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Anti-HIV-1 activity of benzothiadiazine dioxide

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Antiviral assays carried out on the potent benzothiadiazine dioxide (BTD) human cytomegalovirus (HCMV) inhibitors have led us to find marginal but selective anti-HIV-1 activity. Specific pharmacological studies, such as time of addition experiments and assays on specific viral strains with mutations on its reverse transcriptase, have indicated that BTD compounds act as non-nucleoside reverse transcriptase inhibitors. Theoretical calculations showed a butterfly conformation for the active derivatives that are compatible with their mechanism of action. Therefore, BTD derivatives can be considered as potential lead compounds for the treatment of opportunistic HCMV infections in immunocompromised individuals such as AIDS patients.

Keywords: HIV-1, human cytomegalovirus, benzothiadiazine dioxides, non-nucleoside reverse transcriptase inhibitors

Introduction

Human cytomegalovirus (HCMV), a member of the β-herpesvirus family, has been recognised as one of the most important pathogens in immunocompromised individuals (Speich & Van der Bij, 2001; Heininger *et al.*, 2001), particularly when the deficiency affects cell-mediated immune responses (such as in HIV-infected patients), as well as immunosupressed individuals (solid organ and allogenic bone marrow recipients) (Manfredi & Chiodo, 2001).

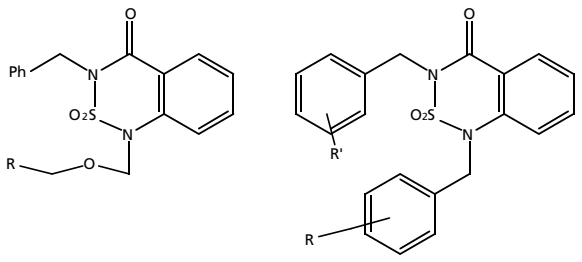
This pathogen is the most common congenital viral infection in humans due to the high prevalence of the virus in the general population, with up to 90% of the urban population infected (Trincado *et al.*, 2000). Although it is well known that highly active antiretroviral therapy has substantially decreased the incidence of HCMV retinitis in AIDS patients (Lee *et al.*, 2001), this change does not indicate that it is no longer of concern (Whitley *et al.*, 1998; Nichols & Boeckh, 2000). On the contrary, HCMV disease in solid organ recipients, newborns, and even in AIDS patients still accounts for considerable morbidity, and drug resistance to the anti-HCMV compounds offers a great challenge for medicinal chemistry research (Emery, 2001).

In our search for new antiviral agents, we initially discovered the 2,1,3-benzothiadiazine dioxide (BTD) modified acyclonucleosides with a marked activity against HCMV and varicella-zoster virus (Martinez *et al.*, 1997; 1999a). In order to obtain further insight into the structural requirements for the biological activity of the BTD family we have carried out several modifications based not only on traditional structure-activity relationship (SAR) studies (Martinez *et al.*, 1999b; 2000a), but also on three-dimensional quantitative SAR methods (Martinez *et al.*, 2000b). These results led us to maintain, and in some cases to improve, the antiviral activity in the second generation of BTD compounds (Figure 1).

The disubstituted BTD derivatives depicted in Figure 1 are potent non-nucleosidic HCMV-inhibitors with a new and interesting inhibition profile acting in the initial stages of the viral replicative cycle (Martinez *et al.*, 2000a,b; 2001).

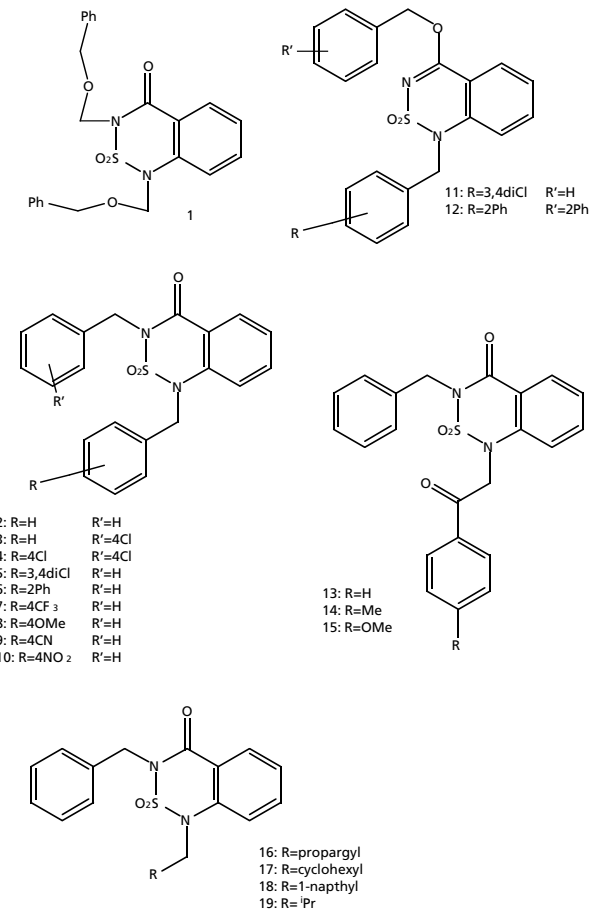
To assess the therapeutic potential of BTD derivatives for the treatment of immunosupressed diseases, such as AIDS, the antiviral screening against HIV has been carried out. Here we report the results on the antiviral evaluation

