

Anti-enterovirus activity and structure–activity relationship of a series of 2,6-dihalophenyl-substituted 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles

Armando M. De Palma ^a, Ward Heggermont ^a, Pieter Leyssen ^a, Gerhard Pürstinger ^c,
Eva Wimmer ^c, Erik De Clercq ^a, Angela Rao ^b, Anna-Maria Monforte ^b, Alba Chimirri ^b,
Johan Neyts ^{a,*}

^a Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

^b Dipartimento Farmaco-Chimico, Università di Messina, Messina, Italy

^c Department of Pharmaceutical Chemistry, Institute of Pharmacy, University of Innsbruck, Austria

Received 21 November 2006

Available online 19 December 2006

Abstract

Despite the fact that enteroviruses are implicated in a variety of human diseases, there is no approved therapy for the treatment of enteroviral infections. Here, a series of 2,6-dihalophenyl-substituted 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles with anti-enterovirus activity is reported. The compounds elicit potent activity against coxsackievirus A9, echovirus 9 and 11 and all six strains of coxsackievirus B. A structure–activity relationship analysis revealed that the presence of substituents at position 6 of the tricyclic system positively influences the antiviral activity, whereas substitutions at position 7 are less favorable. In particular a 6-trifluoromethyl substitution leads to a substantial improvement of the antiviral activity as compared to the unsubstituted structure. Furthermore, an additional introduction of a 2-Cl, 6-F substitution on the phenyl at C-1 results in a further increase of the antiviral activity. Hence, 1-(2-chloro-6-fluorophenyl)-6-trifluoromethyl-1*H*,3*H*-thiazolo[3,4-*a*]benzimidazole results in a dose-dependent inhibition of viral replication with a 50% effective concentration (EC₅₀) of 0.41 μg/ml without any detectable cytotoxicity at the highest concentration (100 μg/ml) tested.

© 2006 Elsevier Inc. All rights reserved.

Enteroviruses belong to the family of picornaviruses and may cause various diseases, ranging from mild respiratory syndromes, herpangina, and hand–foot–mouth-syndrome to life-threatening conditions such as pancreatitis, myocarditis, meningitis, and encephalitis. It is estimated that enteroviruses cause each year 10–15 million (or more) symptomatic infections [1]. In the past, several molecules have been reported to be selective inhibitors of enteroviruses, some of which have entered clinical trials [2]. Pleconaril is the prototype of a series of broad-spectrum picornavirus inhibitors, known as “WIN-compounds” [3]. These compounds block replication by inhibiting host cell attachment and/or uncoating, as a result of binding into a pocket, located beneath the canyon on the virion surface [4–6].

Despite its proven efficacy in several studies [7,8], pleconaril was not approved by the FDA [9]. Another compound that was the subject of clinical trials, is rupintrivir (AG7088), a protease inhibitor with high selectivity against picornavirus 3C protease and low cytotoxicity [10]. In an experimental human rhinovirus (HRV) challenge trial, rupintrivir was able to moderate severity of illness and reduce viral load [11], but in a subsequent natural infection study in patients, the compound failed to demonstrate beneficial effects and was not further developed [12]. Further research efforts to discover an orally bioavailable 3C inhibitor lead to the development of compound 1, a promising candidate for further clinical studies [13]. Next to pleconaril and rupintrivir, which demonstrated *in vivo* activity, *in vitro* inhibition of enterovirus replication has been demonstrated for several synthetic and natural compounds (reviewed in [2]). Despite these efforts, none of the

* Corresponding author. Fax: +32 16 337340.

E-mail address: johan.neyts@rega.kuleuven.be (J. Neyts).

anti-picornavirus compounds has been further developed, and there is no drug approved for the treatment of enteroviral infections [2]. The development of specific anti-enterovirus compounds remains thus an important challenge.

Here, we report on the antiviral activity of a series of 2,6-dihalophenyl-substituted 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles, a class of compounds that was previously reported as non-nucleoside reverse transcriptase inhibitors (NNRTIs) of human immunodeficiency virus type 1 (HIV-1) [14].

Materials and methods

Compounds. A series of 2,6-dihalophenyl-substituted 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazoles was synthesized as described elsewhere [14]. Enviromix was synthesized as reported before [15].

