

Discrepancy between Infectivity and Antigenicity Stabilization of Oral Poliovirus Vaccine by a Capsid-Binding Compound

KOEN ANDRIES,^{1*} BART ROMBAUT,² BART DEWINDT,¹ AND ALBERT BOEYÉ²

Department of Virology, Janssen Research Foundation, B-2340 Beerse,¹ and Department of Microbiology and Hygiene, Vrije Universiteit Brussel, B1090-Brussels,² Belgium

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Two hundred forty pyridazinamine derivatives were tested for the ability to stabilize the antigenicity and infectivity of oral poliovirus vaccine subjected to 45°C for 2 h. Seven compounds stabilized the antigenicity of all three vaccine strains and neutralized the viral particles in a way that is reversible by dilution. Of these, R 77975 (pirodavir) was selected for vaccine potency tests. Sabin type 2 and type 3 strains were subjected to 4, 25, 42, and 45°C for 1 week in the presence and absence of R 77975. Although R 77975 particularly stabilized the infectivity of the most thermolabile vaccine strain (Sabin type 3), the protection did not exceed that of 1 M MgCl₂. When virus was inactivated in the absence of R 77975, the native or N antigenicity changed in H antigenicity. However, in the presence of the capsid-binding compound, N antigenicity was preserved in particles that had lost infectivity.

A number of structurally unrelated antipicornavirus compounds were developed with one common characteristic: they all exert an antiviral activity by direct interaction with the proteins of the viral capsid and thus are commonly known as capsid-binding compounds. Among these are flavans and chalcones (4, 7) as well as several pyridines (8), isoxazoles (10, 15), and pyridazine derivatives (1, 3). For most of these compounds, it has been shown, directly or indirectly, that they bind into the same hydrophobic pocket located at the base of the canyon, within the β barrel of the capsid protein VP1 (2, 5, 12).

The strength of the binding of capsid-binding compounds to susceptible serotypes depends on serotype, drug type and concentration, and time (1). Binding results in a neutralization of the viral infectivity and an inhibition of viral replication. With some serotypes, the neutralization is completely reversible by dilution. With others, the binding is stronger and the neutralization of the viral infectivity can be reversed only by organic solvent extraction, as is the case with neutralization by antibodies (13). With a third group of viruses, the binding results in a neutralization that is not reversible by organic solvent extraction (3).

Binding of capsid-binding compounds to viral particles may also result in a partial stabilization of viral infectivity (reviewed in reference 2) and antigenic character. The infectivity of susceptible picornaviruses can be protected against inactivation by heat or mild acidification (in the case of rhinoviruses). The minimal concentrations required to prevent either acetate or citrate or heat inactivation do not appear to be correlated with the MIC₅₀s, suggesting that inhibition of replication and stabilization are independent events, both resulting from the binding of the drug to the viral capsid (2, 3, 11).

We recently established that the native antigenic character of polioviruses and rhinoviruses can also be stabilized by capsid-binding compounds (9, 11). In the case of polioviruses, the antiviral compounds WIN 51711, an isoxazole derivative produced by Sterling-Winthrop, and R 78206, a pyridazinamine derivative produced by Janssen, were able to stabilize

the native antigenic character of the viral particles for 1 week at up to 42°C. These results confirmed the possible use of capsid-binding compounds as stabilizers of inactivated poliovirus vaccine.

The aim of this work was to investigate the possible use of one or more of these compounds in the improvement of the thermostability of live oral poliovirus vaccine. To be useful, the antigenicity and the infectivity of the viral particles have to be protected against inactivation by heat. Thus, the minimal concentration needed for stabilization should be lower than the concentration having an effect on viral replication and infectivity. Alternatively, the neutralization of the viral particles by the antiviral molecules has to be fully reversible, so that viral particles that are neutralized by the stabilizer regain their infectivity upon dilution of the stabilizer in the gastrointestinal tract.

