den). The samples were applied on the columns under a layer of elution buffer. The effluent from the column was monitored at 2537 Å by LKB Uvicord and 10 min fractions were collected by LKB RadiRac fraction collector directly into the γ-counting vials. All operations were carried out at 4 °C. The column fractions were assayed for ^{109}Cd by a dual channel Nuclear Chicago Spectrometer with an efficiency of 51%. Cd-BP containing fractions were pooled and freeze-dried using VirTis freeze-drier. Polyacrylamide gels were prepared according to the manufacturer's instruction manual (Canalco, USA). Proteins were quantitated by LOWRY's method[11]. Radioactive cadmium-109 was purchased as $^{109}CdCl_2$ carrier free, with radiochemical purity better than 99% from NEN Corporation, Boston, Massachusetts.

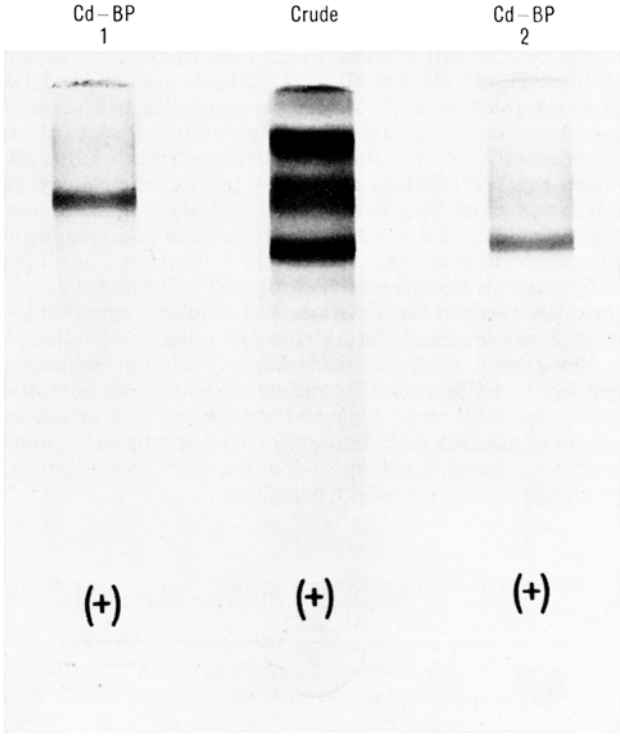

Fig. 4. Disc electrophoresis of Cd-BP (rat liver). Electrophoresis was carried out at room temperature in 7% polyacrylamide gel, using pH 9.5 *Tris*-glycine buffer. The applied current was 5 mA/column. Proteins were fixed in 12% TCA and stained with Coomassie blue.

Results and discussion. The Cd-BP were first induced in rat by s.c. injection of $CdCl_2$[5]. Liver tissue was homogenized and fractionated as described in Figure 1. Figure 2 shows the elution pattern of the soluble fraction on Sephadex G-75 column. Almost all ^{109}Cd was associated with a low molecular weight UV-light absorbing protein peak. The total recovery of ^{109}Cd from the column was better than 98%. Crude Cd-BP containing fractions were pooled and freeze-dried. Salts were removed by gel filtration on a Sephadex G-25 column (2.5 × 50 cm) and eluted with distilled water. Over 95% of the applied protein and ^{109}Cd were recovered in the void volume of the column. The desalted protein fractions were freeze-dried and kept in the dessicated state until further use. About 5 mg of crude protein was dissolved in 1 ml of 0.001 *M Tris* buffer and applied on DEAE-Sephadex column. The proteins were eluted by *Tris*-HCl concentration gradient formed by mixing 0.25 *M Tris* buffer (12 ml/h) with 100 ml of the starting buffer. As shown in Figure 3 two major UV-light absorbing protein peaks containing ^{109}Cd and numbered as Cd-BP 1 and Cd-BP 2 were isolated. Homogeneity of these proteins was verified by disc electrophoresis. As shown in Figure 4, Cd-BP 1 and Cd-BP 2 migrated as single bands having similar mobilities to those separated from the crude protein. To locate the position of ^{109}Cd, unstained gels were sliced into small discs 1 mm in thickness and counted in the γ-spectrometer. Radioactivity was present in the protein staining region.

The procedure described above is useful for analytical purposes, but it can be easily adopted for the preparative scale by appropriate adjustments in the column dimensions. The treatments given to the macromolecules are relatively mild and the principles involved in the isolation procedure can be utilized for the isolation of other metal binding proteins.