Cells and viruses. Vero and HeLa cells were purchased from the American Type Culture Collection (ATCC CCL-81 and ATCC CCL-2). Primary monkey kidney cells were from Diagnostics Hybrids, Athens. Coxsackieviruses A9 and B1-6 were kindly provided by Prof. Marc Van Ranst (University Hospital, KULeuven, Leuven, Belgium). Rhinovirus 2 and 14 and echovirus 9 and 11 were offered by Dr. Koen Andries and poliovirus 1, 2, and 3 by Prof. Bart Rombaut.

Antiviral assay. The antiviral activity of the selected compounds was determined using an MTS-based CPE reduction assay and was expressed as the 50% effective concentration (EC₅₀), or the concentration of compound that inhibits virus-induced cytopathic effect formation by 50%. Cells, grown to confluency in 96-well plates, were infected with 100 CCID₅₀ of virus, one CCID₅₀ being the 50% cell culture infective dose. After an adsorption period of two hours at 37 °C (35 °C for rhinovirus), virus was removed and serial dilutions of the compounds were added. The cultures were further incubated at 37 °C (35 °C) for 3 days, until complete CPE was observed in the infected and untreated virus control (VC). After removal of the medium, 90 μl medium and 10 μl MTS/PMS (Promega, Leiden, The Netherlands) were added to each well. After an incubation period of 2 h at 37 °C (35 °C) the optical density of each well was read at 498 nm in a microplate reader. CPE values were calculated as follows: % CPE = $[\text{OD}_{\text{VC}} - \text{OD}_{\text{CVB3+Compound}}] / [\text{OD}_{\text{CC}} - \text{OD}_{\text{VC}}]$. In these formulae, OD_{CC} corresponds to the optical density of the uninfected and untreated cell cultures, OD_{VC} represents the infected and untreated cell cultures and OD_{CVB3+Compound} are CVB3-infected cell cultures, treated with a given concentration of compound.

Cytotoxic assay. The cytotoxicity of the compounds was evaluated by the MTS-method and the 50% cytotoxic concentration (CC₅₀) was calculated. Briefly, the same experimental set-up was used as for the antiviral assay, but for cytotoxicity determination, uninfected cultures were incubated with serial dilution of compound for three days at 37 °C. The cytotoxic activity was calculated using the following formula: % CPE = $[\text{OD}_{\text{CC}} - \text{OD}_{\text{Compound}}] / \text{OD}_{\text{CC}}$, where OD_{CC} corresponds to the optical density of the uninfected and untreated cell cultures and OD_{Compound} are uninfected cultures, treated with compound.

Results

A large scale antiviral screening effort resulted in the identification of several thiazolobenzimidazoles as selective inhibitors of coxsackievirus B replication in a multi-cycle viral growth assay. The general structure of the compounds studied is depicted in Fig. 1. This class of compounds shares a common 1*H*,3*H*-thiazolo[3,4-*a*]benzimidazole scaffold with a 2,6-dihalophenyl substitution at C-1. This substitution consists of either a (i) -2,6-F₂, (ii) -2,6-Cl₂ or (iii) -2-Cl,6-F. Either of these substitutions was combined

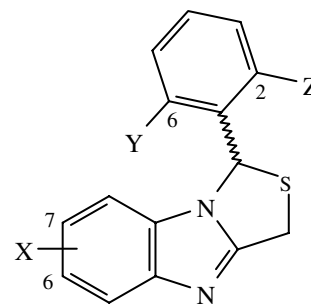


Fig. 1. Structural formulae of thiazolobenzimidazoles.

with a (i) -CF₃, (ii) -OCH₃ or (iii) -F substituent in position 6 or 7 of the thiazolobenzimidazole (Table 1). The 6- or 7-unsubstituted analogues were included as well.

