purification. All solvents used were anhydrous and used as supplied by Aldrich. Crude products of the reactions were purified by means of flash-column chromatography (silica gel 60; 230–400 mesh), using as the eluent the ethyl acetate/hexane (2:1) system, and then crystallized from ethyl acetate.

Thiophthalimide (1h). Thiourea (1h) was synthesized according to a previously published procedure (Orezsko et al., 2001) from commercial phthalimide using Lawesson’s reagent, i.e., 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide. Yield 67%; m.p. 198°C.

2.58 (m, 1H, CH-CN), 3.98 (m, 2H, CH2N), 4.26 (m, 1H, CHO), 6.05 (t, CH), 7.46 (s, 1H, CH), 11.29 (s, NH).

The yield of white crystals was 193 mg (34%); m.p. 185°C. Spectroscopic data for 2a: FT-IR (KBr) cm⁻¹: 3685 (NH), 3698 (NIH), 2113 (CN), 1777 (C=O), 1724 (C=O), 1617 (C=N), 1H NMR (CDCl₃) δH 1.25–2.65 (m, 15Hadam.), 1.66 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CHO), 4.22 (m, 1H, CHO), 6.05 (t, CH), 7.46 (s, 1H, CH), 8.07–8.44 (m, 3H₃), 11.21 (s, NH).

5'-N-(4',5'-Dichlorophthaloyl)-3'-azido-2',3'-dideoxythymidine (2b). The compound was prepared as described for 2a above from 4,5-dichlorophthalimide 1b (10 mmol) in THF (25 ml) at room temperature. The yield of white crystals was 225 mg (45%); m.p. 165°C. Spectroscopic data for 2b: FT-IR (KBr) cm⁻¹: 3691 (NH), 3698 (NIH), 2106 (CN), 1770 (C=O), 1709 (C=O), 1606 (C=N), 1H NMR (CDCl₃) δH 1.25–2.65 (m, 15Hadam.), 1.66 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CHO), 3.97 (m, 2H, CH₂N), 4.22 (m, 1H, CHO), 6.05 (t, CH), 7.46 (s, 1H, CH), 8.07–8.44 (m, 3H₃), 11.21 (s, NH).

5'-N-(3',4',5',6'-Tetrachlorophthaloyl)-3'-azido-2',3'-dideoxythymidine (2d). The compound was prepared as described for 2a, above, from 3,4,5,6-tetrachlorophthalimide 1c (10 mmol) in THF (25 ml) at room temperature. The yield of white crystals was 193 mg (34%); m.p. 185°C. Spectroscopic data for 2d: FT-IR (KBr) cm⁻¹: 3698 (NH), 3691 (NIH), 2113 (CN), 1777 (C=O), 1724 (C=O), 1617 (C=N), 1H NMR (CDCl₃) δH 1.70 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CH-CN), 3.98 (m, 2H, CH₂N), 4.22 (m, 1H, CHO), 6.05 (t, CH), 7.45 (s, 1H, CH), 11.29 (s, NH).

5'-N-(3',4',5',6'-Tetrabromophthaloyl)-3'-azido-2',3'-dideoxythymidine (2c). The compound was prepared as described for 2a, above, from 3,4,5,6-tetrabromophthalimide 1d (10 mmol) in THF (25 ml) at room temperature. The yield of white crystals was 125 mg (25%); m.p. 245°C. Spectroscopic data for 2c: FT-IR (KBr) cm⁻¹: 3688 (NH), 3698 (NIH), 2113 (CN), 1777 (C=O), 1728 (C=O), 1617 (C=N), 1H NMR (CDCl₃) δH 1.25 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CH-CN), 3.98 (m, 2H, CH₂N), 4.22 (m, 1H, CHO), 6.05 (t, CH), 7.43 (s, 1H, CH), 11.29 (s, NH).