Figure 1. Benzothiadiazine dioxides human cytomegalovirus-inhibitors



R′ and R represent, for example, H, 4NO_2 or 4OMe.

Figure 2. BTD derivatives evaluated against HIV-1 and HIV-2



against HIV of some of the previously described BTD compounds (**1–19**) (Figure 2).

Materials and methods

Cells

MT-4 (Miyoshi *et al.*, 1982) and CEM (Foley *et al.*, 1965) cells were grown and maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 0.1% sodium bicarbonate and 20 μg of gentamicin/ml.

Antiretroviral evaluation

HIV-1 (HTLV-IIIb) was kindly provided by Dr RC Gallo (when at the National Institutes of Health, Betheseda, Md., USA). Virus stocks were prepared from the supernatants of HIV-1-infected MT-4 cells. HIV-2 (strain ROD) was provided by Dr L Montagnier (Pasteur Institute, Paris, France) and virus stocks were prepared from the supernatants of HIV-2-infected MT-4 cells. CEM cells were obtained from the American Tissue Culture Collection (Rockville, Md., USA). CEM cells were infected as follows: 4×10^5 cells/ml were infected with HIV-1 or HIV-2 at ~100 $CCID_{50}$ (50% cell culture infective dose) per ml of cell suspension. Then 100 μl of the infected cell suspension were transferred to 96-well microtitre plate wells and mixed with 100 μl of the appropiate dilutions of the test compounds. After 4 days the HIV-infected cell cultures were examined microscopically for giant cell formation.

Cytotoxicity assays

Cytotoxicity measurements were based on the inhibition of MT-4 and CEM cell growth. MT-4 or CEM lymphocytes were seeded at a rate of 5×10^3 cells/well microtitre plates and allowed to proliferate for 24 h. Different concentrations of the test compounds were then added (in duplicate), and after 3 days of incubation at 37°C in 5% CO_2 atmosphere, the cell number was determined with a coulter counter. Cytotoxicity is expressed as CC_{50}, which represents the compound concentration required to reduce cell growth by 50%.

Results

In vitro anti-HIV activity

The anti-HIV evaluation of BTD **1–19** against HIV-1 (strain III_B) and HIV-2 (strain ROD) in two different cell lines of human T-lymphocytes (CEM and MT-4 cells respectively) were performed following standard procedures (De Clercq, 1994; Pauwels *et al.*, 1988). Their antiviral activity values expressed as the 50% of effective concentration (EC_{50}) are collected in Table 1.

Several compounds were found to inhibit the replication of HIV-1 and HIV-2 at a concentration that was only two- to fivefold below the cytotoxic concentration for the host cells. However, some other derivatives, such as **3**, **6**, **17** and **19**, showed selectivity indices with values up to 10 against HIV-1 and were completely inactive against HIV-2 which point to a virus specific action.

Time of addition experiments

This experiment investigates which step of the replication cycle of HIV is inhibited by an anti-HIV compound. It determines how long after infection the addition of the compound can be postponed before losing its antiviral activity in one replication cycle. Reference compounds with a known mode of action were included. In this case we used dextran sulfate, a polyanion which is known to interfere with binding of the virus to the cell; the nucleoside analogue zidovudine (AZT), which inhibits the reverse transcription process; and ritonavir, which is an inhibitor of the proteolytic cleavage. Inhibition of viral replication was

Table 1. Anti-HIV activity (HIV-1, strain III_B and HIV-2 strain ROD) of BTD derivatives **1–19** in CEM and MT-4 cells