Compounds with the highest antiviral potencies all carried a substitution at position 6 or 7 of the thiazolobenzimidazole (4a–12a and 4b–12b), whereas the unsubstituted analogues (1–3) exhibited rather weak or no activity at all (Table 1). When comparing the 6-substituted (4a–12a) with the 7-substituted (4b–12b) molecules, congeners that carried a 6-substitution proved generally more potent than the corresponding 7-substituted analogues. In fact only the -OCH₃ substitution combined with -2-Cl,6-F resulted in a significant increase in antiviral potency when this substitution was located at position 7 (9b vs. 9a). A 6-OCH₃ substitution of the thiazolobenzimidazole engendered one molecule (7a) with an EC₅₀ value of 2.6 ± 0.3 μg/ml, whereas the corresponding 7-substituted analogue (7b) exhibited moderate activity (13 ± 2 μg/ml). No other 6- or 7-OCH₃ substitutions resulted in compounds with marked anti-CVB3 activity. Substitution with 6-F or 7-F, yielded an analogue with strong anti-CVB3 activity (12a, EC₅₀ = 2.5 ± 0.1 μg/ml), whereas the other analogues elicited only weak or no antiviral activity. Noteworthy is that the corresponding 7-substituted analogue (12b) exhibited no antiviral activity at all.

The most potent compounds (4a–6a) of this series shared one structural feature, i.e. the 6-CF₃ substitution. In combination with either of the three dihalophenyl substitutions, this resulted in analogues with EC₅₀ values ranging between 0.41 and 1.9 μg/ml. Also these congeners lost activity when the 6-CF₃ substitution was switched to position 7 (4b–6b). The most potent compound (6a, EC₅₀ = 0.41 ± 0.15 μg/ml) of this series, was achieved by combining the 6-CF₃ substitution on the thiazolobenzimidazole with the -2-Cl,6-F-phenyl at C-1. There was no clear structure–activity relation for the potential adverse effects of the analogues on the uninfected host cell. Interestingly, the most potent compounds (4a and 6a) did not cause any detectable toxicity at the highest concentration tested (100 μg/ml). As is also evident from the dose–response curves (Fig. 2A), compounds 4a and 6a emerged as the most potent and selective inhibitors in this series. These molecules inhibited viral (CBV3) replication by 100% at concentrations that had no effect on the uninfected host cell. Selectivity indices of >83 and >244, respectively,

Table 1
Antiviral and cytotoxic activities of several thiazolobenzimidazoles against coxsackievirus B3 replication in Vero cells

| Position | X | Y | Z | Compound | EC ₅₀ ^a (μg/ml) | CC ₅₀ ^b (μg/ml) | SI ^c |
|------------|-------------------|-----|-----|-------------|---------------------------------------|---------------------------------------|-----------------|
| — | -H | -F | -F | 1 | 17 ± 3 | >100 | >5.9 |
| | | -Cl | -Cl | 2 | 50 ± 26 | >100 | >2.0 |
| | | -Cl | -F | 3 | >100 | >100 | 1 |
| 6-X | -CF ₃ | -F | -F | 4a | 1.2 ± 0.7 | >100 | >83 |
| | | -Cl | -Cl | 5a | 1.9 ± 0.6 | 13 ± 0 | 7.1 |
| | | -Cl | -F | 6a | 0.41 ± 0.15 | >100 | >244 |
| | -OCH ₃ | -F | -F | 7a | 2.6 ± 0.3 | 16 ± 3 | >6.2 |
| | | -Cl | -Cl | 8a | >100 | >100 | 1 |
| | | -Cl | -F | 9a | >100 | >100 | 1 |
| | -F | -F | -F | 10a | 33 ± 14 | >100 | >3.1 |
| | | -Cl | -Cl | 11a | >13 | 13 ± 0 | <1 |
| | | -Cl | -F | 12a | 2.5 ± 0.1 | 13 ± 0 | 5.4 |
| 7-X | -CF ₃ | -F | -F | 4b | 12 ± 1 | >100 | >8.6 |
| | | -Cl | -Cl | 5b | >100 | >100 | 1 |
| | | -Cl | -F | 6b | 2.6 ± 0.1 | 13 ± 0 | 5.0 |
| | -OCH ₃ | -F | -F | 7b | 13 ± 2 | >100 | 7.5 |
| | | -Cl | -Cl | 8b | >100 | >100 | 1 |
| | | -Cl | -F | 9b | 13 ± 2 | >100 | 7.9 |
| | -F | -F | -F | 10b | 19 ± 8 | >100 | 5.4 |
| | | -Cl | -Cl | 11b | >100 | >100 | 1 |
| | | -Cl | -F | 12b | >35 | 35 ± 19 | <0.35 |
| Enviroxime | | | | 0.25 ± 0.11 | 63 ± 2 | 253 | |

Data are mean values ± SD for at least three independent experiments.