5'-N-(3',4',5',6'-Tetrafluorophthaloyl)-3'-azido-2',3'-dideoxythymidine (2e). The compound was prepared as described for 2a, above, from 3,4,5,6-tetrafluorophthalimide 1e (10 mmol) in THF (25 ml) at room temperature. The yield of white crystals was 239 mg (32%); m.p. 240°C. Spectroscopic data for 2e: FT-IR (KBr) cm⁻¹: 3695 (NH), 2112 (CN), 1773 (C=O), 1725 (C=O), 1606 (C=N), 1H NMR (CDCl₃) δH 1.68 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CH-CN), 3.98 (m, 2H, CH₂N), 4.46 (m, 1H, CHO), 6.05 (t, CH), 7.45 (s, 1H, CH), 11.29 (s, NH).

5'-N-(4',5'-Nitrophthaloyl)-3'-azido-2',3'-dideoxythymidine (2f). The compound was prepared as described for 2a, above, from 3-nitrophthalimide 1f (10 mmol) in THF (25 ml) at room temperature. The yield of white crystals was 162 mg (33%); m.p. 226°C. Spectroscopic data for 2f: FT-IR (KBr) cm⁻¹: 3691 (NH), 2111 (CN), 1777 (C=O), 1727 (C=O), 1606 (C=N), 1H NMR (CDCl₃) δH 1.57 (s, 3H, CH₃), 2.37 (m, 2H, CH₂), 2.58 (m, 1H, CH-CN), 3.98 (m, 2H, CH₂N), 7.45 (s, 1H, CH), 11.29 (s, NH).
HIV-2 were added to the cell cultures at 100 times the 50% titration of virus-induced giant cell formation. HIV-1 and post-infection was based on the microscopic examination.

IR (KBr) cm⁻¹: 3684 (NH), 2111 (CN), 1784 (C=O), 1727 cm⁻¹ (C=N);

1H NMR (CDCl₃) δ: 3.98 (m, 2H, CH₂N), 4.46 (m, 1H, CHO), 6.05 (s, CH), 7.45 (s, 1H, CHO). 8.05–8.55 (m, 3H), 11.25 (s, NH).

Reverse transcriptase assay. For determination of the 50% inhibitory concentrations (IC₅₀) of the test compounds, the RT assay was performed as described previously (Pelemans et al., 2001). A fixed concentration of the radio-labelled substrate [³H]dTTP (specific radioactivity 49 Ci/mmol; Amersham Pharmacia Biotech) and a fixed concentration of the template-primer poly(rA).oligo(dT₁₂–₁₈) (0.1 mM; Amersham Pharmacia Biotech) were used in the reaction mixture containing a variety of drug concentrations. The IC₅₀ for each test compound was determined as the compound concentration that inhibited recombinant RT activity by 50%.

Results

Chemistry

All 5'-N-phthaloyl derivatives of AZT were obtained from 3'-azido-2',3'-dideoxythymidine and unsubstituted phthalimides in THF via the Mitsunobu reaction (Mitsunobu, 1981). The synthetic pathway is shown in Figure 1. The starting imides 1a–g were commercial products but on the other hand, a thioimide 1h was synthesized, as described in a previous publication (Orzeszko et al., 2001) from phthalimide using Lawesson's reagent. The thionation reaction was performed in boiling toluene and the crude product was crystallized with benzene/hexane. 4-[(Adamantyl-1-yl)methyleneoxy carbonyl]phthalimide 1i was obtained in a previous publication (Olive et al., 2000). The purity of compounds 2a–h was controlled chromatographically by HPLC analysis and characterized by FT-IR and 1H-NMR spectroscopy, as well as by elemental analysis.

Antiviral activity assays. The activity against HIV-1 and HIV-2 in CEM cell cultures was examined at 4 to 5 days post-infection and was based on the microscopic examination of virus-induced giant cell formation. HIV-1 and HIV-2 were added to the cell cultures at 100 times the 50% cell culture infective dose (CCID₅₀). The inhibitory effect of the test compounds on MSV-induced transformation of murine embryo fibroblast C127/3T3 cell cultures was examined microscopically at day 6 post infection. MSV was added at 75 focus-forming units to the cell cultures. The procedures for the antiviral evaluations have been previously described in detail (Balzarini et al., 1991).

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in the phthalimide ring, showed a considerably increased (≥10-fold) antiviral potency (EC\textsubscript{50} ≥ 0.06–0.11 μM) when compared with the unsubstituted 2a derivative; that is, at an antiviral potency that was only 10- to 20-fold lower than that of the parent compound AZT (Table 1).