	CEM			MT-4		
	EC_{50} (μM)*		CC_{50} (μM)†	EC_{50} (μM)*		CC_{50} (μM)†
No.	HIV-1 (III_B)	HIV-2 (ROD)		HIV-1(III_B)	HIV-2 (ROD)	
1	25.0 ±7.1	25.0 ±7.1	27.7 ±4.3	>44	>44	44.2 ±9.4
2	25.0 ±2.8	>50	108 ±51.5	20.3 ±0.9	>125	>125
3	5.0 ±2.6	>10	27.8 ±0.1	18.4 ±10.5	>38	37.6 ±8.6
4	>10	7.5 ±3.5	35.8 ±3.3	>125	>125	>125
5	>2	>2	16.4 ±3.4	>125	>125	>125
6	2.0 ±0.9	18.0 ±17.0	82.0 ±11.7	12.0 ±4.0	>125	>125
7	6.5 ±0.7	>10	14.7 ±0.3	>125	>125	>125
8	>10	>10	21.9 ±2.1	>125	>125	>125
9	8.0 ±2.8	>10	19.4 ±0.1	241.9 ±2.1	>125	>125
10	5.0 ±1.4	>10	22.4 ±0.1	>104	>104	>104
11	>10	>10	30.5 ±0.3	>125	>125	>125
12	>50	>50	>250	>68	>68	67.5 ±48.2
13	>10	>10	23.2 ±0.2	66.2 ±6.5	>60	66.2 ±6.5
14	>10	>10	22.8 ±3.3	>125	>125	>125
15	>10	>10	24.7 ±4.7	>125	>125	>125
16	>10	>10	28.7 ±5.9	60.1 ±5.2	>68	60.1 ±5.2
17	3.0 ±1.4	>10	21.3 ±5.3	8.5 ±0.3	>125	>125
18	>10	>10	>10	>125	>125	>125
19	4.5 ±2.1	>10	21.6 ±1.4	8.1 ±1.0	>68	71.0 ±8.5

*EC_{50}, 50% effective concentration.
†CC_{50}, 50% cytotoxic concentration.

measured as the logarithm of viral antigen production p24 (log p24), in function of time post infection at which the compound was added to a culture of infected HIV-1 MT-4 cells. The results are shown in (Figure 3). Addition of BTD **3** can be postponed for 3 h after infection, showing the same graphical profile in this experiment of the reverse transcriptase (RT) inhibitor AZT. These data suggest a viral interaction of BTD **3** proximately at the moment of reverse transcription of HIV.

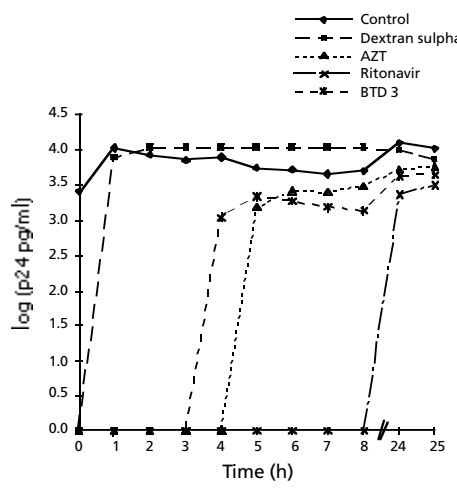
Inhibitory activity of BTD against NNRTI-resistant mutant HIV-1 strain

To determine the BTD binding site to RT, BTD **3**, as well as AZT (nucleoside reverse transcriptase inhibitor [NRTI]), nevirapine (non-nucleoside reverse transcriptase inhibitor [NNRTI]) and indinavir (protease inhibitor), were evaluated against an HIV-1 strain with specific mutations in its RT (K103N and Y181C) that make it resistant to NNRTIs. AZT and indinavir retain their antiviral activity (EC_{50} values of 0.004 and 0.018 μM, respectively), whereas BTD **3** and nevirapine markedly lost their capacity to inhibit HIV-1 replication (EC_{50} values >50 and >15 μM, respectively). This fact points out that BTD derivatives exert their anti-HIV action by interaction with the HIV RT in the same allosteric pocket as the known NNRTIs.