Since heat treatment of poliovirus was known to cause a parallel loss of antigenicity and infectivity (6), antigenicity determinations were initially thought to represent an easy alternative to the virus titrations involved in the estimation of biological inactivation. Therefore, a selection of 240 pyridazinamine derivatives was initially tested for the ability to reduce the loss of the N antigenicity of radiolabelled Sabin type 2 virus. Monovalent virus stocks of the commercially available Sabin vaccine (kindly provided by Smith Kline Beecham) were used as seed to produce virus stocks in HeLa cells in one or two additional passages. A protein A-aided microimmunoprecipitation technique was used to estimate the loss of N antigenicity and the simultaneous increase in H antigenicity of radioactively labelled virions after heating in the presence or absence of the antiviral compounds (11, 14). Briefly, stock solutions (10 mg/ml) of compounds were made in dimethyl sulfoxide, serially diluted in phosphate-buffered saline (PBS) (137 mM NaCl-2.7 mM KCl-9.5 mM sodium phosphate, [pH 7.2] supplemented with 0.1% bovine serum albumin), and mixed with radiolabelled virus. After incubation of the mixture for 2 h at 45°C, the MSC₅₀, defined as the concentration of compound required to protect 50% of the virus against thermodenaturation, was determined.

Thirty-six compounds proved able to stabilize at least 90% of the N antigenicity of Sabin type 2 virions. Of those, 19 compounds stabilized the Sabin type 1 and type 3 strains to the

* Corresponding author. Mailing address: Department of Virology, Janssen Research Foundation, Turnhoutseweg 30, B2340 Beerse Belgium. Phone: (32) 14-602033. Fax: (32) 14-602841. Electronic mail address: andriesk@jrf.be.

TABLE 1. Comparison of MIC₅₀s (inhibition of replication) and MSC₅₀s (stabilization for 2 h at 42°C) of selected compounds

Compound no.	MIC ₅₀ (µg/ml)			MSC ₅₀ (µg/ml)			MSC ₅₀ /MIC ₅₀ ratio		
	Sabin type 1	Sabin type 2	Sabin type 3	Sabin type 1	Sabin type 2	Sabin type 3	Sabin type 1	Sabin type 2	Sabin type 3
75761	0.012	0.001 ^a	0.006 ^a	0.01	0.05	1.80	0.8	36	281
77089	0.041	0.011 ^a	0.008 ^a	0.05	0.08	5.70	1.2	7.3	760
77805	0.080	0.033	0.009 ^a	0.02	0.30	2.00	0.3	9.1	233
77975	0.91	0.27	0.049	0.83	1.80	1.80	0.9	6.7	37
78206	0.018	0.002 ^a	0.003 ^a	0.02	0.08	0.20	0.9	41	74
79083	0.46	0.088	0.047 ^a	0.07	3.63	>10	0.2	41	>215
79654	0.97	0.034	0.028	0.26	3.31	0.50	0.3	97	18
79892	3.9	0.91	1.2	0.85	1.60	10.00	0.2	1.8	9
80619	0.75	0.14	0.16 ^a	0.72	0.33	8.70	1.0	2.3	53
80671	1.1	0.033	0.048	0.55	0.02	0.30	0.5	0.6	6
80790	0.008	0.057	0.031	0.05	0.44	5.20	6.1	7.7	168
81586	6.4	0.082	0.11 ^a	0.04	0.44	6.20	0.0062	5.4	59
81974	0.040 ^a	0.004 ^a	0.012	0.01	2.29	>10	0.3	520	>870
82461	7.0	5.8	0.93	3.20	8.50	2.50	0.5	1.5	3
83774	0.11	0.044	0.007 ^a	0.05	0.36	0.55	0.5	8.2	79
84028	0.12	0.063	0.007 ^a	0.07	0.31	0.63	0.6	4.9	86
84199	7.2	1.3	0.40	0.79	0.46	2.80	0.1	0.4	7
84736	0.25	0.36	0.034 ^a	0.32	0.40	3.20	1.3	1.1	96
88597	0.91	0.23	0.030 ^a	0.25	0.26	1.30	0.3	1.1	44

^a Irreversible virus-drug binding.

same extent. The other compounds failed to stabilize one or more Sabin strains to a sufficient degree and were barred from further testing.

For each of the 19 compounds that passed the initial screening tests, the minimal stabilizing concentration (MSC₅₀) and the minimum antiviral concentration (MIC₅₀) were determined for each of the three Sabin strains (Table 1). The MIC₅₀s, defined as the 50% inhibitory concentrations for cytopathogenicity, were determined by standard techniques (3). Of the 19 compounds, none actually had MSC₅₀s lower than the MIC₅₀s for all three Sabin strains (Table 1). The MSC₅₀s were generally well above, and quantitatively not correlated with, the MIC₅₀s (Pearson $r^2 = 0.03$). The MSC₅₀/MIC₅₀ ratios varied from 0.001 to approximately 1,000, confirming that under the tested conditions, capsid stabilization and inhibition of virus replication are two unrelated effects of capsid-binding compounds. Interestingly, the ratios were clearly higher for the Sabin type 3 strain than for the type 1 or 2 strain. This may be related to the higher thermosensitivity of Sabin type 3 (Fig. 1).