### Discussion

None of the compounds proved inhibitory to HIV-2 in CEM/Tk- cell cultures. The CEM/Tk- cells are deficient for cellular thymidine kinase activity and unable to phosphorylate thymidine nucleoside analogues such as AZT. This means that the 5'-'N-phthaloyl derivatives of AZT need to be obligatorily converted to AZT before being further phosphorylated to their metabolically active AZT triphosphate derivatives. This conclusion was corroborated by our findings that compounds 2a, 2e, and 2i did not inhibit HIV-1 RT at 200 μM under conditions where AZT-TP inhibited the reaction at an IC\textsubscript{50} of 0.02 μM (Balzarini et al., 1988). Thus, the compounds evaluated in this study should be considered prodrugs of AZT and not as acting in their own right.

Given the exquisite activity of AZT against HIV, the micromolar antiviral activity of the phthaloyl derivatives of AZT suggest that the release of AZT from the prodrug molecules is not very efficient in the cell cultures (at most 0.05 to 0.5%). It can be hypothesized that 2i may result in a better AZT release than the other compounds; however, it is also possible that this compound is much better taken up by the intact cells, due to the presence of the adamantane moiety on the molecule, and then more efficiently converted intracellularly to AZT. Due to the lack of radio-labeled drug, profound cellular uptake studies could not be performed. However, the adamantane derivative 2i has a shorter Rt value on reversed phase (C18) HPLC than its unsubstituted derivative 2a. Therefore, we have no solid evidence for a better uptake of 2i versus 2a to explain its higher antiviral efficacy. However, 2a and its corresponding adamantane derivative 2i have been exposed to concentrated CEM cell extracts and to 20% bovine serum in PBS. Interestingly, whereas incubation of 2a in the presence of CEM cell extract and bovine serum did not result in measurable release of AZT by HPLC analysis, 2i was clearly partially converted to AZT within 20 hrs of incubation. These observations may explain why 2i inhibits HIV-1 and HIV-2 in CEM cell cultures more than 2a. The higher extra- and intracellular metabolic instability may therefore be at least one contributing factor to the increased antiviral activity of adamantane derivative 2i.

When the compounds were evaluated for their antiviral properties against MSV-induced transformation of murine fibroblast cell cultures, a close correlation between anti-HIV activity and anti-MSV activity was observed (Table 2, Fig. 2), pointing to a similar mechanism of antiviral action for MSV and HIV. Again, as observed for HIV, the compounds were markedly less inhibitory to MSV than the parent molecule AZT.

In conclusion, a series of 5'-'N-phthaloyl-AZT derivatives exhibited activity against HIV and MSV, although at a much lower extent than the parent compound. Although not superior to AZT, it may be worth investigating the compounds' pharmacological properties in vivo compared with those of AZT (for example, blood-brain barrier penetration, plasma half-life, liver metabolism etc.). Therefore,
investigation of the derivatives’ in vivo efficacy and toxicity profile compared to the parent compound AZT in animal models should be performed to estimate the potential value of the phthalimide derivatives of AZT to treat HIV infection.

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Table 2. Inhibitory effect of compounds against MSV-induced transformation of murine embryo C3H10T1/2 fibroblasts in cell culture

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 µM</th>
<th>MIC(µM)</th>
</tr>
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<tbody>
<tr>
<td>2a</td>
<td>2.6</td>
<td>465</td>
</tr>
<tr>
<td>2b</td>
<td>11.0</td>
<td>401</td>
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<tr>
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<td>321</td>
</tr>
<tr>
<td>AZT</td>
<td>0.008</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*50% effective concentration or compound concentration required to inhibit MSV-induced transformation of C3H10T1/2 cell cultures by 50%. *Minimal inhibitory concentration or compound concentration required to cause a microscopically visible alteration of cell morphology.

Figure 2. Correlation between the antiviral effects of the test compounds 2a to 2i and AZT against HIV-1 and MSV*

* The numbering of the compounds (a to i) in the graph corresponds to the numbering in Figure 1 (2a to 2i).

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