Molecular modeling of BTD **3**

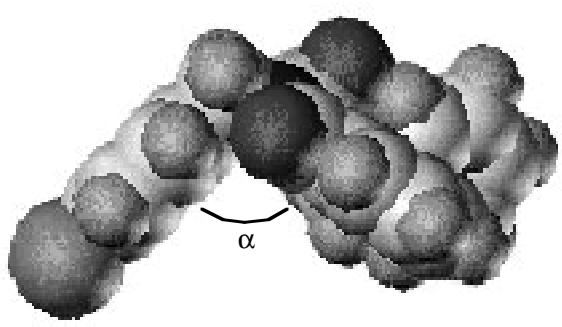
A preliminary conformational study of BTD **3** was carried out using *ab initio* Hartree–Fock methods. The molecular geometry was fully optimized with HF/3-21G* and HF/6-21G*, using Gaussian 94 program (Frisch *et al*., 1995), to determine the optimum position of the two aromatic systems. The optimized geometry shows a disposition of the aromatic system in agreement with a butterfly-like configuration with a valence angle α between the two rings of 111° (Figure 4).

Figure 3. Effect of the time addition of BTD **3** and control drugs on MT-4 cells infected by HIV-1



AZT, zidovudine; BTD, benzothiadiazine dioxide

Figure 4. Optimized geometry of BTD 3



Discussion

BTDs are a promising new class of anti-HCMV inhibitors that have shown a marginal but selective anti-HIV-1 activity, representing an important hit among the antiviral drugs. Combination of anti-HCMV and anti-HIV activities in a single compound could prove an efficient strategy for the treatment of acute HCMV infection in AIDS patients, while simultaneously combating the primary HIV infection.

The anti-HIV-1 data shown in Table 1 led us to establish preliminary SARs. The length of the linker between the aromatic ring attached to the N1 of the thiadiazine framework is crucial for activity (see the lack of activity of compounds **1** and **13–15** versus **2–10**), pointing to additional interactions with the viral enzyme. The activity found in BTDs **17** and **19** opens the possibility of changing the electronic nature of substituents attached to N1 for increased anti-HIV-1 activity. However, for the development of single compounds that retain both antiviral activities (anti-HCMV and anti-HIV-1), the structure-activity relationships derived for the first antiviral activity found in these compounds should also be considered (Martinez *et al.*, 2000a).

Regarding the anti-HIV-1 activity described here, the time of addition experiments allowed us to establish the point in the HIV-1 replicative cycle at which BTD derivatives could act. The behaviour found for BTD **3** is the same as that shown for the known RT inhibitor AZT (Figure 3), suggesting a viral interaction approximately at the moment of reverse transcription of HIV. Furthermore, the lack of anti-HIV-1 activity of BTD **3** on viral strains with specific mutations on its RT that make it resistant to NNRTIs, points to a mechanism of action similar to those of the NNRTIs.

NNRTIs bind to RT in a largely hydrophobic pocket whose shape varies only slightly for different NNRTIs. Thus, complementarity with the pocket shape, rather than with the pocket charge, is the crucial factor in determining the binding mode of many NNRTIs. In this context, it is well known that NNRTIs comprise a structurally diverse family sharing some common three-dimensional feature. Their shape is a butterfly-like conformation that fits well into a complementary cavity in the enzyme. In general, NNRTIs can adopt this shape with two hydrophobic moieties connected by a linker group that allows the two wings to bend with an angle of 110–115° (Mager, 1996). The molecular modelling performed on BTD **3** showed a conformation compatible with this bioactive configuration.

In summary, we have shown an anti-HIV-1 NNRTI profile of BTD HCMV inhibitors. These compounds can be considered as potential leads in the treatment of opportunistic infections in immunocompromised individuals, such as AIDS patients. Further studies are in progress to optimize this new therapeutic action found on BTD derivatives.

Acknowledgements

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References

De Clercq E (1994). *In vitro* and *ex vivo* test systems to rationalize drug design and delivery. In *Minutes of an European Symposium*; pp. 108–125. Edited by D Crommelin, P Couvreur & D Duchêne. Paris: Editions de Santé.

Emery VC (2001). Progress in understanding cytomegalovirus drug resistance. *Journal of Clinical Virology* **21:**223–228.

Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA & McCarthy RE (1965). Continous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18:**522–529.

Frisch MJ, Trucks GW, Schlegel HB, Gill PMW, Johnson BG, Robb MA, Cheeseman JR, Keith T, Petersson GA, Montgomery JA, Raghavachari K, Al-Laham MA, Zakrzewski VG, Ortiz JV, Foresman JB, Peng CY, Ayala PY, Chen W, Wong MW, Andres JL, Replogle ES, Gomperts R, Martin RL, Fox DJ, Binkley JS, Defrees DJ, Baker J, Stewart JP, Head-Gordon M, Gonzalez C & Pople JA (1995). *Gaussian 94, revision D.4.* Pittsburgh: Gaussian.