^a 50% effective concentration.

^b 50% cytotoxic concentration.

^c SI, selectivity index.

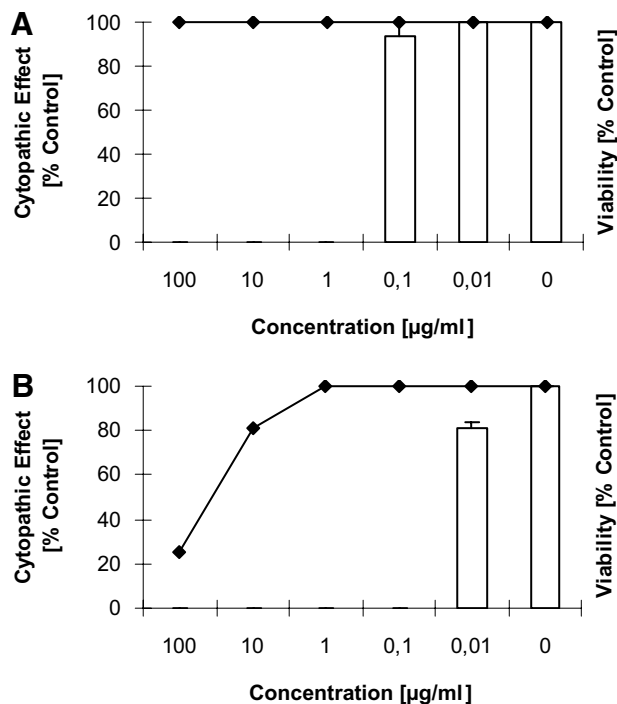


Fig. 2. Effect of compound **6a** (A) and the reference compound enviroxime (B) on CVB3-induced cytopathic effect (bars) and cytotoxicity (lines) in Vero cells. Data are average values ± SD for four independent experiments.

were calculated. Enviroxime, another benzimidazole analogue acting as a broad-spectrum anti-picornavirus replication inhibitor [16], was included as a reference compound (Table 1 and Fig. 2B). The antiviral activity of enviroxime was slightly more pronounced than that of compound **6a**, but enviroxime resulted in 50% cytotoxicity at a concentration of 63 μg/ml. The activities of compounds **4a** and **6a** were also evaluated against a number of other enteroviruses and rhinoviruses (Table 2). Both compounds proved active against other CVB strains as well as against coxsackievirus A9, and echovirus 9 and 11. No activity was observed against rhinovirus 2 and 14 and polioviruses.

Discussion

Picornaviruses, and entero- and rhinoviruses in particular, are involved in a range of clinical manifestations, including self-limiting illnesses like febrile colds and sore throats, but may also cause more serious diseases such as pancreatitis, meningitis, and encephalitis [1]. Despite this, no specific therapy is available, and the search for replication inhibitors of enteroviruses thus remains compulsory. We here report on a class of substituted *1H,3H*-thiazolo[3,4-*a*]benzimidazoles of which some congeners exhibit potent and selective activity against enteroviruses, as determined by an MTS-based cell protection assay. Regardless of the nature of the substituent, it should be emphasized that 6-or 7-substitution of the thiazolobenzimidazole is

Table 2
Antiviral activity of compounds **4a** and **6a** against a selected panel of picornaviruses

| | Compound 4a EC ₅₀ ^a (μg/ml) | Compound 6a EC ₅₀ ^a (μg/ml) |
|--------------------------------|---|---|
| Coxsackievirus A9 ^b | <4.0 ^f | <4.0 ^f |
| Coxsackievirus B1 ^c | 5.8 ± 4.7 | 6.1 ± 5.0 |
| Coxsackievirus B2 ^c | 1.1 ± 0.7 | 0.42 ± 0.02 |
| Coxsackievirus B3 ^c | 1.2 ± 0.7 | 0.41 ± 0.15 |
| Coxsackievirus B4 ^c | 1.0 ± 0.8 | 0.83 ± 0.15 |
| Coxsackievirus B5 ^c | 1.4 ± 0.8 | 1.2 ± 0.8 |
| Coxsackievirus B6 ^c | 1.3 ± 0.7 | 0.55 ± 0.30 |
| Echovirus 9 ^b | <4.0 ^f | <4.0 ^f |
| Echovirus 11 ^b | <4.0 ^f | <4.0 ^f |
| Rhinovirus 2 ^d | >100 | >100 |
| Rhinovirus 14 ^d | >100 | 56 ± 32 |
| Poliovirus 1 ^c | >100 | >100 |
| Poliovirus 2 ^c | >100 | >100 |
| Poliovirus 3 ^c | >100 | >100 |

Data are mean values ± SD for at least three independent experiments.