Résumé. Un procédé pour l'isolation des protéines combinées au Cd radioactif (^{109}Cd) a été mis au point. La méthode ne détruit pas les protéines. D'autres protéines ayant des affinités pour les métaux lourds pourront ainsi être isolées.

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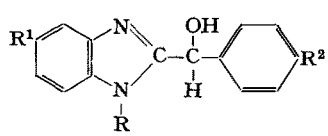
The High Activity of Fluorohydroxybenzylbenzimidazoles Against Some Small RNA-Viruses

O'SULLIVAN, LUDLOW, PANTIC and WALLIS[1] reported that the introduction of a 5-fluoro substituent into 2-(α-hydroxybenzyl)benzimidazole (HBB; Ia) produced a notable increase in protective activity towards tissue cultures infected with poliovirus. Information is now given on this fluoro derivative (5-FHBB; Ib) and on two new highly active fluoro compounds, 2-(α-hydroxy-*p*-fluorobenzyl)benzimidazole (HFBB; Ic) and 5-fluoro-2-(α-hydroxybenzyl)-1-propylbenzimidazole (FPHBB; Id). The latter is by far the most active of all the antipicornaviral benzimidazoles hitherto investigated.



Ia; R = R^1 = R^2 = H
Ib; R = R^2 = H, R^1 = F
Ic; R = R^1 = H, R^2 = F
Id; R = Pr, R^1 = F, R^2 = H
Ie; R = Pr, R^1 = R^2 = H

The compounds were tested with polioviruses 1, 2 and 3 and coxsackievirus A21 in ERK (human) cell line monolayers and with coxsackievirus A9 in secondary MK monolayers. The ERK cells, on subculturing, were grown in slowly revolving tubes at 37 °C for 3 days in

Eagle's basal medium (1 ml) containing calf serum (10%) and $NaHCO_3$ (0.088% w/v) and then in fresh medium (2 ml) containing serum (10%), $NaHCO_3$ (0.176%) and, if desired, virus and compound. In determinations of inhibition of viral multiplication[2], $NaHCO_3$ (0.176%) was present throughout, but serum was absent during the 16 h viral multiplication period and only 5% was present in the subsequent viral titrations. The MK cells were grown for 1 day in Eagle's basal medium (0.5 ml) containing foetal bovine serum (10%) and $NaHCO_3$ (0.044%) and for 4 days in Eagle's basal medium (2 ml) containing foetal bovine serum (2%) and $NaHCO_3$ (0.176%). This was then replaced by similar medium containing virus and compound. Benzyl-penicillin (100 U/ml) and streptomycin (100 µg/ml) were present in all cases.

Toxicities. ERK cells were grown in serial dilutions of each compound. The maximum tolerated concentration (MTC; Table I) was the highest concentration having no visual effect on cell morphology or multiplication for up to $4^1/_2$ days. Compounds were less toxic to MK than to ERK cells.

Inhibition of cytopathic effect (CPE). Cultures were simultaneously infected with a range of virus concentrations and treated with compounds at appropriate fractions of their (ERK cell) MTC's, HBB (Ia) at 50% MTC and PHBB (Ie) at $12^1/_2$% MTC were included as standards. Time for the 50% CPE end-point of each tube was noted. Data from one of the poliovirus 1, 2 and 3 and coxsackievirus A9 concentrations are portrayed in Table I and from 4 coxsackie A21 concentrations in Table II.

Inhibition of viral multiplication. Cells, infected with 400 TCD_{50} units, were incubated with a range of compound concentrations for 16 h. After repeated freezing and thawing, TCD_{50}'s were determined. The increase in virus concentration over that of cell-free controls was plotted against compound concentration and the compound concentration giving 75% inhibition of viral replication thus obtained (Table III).

Activities. The MTC's of the fluoro derivatives were only 6 to 38% less than those of their non-fluorinated analogues (Table I). However, the presence of the fluorine substituent markedly increased both the protection of cells against viral CPE (Tables I and II) and the inhibition of viral multiplication (Table III). In the experiment of Table II, 40 µ*M* PHBB gave little, and 105 µ*M* HBB gave no inhibition of CPE at any of the tested viral concentrations. The inhibition of poliovirus 2 replication illustrates the effect of the new substituent; thus Table III shows that FPHBB has 50 times the molar activity of HBB and over 10 times that of PHBB. Hitherto, the latter was amongst the most active of the selective anti-picornaviral agents[2,3].

At concentrations up to 35 µ*M*, FPHBB did not accelerate the thermal inactivation that occurred when poliovirus 2 was incubated at 37 °C in the absence of cells.