Heininger A, Jahn G, Engel C, Notheisen T, Unertl K & Hamprecht K (2001). Human cytomegalovirus infections in non-immunosuppressed critically ill patients. *Critical Care Medicine* **29:**541–547.

Lee TA, Sullivan SD, Veenstra DL, Ramsey SD, Steger PJ, Malinverni R, Pleil AM & Williamson T (2001). Economic evaluation of systemic treatments for cytomegalovirus retinitis in patients with AIDS. *Pharmacoeconomics* **19:**535–550.

Mager PP (1996) Evidence of a butterfly-like configuration of structurally diverse allosteric inhibitors of the HIV-1 reverse transcriptase. *Drug Design and Discovery* **14:**241–257.

Manfredi R & Chiodo F (2001). Management of relapsing cytomegalovirus disease in transplant recipients: diagnostic and therapeutic dilemmas. Management of relapsing cytomegalovirus disease in transplant recipients: diagnostic and therapeutic dilemmas. *Transplantation Infective Diseases* **3:**51–53.

Martinez A, Esteban AI & De Clercq E (1997). Benzothiadiazine dioxide acyclonucleosides as lead compounds for the development of new agents against human cytomegalovirus and varicella-zoster virus infections. *Bioorganic Medicinal Chemistry Letters* **7:**1031–1032.

Martinez A, Esteban AI, Castro A, Gil C, Conde S, Andrei G, Snoeck R, Balzarini J & De Clercq E (1999a). Novel potential agents for human cytomegalovirus infection: synthesis and antiviral activity evaluation of benzothiadiazine dioxide acyclonucleosides. *Journal of Medicinal Chemistry* **42:**1145–1150.

Martinez A, Gil C, Perez C, Castro A, Prieto C & Otero J (1999b). Chlorophenylmethyl benzothiadiazines dioxides derivatives: Potent human cytomegalovirus inhibitors. *Bioorganic Medicinal Chemistry Letters* **9:**3133–3136.

Martinez A, Gil C, Perez C, Castro A, Prieto C, Otero J, Andrei G, Snoeck R, Balzarini J & De Clercq E (2000a). Nonnucleoside human cytomegalovirus inhibitors: synthesis and antiviral evaluation of (chlorophenylmethyl)benzothiadiazine dioxide derivatives. *Journal of Medicinal Chemistry* **43:**3267–3273.

Martinez A, Gil C, Abasolo MI, Castro A, Bruno AM, Perez C, Prieto C & Otero J (2000b). Benzothiadiazine dioxide dibenzyl derivatives as potent human cytomegalovirus inhibitors: synthesis and comparative molecular field analysis. *Journal of Medicinal Chemistry* **43:**3218–3225.

Martinez A, Castro A, Gil C & Perez C (2001). Recent strategies in the development of new human cytomegalovirus inhibitors *Medicinal Research Reviews* **21:**227–244.

Miyoshi I, Taguchi H, Kobonishi I, Yoshimoto S, Ohtsuki Y, Shiraishi Y & Agaki T (1982). Type C virus-producing cell lines derived from adult T cell leukemia. *Gann Monographies* **28:**219–228.

Nichols WG & Boeckh M (2000). Recent advances in the therapy and prevention of CMV infections. *Journal of Clinical Virol*ogy **16:**25–40.

Pauwels R, Balzarini J, Baba H, Snoeck R, Schols D, Herdewijn P, Desmyter J & De Clercq E (1988). Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *Journal of Virology Methods* **20:**309–321.

Speich R & Van der Bij W (2001). Epidemiology and management of infections after lung transplantation. *Clinical Infectious Diseases* **33:**S58–S65.

Trincado DE, Scott GM, White PA, Hunt C, Rasmussen L & Rawlinson WD (2000). Human cytomegalovirus strains associated with congenital and perinatal infections. *Journal of Medical Virology* **61:**481–487.

Whitley RJ, Jacobson MA, Friedberg DN, Holland GN, Jabs DA, Dieterich DT, Hardy WD, Polis MA, Deutsch TA, Feinberg J, Spector SA, Walmsley S, Drew WL, Powderly WG, Griffiths PD, Benson CA & Kessler HA (1998). Guidelines for the treatment of cytomegalovirus diseases in patients with AIDS in the era of potent antiretroviral therapy: recommendations of an international panel. International AIDS Society-USA. *Archives of Internal Medicine* **158:**957–969.

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