^a 50% effective concentration.

^b Cultured in primary monkey kidney (PMK) cells at 37 °C.

^c Cultured in Vero cells at 37 °C.

^d Cultured in HeLa cells at 35 °C.

^e Cultured in HeLa cells at 37 °C.

^f 100 % inhibition of viral replication at the lowest (4 μg/ml) concentration tested.

mandatory to obtain potent antiviral activity, as compared to the unsubstituted compound. The most active compounds (**4a** and **6a**) were obtained by introduction of a -CF₃ substitution at position 6 of the thiazolobenzimidazole, combined with either a -2,6-F₂, or -2-Cl,6-F. Derivative **6a**, the most potent congener of the series, exhibited a selectivity comparable to that of enviroxime, but, unlike enviroxime, was not cytotoxic at the highest concentration tested. No clear structure–activity relationship of the compounds could be established regarding adverse effects of the compounds on the host cells. Only six of the 21 studied compounds showed cytotoxicity below the maximum concentration tested.

Several thiazolobenzimidazoles were previously reported to act as NNRTIs of HIV-1 [14]. It could be hypothesized that the mode of action of the compounds against enteroviruses may be, akin to the situation for HIV, inhibition of the viral polymerase. However, since (i) the compounds are only active against HIV-1 and not against HIV-2, (ii) there is more similarity between the HIV-1 and the HIV-2 polymerase than between the HIV-1 polymerase and the enteroviral polymerase and (iii) there is no parallel between the structure–activity of the anti-HIV-1 and the anti-enterovirus activity, it is highly unlikely that the molecular mechanism of action against retro- and enteroviruses is identical. In fact, the most potent anti-HIV-1 compound proved inactive against coxsackievirus and vice versa. The chemical core of this series is derived from a benzimidazole structure. Several other picornavirus inhibitors also contain a benzimidazole scaffold, three of which are well characterized: enviroxime, which targets the 3A protein and HBB [2-(hydroxybenzyl)-benzimid-

azole] and MRL-1237 [1-(4-fluorophenyl)-2-(4-imino-1,4-dihydropyridin-1-yl) methylbenzimidazole hydrochloride], which target the viral 2C protein [16–19]. The precise mechanism of the anti-enterovirus activity of the thiazolobenzimidazoles remains however to be studied.

Acknowledgments

This work was supported by the VIZIER integrated project (LSHG-CT-2004-511960) from the European Union 6th PCRDT. We thank Mrs. M. Stuyck for excellent technical assistance.