Preparation of the o-nitroanilines and o-phenylenediamines. 1. 4-Fluoro-2-nitroaniline, m.p. 93–94°, was obtained in 48% yield from *p*-fluoroaniline by nitration using copper nitrate[4]. Reduction with zinc and sodium hydroxide gave the 4-fluorodiamine (47% yield), m.p. 93–94° (from benzene-light petroleum)[5].

Table I. Protection afforded by compounds illustrated by percentage remission times for infected cells[a]

Compound	Tested µ-molarity	MTC[b] (µ*M*)	Virus Polio 1[b]	Polio 2[b]	Polio 3[b]	Coxsackie A9[c]
HBB (Ia)	105	210	0	40	0	50
PHBB (Ie)	10	80	7	47	7	64
HFBB (Ic)	65	130	7	60	7	86
5-FHBB (Ib)	40	160	7	53	7	86
FPHBB (Id)	80	–	10	73	10	100
	9.4	75	89	100	40	100
	18.8	–	100	100	53	100
	37.5	–	100	100	100	100

[a] Defined by $100\,\Delta t/\Delta t_0$, where Δt is the time interval between 50% CPE for compound-treated infected cells and for the corresponding untreated infected controls and Δt_0 is the difference in time between 50% cell death of non-infected and 50% CPE of infected controls. Cell life of non-infected and infected cells were 4.5 and 0.75 days from start of experiment (virus inoculations were approximately 10^5 TCD_{50} units). [b] In ERK cells. [c] In MK cells.

Table II. Protective effect of FPHBB (Id) against coxsackie A21 infection of ERK cells[a]

FPHBB concentration (µ*M*)	Virus inocula ($log_{10}TCD_{50}$ units per tube) 5.4	4.4	3.4	2.4
0	0 (12[b])	0 (30[b])	0 (48[b])	0 (66[b])
9.4	0	8	10	14
18.8	6	15	20	43
37.5	13	23	50	100

[a] Given as percentage remission times (see footnote[a] to Table I). [b] Time in h to reach 50% CPE in controls (uninfected controls were 50% dead $4^1/_2$ days from the start of the experiment).

Table III. Micromolarities giving 75% inhibition in viral multiplication

Compound	Virus[a] Polio 1	Polio 2	Polio 3	Coxsackie A21
HBB (Ia)	160	35	160	
PHBB (Ie)	9	7.5	22.5	
HFBB (Ic)	35	10.5	36	
5-FHBB (Ib)	30	10	25	
FPHBB (Id)	2	0.7	1.5	15

[a] Poliovirus strains used in these present studies were L Sc 2 ab, P 712 Ch 2 ab, and Leon 12 ab.

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2. Propylamine (0.2 mole) was added to 2,5-difluoronitrobenzene (0.08 mole) in ethanol (24 ml). Crystals separated on standing, which formed red plates (from ethanol) of 4-fluoro-2-nitro-N-propylaniline (89.5% yield), m.p. 65–66° (Anal.-Found: C, 54.5; H, 5.50; N, 14.0. $C_9H_{11}FN_2O_2$ requires C, 54.5; H, 5.59; N, 14.1%).

This product (0.017 mole) was mixed with granulated tin (3.8 g), concentrated hydrochloric acid (9.0 ml) was slowly added, and then the mixture was warmed to 100 °C for 40 min. After cooling, it was made alkaline, extracted with ether and the combined ethereal extracts extracted with *M* hydrochloric acid (total of 50 ml). Dissolved ether was removed and the combined acidic extracts (containing the diamine) were immediately condensed with mandelic acid.

Preparation of the benzimidazole derivatives. 1. *o*-Phenylenediamine (0.01 mole), *p*-fluoromandelic acid (0.015 mole) and *M* hydrochloric acid (25 ml) were heated under reflux for 6 h. The reaction mixture was cooled, treated with concentrated ammonium hydroxide and the precipitate collected. The latter gave 2-(α-hydroxy-*p*-fluorobenzyl)-benzimidazole (HFBB) (75.4% yield) as microcrystals from benzene, m.p. 105–106°; *M* (mass-spectrum), 242; τ [Me_2SO] −2.20br (1H, NH), 2.4–2.6 (4H, m, aromatic), 2.7–2.9 (4H, m, aromatic), 3.46 (1H, d, *J* 4Hz, OH), 4.06 (1H, d, *J* 4Hz, CH-Ph). The hydrochloride crystallized from ethanol-ether as prisms, m.p. 285° (dec.) (Anal.-Found: C, 60.2; H, 4.43; N, 10.1. $C_{14}H_{12}ClFN_2O$ requires C, 60.3; H, 4.34; N, 10.1%).