As we had to proceed with compounds that stabilized the oral poliovirus vaccine strains at concentrations that are higher than the concentrations having an effect on viral infectivity, the effect on the viral infectivity would have to be reversible by dilution to be useful as an adjuvant to oral poliovirus vaccine. The reversibility of the virus neutralizing effect on the three poliovirus serotypes was studied by using previously described methods (3). High-titered virus samples that were treated with compound and found to have a lower virus titer than the untreated controls were considered to contain virus, neutralized by the compound, in a way irreversible by dilution.

Twelve of the nineteen compounds neutralized one or more of the three Sabin strains in a way that was irreversible by dilution (Table 1). The reversibility of the binding was unpredictable. For instance, R 78206 bound irreversibly to poliovirus serotypes 2 and 3 (resulting in a loss of infectivity of about 2 log₁₀). In contrast, the binding of R 77975 was fully reversible, despite the fact that these two molecules are close chemical analogs (Fig. 2).

R 77975 (pirodavir) was finally selected for further study, as

it stabilized the three Sabin strains as well as the other remaining compounds did and because it is currently undergoing active clinical trials.

The ability of R 77975 to stabilize the infectivity and antigenicity of Sabin types 2 and 3 was tested upon incubation of virus-drug mixtures for 2 h, 1 day, 3 days, 5 days, and 1 week at 4, 25, 42, and 45°C. Virus pools (monovalent bulk vaccine passaged once in HeLa cells) at approximately 10^{7.0} infectious units per ml in PBS including 2% fetal calf serum and 0.1% Tween 80 were mixed with either 10 µg of R 77975 per ml (the maximal soluble concentration in an aqueous solution) or 1 M MgCl₂ or PBS and then incubated for 1 h to allow for optimal drug binding. Samples were then incubated in tightly stoppered plastic vials and immersed for 1 week in a water bath at the appropriate temperature. Samples from the same vials were evaluated by both antigenicity assays and infectivity assays. The infectivity assay was performed according to a World Health Organization procedure (16). Samples were diluted 1:3 in 0.666 ml of PBS, and 100 µl of each dilution was added to each of 8 wells of a 96-well microtiter plate (Nunc 1-67008A) with a freshly seeded HeLa cell suspension (12,000 cells per well). Plates were incubated in a 5% CO₂ atmosphere at 33°C for 7 days and microscopically evaluated on days 3 and 7 postinfection. Titers were calculated according to the Reed and Muench method.

At 4 and 25°C, no significant loss (<0.1 log₁₀) of infectivity or antigenicity of Sabin type 2 or 3 was observed over the 7-day observation period (results not shown). At 42°C, the infectivity of Sabin type 3 fell rapidly over the first 24 h of incubation and more slowly thereafter (Fig. 1A). R 77975 reduced the loss of infectivity to about 1.5 log₁₀ and stabilized the antigenicity almost completely. At 45°C (Fig. 1B), the infectivity and antigenicity dropped below detection within the first 24 h in the absence and after 7 days in the presence of R 77975. MgCl₂ at a concentration of 1 mM was equipotent in its effects on viral infectivity. Unfortunately, the loss of antigenicity in the presence of MgCl₂ could not be assessed because of the effects of MgCl₂ on virus aggregation. The rate of loss of native antigenicity in the presence of R 77975 was again much slower than the loss in infectivity. Similar results were obtained for the