References

- [1] H.A. Rotbart, Treatment of picornavirus infections, *Antiviral Res.* 53 (2002) 83–98.
- [2] D.L. Barnard, Current status of anti-picornavirus therapies, *Curr. Pharm. Des.* 12 (2006) 1379–1390.
- [3] N.R. Florea, D. Maglio, D.P. Nicolau, Pleconaril, a novel antipicornaviral agent, *Pharmacotherapy* 23 (2003) 339–348.
- [4] J.J. McSharry, L.A. Caliguiri, H.J. Eggers, Inhibition of uncoating of poliovirus by arildone, a new antiviral drug, *Virology* 97 (1979) 307–315.
- [5] D.C. Pevear, M.J. Fancher, P.J. Felock, M.G. Rossmann, M.S. Miller, G. Diana, A.M. Treasurywala, M.A. McKinlay, F.J. Dutko, Conformational change in the floor of the human rhinovirus canyon blocks adsorption to HeLa cell receptors, *J. Virol.* 63 (1989) 2002–2007.
- [6] N. Vaidehi, W.A. Goddard III, The pentamer channel stiffening model for drug action on human rhinovirus HRV-1A, *Proc. Natl. Acad. Sci. USA* 94 (1997) 2466–2471.
- [7] F.G. Hayden, D.T. Herrington, T.L. Coats, K. Kim, E.C. Cooper, S.A. Villano, S. Liu, S. Hudson, D.C. Pevear, M. Collett, M. McKinlay, Efficacy and safety of oral pleconaril for treatment of colds due to picornaviruses in adults: results of 2 double-blind, randomized, placebo-controlled trials, *Clin. Infect. Dis.* 36 (2003) 1523–1532.
- [8] H.A. Rotbart, A.D. Webster, Treatment of potentially life-threatening enterovirus infections with pleconaril, *Clin. Infect. Dis.* 32 (2001) 228–235.
- [9] K. Senior, FDA panel rejects common cold treatment, *Lancet Infect. Dis.* 2 (2002) 264.
- [10] A.K. Patick, S.L. Binford, M.A. Brothers, R.L. Jackson, C.E. Ford, M.D. Diem, F. Maldonado, P.S. Dragovich, R. Zhou, T.J. Prins, S.A. Fuhrman, J.W. Meador, L.S. Zalman, D.A. Matthews, S.T. Worland, In vitro antiviral activity of AG7088, a potent inhibitor of human rhinovirus 3C protease, *Antimicrob. Agents Chemother.* 43 (1999) 2444–2450.
- [11] F.G. Hayden, R.B. Turner, J.M. Gwaltney, K. Chi-Burris, M. Gersten, P. Hsyu, A.K. Patick, G.J. Smith III, L.S. Zalman, Phase II, randomized, double-blind, placebo-controlled studies of rupintrivir nasal spray 2-percent suspension for prevention and treatment of experimentally induced rhinovirus colds in healthy volunteers, *Antimicrob. Agents Chemother.* 47 (2003) 3907–3916.
- [12] A.K. Patick, Rhinovirus chemotherapy, *Antiviral Res.* 71 (2006) 391–396.
- [13] A.K. Patick, M.A. Brothers, F. Maldonado, S. Binford, O. Maldonado, S. Fuhrman, A. Petersen, G.J. Smith III, L.S. Zalman, L.A. Burns-Naas, J.Q. Tran, In vitro antiviral activity and single-dose pharmacokinetics in humans of a novel, orally bioavailable inhibitor of human rhinovirus 3C protease, *Antimicrob. Agents Chemother.* 49 (2005) 2267–2275.
- [14] A. Chimirri, S. Grasso, P. Monforte, A. Rao, M. Zappala, A.M. Monforte, C. Pannecouque, M. Witvrouw, J. Balzarini, E. De Clercq, Synthesis and biological activity of novel 1*H*,3*H*-thiazolo[3,4-*a*]benz-

- imidazoles: non-nucleoside human immunodeficiency virus type 1 reverse transcriptase inhibitors, *Antivir. Chem. Chemother.* 10 (1999) 211–217.
- [15] J.H. Wikel, C.J. Paget, D.C. DeLong, J.D. Nelson, C.Y. Wu, J.W. Paschal, A. Dinner, R.J. Templeton, M.O. Chaney, N.D. Jones, J.W. Chamberlin, Synthesis of syn and anti isomers of 6-[[hydroxyimino]phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1H-benzimidazol-2-amine. Inhibitors of rhinovirus multiplication, *J. Med. Chem.* 23 (1980) 368–372.
- [16] B.A. Heinz, L.M. Vance, The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus, *J. Virol.* 69 (1995) 4189–4197.
- [17] D. Hadaschik, M. Klein, H. Zimmermann, H.J. Eggers, B. Nelsen-Salz, Dependence of echovirus 9 on the enterovirus RNA replication inhibitor 2-(alpha-Hydroxybenzyl)-benzimidazole maps to nonstructural protein 2C, *J. Virol.* 73 (1999) 10536–10539.
- [18] M.P. Langford, W.A. Ball, J.P. Ganley, Inhibition of the enteroviruses that cause acute hemorrhagic conjunctivitis (AHC) by benzimidazoles; enviroxime (LY 122772) and envirodone (LY 127123), *Antiviral Res.* 27 (1995) 355–365.
- [19] H. Shimizu, M. Agoh, Y. Agoh, H. Yoshida, K. Yoshii, T. Yoneyama, A. Hagiwara, T. Miyamura, Mutations in the 2C region of poliovirus responsible for altered sensitivity to benzimidazole derivatives, *J. Virol.* 74 (2000) 4146–4154.