2. 5-Fluoro-2-(α-hydroxybenzyl)benzimidazole (5-FHBB) was obtained in a similar manner from 4-fluoro-*o*-phenylenediamine (1 mole) and mandelic acid (1.3 mole), but in 33.3% yield. The hydrochloride separated from the reaction mixture and was collected, dissolved in water, and the solution treated with charcoal. After addition of concentrated ammonium hydroxide, the precipitated base was crystallized from aqueous methanol as plates, m.p. 207.5–208.5° (Anal.-Found: C, 69.2; H, 4.59; N, 11.5; *M* [mass-spectrum], 242. $C_{14}H_{11}FN_2O$ requires C, 69.4; H, 4.58; N, 11.6%; *M*, 242); τ [Me_2SO] −2.45 br (1H, NH), 2.4–2.8 (7H, m, aromatic), 3.03 (1H, td, *J* 9 and 3Hz, H-6), 3.47 br (1H, s, OH), 4.07 (1H, s, CH-Ph).

3. 5-Fluoro-1-propyl-2-(α-hydroxybenzyl)benzimidazole (FPHBB) was obtained from 2-amino-4-fluoro-1-propylamine (1 mole) and mandelic acid (1 mole) using the procedure described for the *p*-fluoro derivative. The yield was 45.5% from the nitropropylaniline. The 5-fluoro-1-propyl compound crystallized as plates from aqueous methanol, m.p. 169–169.5° (Anal.-Found: C, 72.1; H, 5.90; N, 10.0. $C_{17}H_{17}FN_2O$ requires C, 71.8; H, 6.03; N, 9.9%).

Clearly FPHBB is an outstanding compound of unrivalled inhibiting activity and comparatively low cytotoxicity. Its potential is enhanced by its accessibility from a 3-stage synthesis with an overall yield of 40.7%. We are continuing our study of the spectrum of antiviral activity of these fluoro compounds, their mode of action and the possible development of resistant virus strains.

Zusammenfassung. Fluorierte Derivate des 2-(α-Oxybenzyl)-benzimidazol hemmen die Vermehrung des Poliovirus, 1, 2 und 3, sowie diejenige des Coxsackievirus A9 und A21.

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The Major Carotenoid Pigments of Six Species of Barnacle

In the blue oceanic barnacle *Lepas fascicularis* a preponderance of astaxanthin has been found, associated with the characteristic blue carotenoprotein[1,2]. The littoral stalked species *Pollicipes polymerus* also contains large amounts of astaxanthin and its esters, although lutein is the second major pigment, rather than zeaxanthin as in *L. fascicularis*[3]. In contrast the parasitic rhizocephalan species *Sacculina carcini* only contains β-carotene[4]. Three further stalked species, in addition to more specimens of *L. fascicularis*, and 2 sessile species from very different environments have been investigated.

Lepas anatifera, L. fascicularis and *L. pectinata* were obtained during cruises of RRS Discovery in the eastern north Atlantic in 1967, 1968 and 1969 and *Conchoderma virgatum* were removed from the ships hull at the same time. All other animals were deep-frozen at −20 °C on board ship until they could be transported back to the laboratory for analysis. *Chthamalus fragilis* were kindly obtained by Dr. P. Bacon of the University of the West Indies and flown live to England and *Elminius modestus* were grown on settling plates at the Admiralty exposure trials station, Portsmouth, through the courtesy of Mr. D. Houghton.

It proved impracticable to remove gut contents before the pigments were extracted, but the sessile species were scrubbed before removal from their settlement sites in order to remove any adventitious plant material on the external surfaces of their plates. All pigments were extracted, identified, and quantitative determinations made, as described earlier[5]. *Chthamalus* and *Elminius* extracts were saponified before separation in order to remove relatively large amounts of lipid and some chlorophyll products.

The results of the analysis of the various species are given in the Table. Although the stalked species have a carotenoid content some 10 times that of the sessile species when calculated on a fresh weight basis the values would probably be much more nearly equivalent were the weights of the animals' calcareous plates not included. The content of astaxanthin in *L. fascicularis* is rather lower than in the earlier analysis[2], and this is almost certainly correlated with the almost total absence of blue somatic coloration and blue eggs in the animals used in the present investigation. There is a consequent relative increase in the content of zeaxanthin and its esters, and there is a significant proportion of fucoxanthin.

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