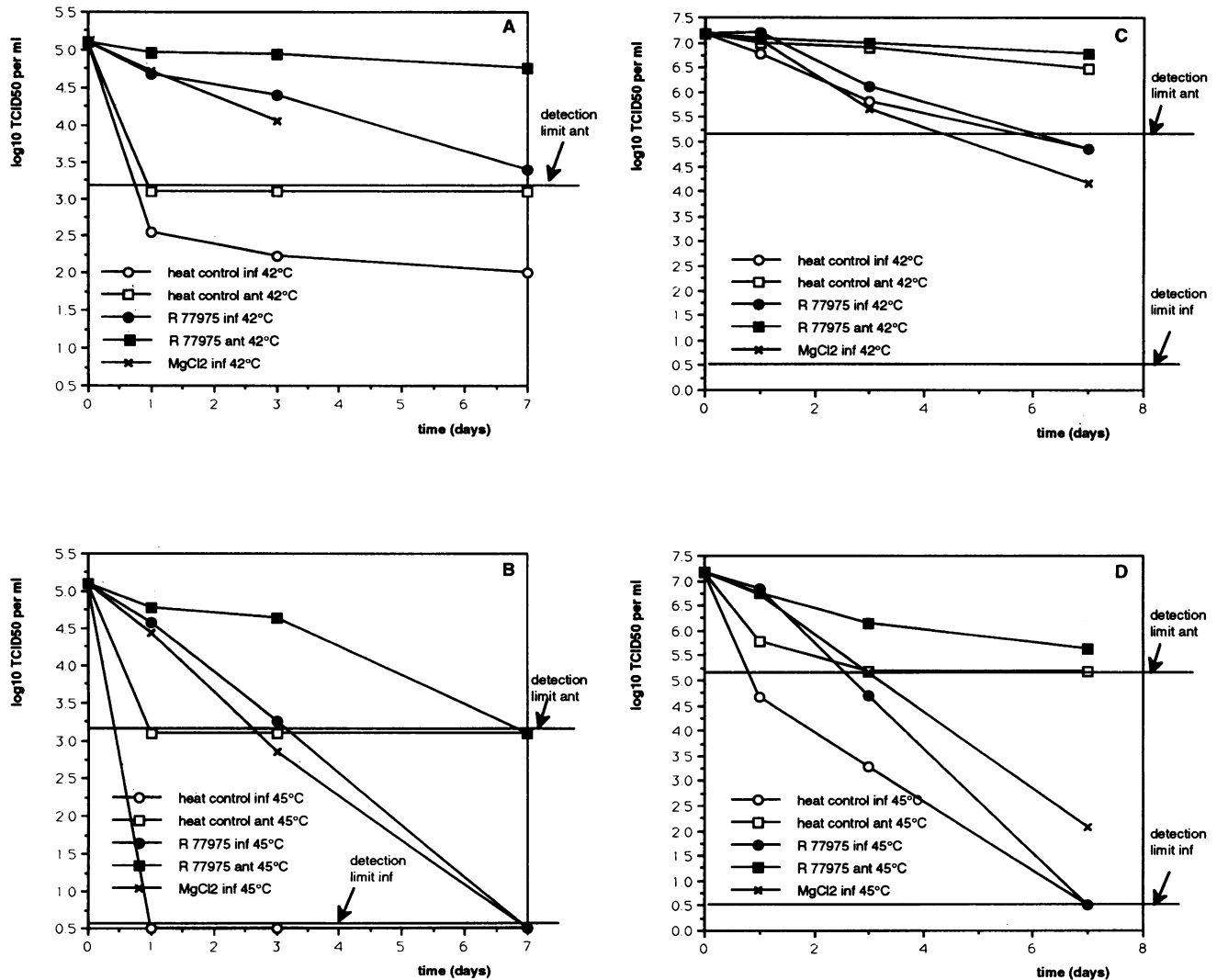


FIG. 1. Infectivity (inf) and antigenicity (ant) assays of poliovirus type 3 at 42°C (A), type 3 at 45°C (B), type 2 at 42°C (C), and type 2 at 45°C (D).

Sabin type 2 strain (Fig. 1C and D), except for the fact that Sabin type 2 itself was less thermolabile than Sabin type 3. Six additional compounds that exhibited reversible inhibition (Table 1) were tested for the ability to stabilize the antigenicity of the Sabin type 3 strain at 42°C for 3 days and proved not to be superior to R 77975 in their stabilizing properties (results not shown).

The observed discrepancy between the residual infectivity and antigenicity was unsuspected. Many viral particles were

stabilized in their native antigenic composition by R 77975, even though they had lost their infectivity. The cause of this discrepancy between infectivity and antigenicity is the subject of further study. By its nature, this discrepancy limits the possible use of R 77975 to the stabilization of inactivated poliovirus vaccines.

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REFERENCES

1. Andries, K., B. Dewindt, M. De Brabander, R. Stokbroeckx, and P. A. J. Janssen. 1988. In vitro activity of R 61837, a new antirhinovirus compound. *Arch. Virol.* **101**:155-167.
2. Andries, K., B. Dewindt, J. Snoeks, and R. Willebrords. 1989. Lack of quantitative correlation between inhibition of replication of rhinoviruses by an antiviral drug and their stabilization. *Arch. Virol.* **106**:51-61.

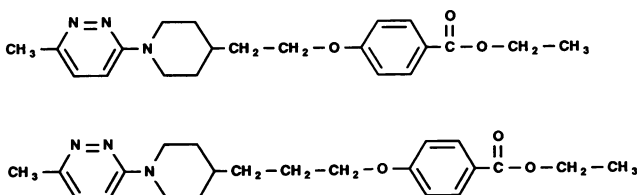


FIG. 2. Chemical structures of R 77975 (top) and R 78206 (bottom).

3. **Andries, K., B. Dewindt, J. Snoeks, R. Willebrords, K. Van Eemeren, R. Stokbroekx, and P. A. J. Janssen.** 1992. In vitro activity of pirodavir (R 77975), a substituted phenoxy-pyridazinamine with broad-spectrum antipicornaviral activity. *Antimicrob. Agents Chemother.* **36**:100–107.
4. **Bauer, D. J., J. W. T. Selway, J. F. Batchelor, M. Tisdale, I. C. Caldwell, and D. A. B. Young.** 1981. 4',6-Dichloroflavan (BW683C), a new anti-rhinovirus compound. *Nature (London)* **292**:369–370.
5. **Chapman, M. S., I. Minor, M. G. Rossmann, G. D. Diana, and K. Andries.** 1991. Human rhinovirus 14 complexed with antiviral compound R 61837. *J. Mol. Biol.* **217**:454–463.
6. **Hinuma, Y., S. Katagiri, M. Fukuda, K. Fukushi, and Y. Watanabe.** 1965. Kinetic studies on the thermal degradation of purified poliovirus. *Biken J.* **8**:143–145.
7. **Ishitsuka, H., Y. N. Ninomiya, C. Ohsawa, M. Fujitsu, and Y. Suhura.** 1982. Direct and specific inactivation of rhinovirus by chalcone Ro 09-410. *Antimicrob. Agents Chemother.* **22**:617–621.
8. **Kenny, M. T., J. K. Dulworth, T. M. Bargar, H. L. Torney, M. C. Graham, and A. M. Manelli.** 1986. In vitro antiviral activity of the 6-substituted 2-(3',4'-dichlorophenoxy)-2H-pyrano[2,3-*b*]pyridines MDL 20,610, MDL 20,646, and MDL 20,957. *Antimicrob. Agents Chemother.* **30**:516–518.
9. **Moeremans, M., M. De Raeymaeker, G. Daneels, M. De Brabander, F. Aerts, C. Janssen, and K. Andries.** 1992. Study of the binding parameters of R 61837 to HRV9 and immunobiochemical evidence for a capsid stabilizing activity of the compound. *Antimicrob. Agents Chemother.* **36**:417–424.
10. **Otto, M. J., M. P. Fox, M. J. Fancher, M. F. Kuhrt, G. D. Diana, and M. A. McKinlay.** 1985. In vitro activity of WIN 51711, a new broad-spectrum antipicornavirus drug. *Antimicrob. Agents Chemother.* **27**:883–886.
11. **Rombaut, B., K. Andries, and A. Boeyé.** 1991. A comparison of WIN 51711 and R 78206 as stabilizers of poliovirus virions and procapsids. *J. Gen. Virol.* **72**:2153–2157.
12. **Smith, T. J., M. J. Kremer, M. Lou, G. Vriend, E. Arnold, G. Kamer, M. G. Rossmann, M. A. McKinlay, G. D. Diana, and M. J. Otto.** 1986. The site of attachment in human rhinovirus 14 for antiviral agents that inhibit uncoating. *Science* **233**:1286–1293.
13. **Svehag, S. E.** 1963. Reactivation of neutralized virus by fluorocarbon: mechanism of action and demonstration of reduced reactivity with time of virus-antibody interaction. *Virology* **21**:174–182.
14. **Vrijnsen, R., B. Rombaut, and A. Boeyé.** 1983. A simple quantitative protein A micro-immunoprecipitation method; assay of antibodies to the N and H antigens of poliovirus. *J. Immunol. Methods* **59**:217–220.
15. **Woods, M. G., G. D. Diana, M. C. Rogge, M. J. Otto, F. J. Dutko, and M. A. McKinlay.** 1989. In vitro and in vivo activities of WIN 54954, a new broad-spectrum antipicornavirus drug. *Antimicrob. Agents Chemother.* **33**:2069–2074.
16. **World Health Organization.** 1990. Annex 1, appendix 2, assay method for the determination of the virus content of poliomyelitis vaccine (oral). WHO Technical Report Series no. 800. World Health Organization, Geneva.