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DERIVATIVES OF BENZO(C)FLUORENE. VI. ANTIVIRAL AND INTERFERON-INDUCING ACTIVITIES OF THREE BENZO(C)FLUORENONE DERIVATIVES IN MICE

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Summary. — The antiviral effect of 3 derivatives of benzo(c)fluorenone against encephalomyocarditis (EMC) and vaccinia viruses in mice was compared with that of tilorone hydrochloride (THCl). All 3 derivatives were effective against either virus following single-dose oral application as well as after 4-times repeated subcutaneous (s.c.) treatment, but following oral administration only the VÚFB 14162 derivative exerted an antiviral effect corresponding to that of THCl. After s.c. application, however, VÚFB 14162 derivative was less toxic than THCl. Two benzo-(c)fluorenone derivatives, namely VÚFB 14162 and 13431, induced in mouse sera the levels of interferon (IFN), which production kinetics and hyporeactivity phenomenon were comparable with those induced by THCl. Because no IFN was found following administration of the third VÚFB 13371 derivative, its antiviral effect consisted probably in an other than IFN-inducing mechanism.

Key words: benzo(c)fluorenone derivatives; antiviral effects; mice; interferon induction; hyporeactivity phenomenon

Introduction

Tilorone (bis-diethylaminoethyl-fluorenone) represents the first synthetic compound inducing in mice high levels of IFN (Krueger and Mayer, 1970; Mayer and Krueger, 1970). It was found effective against many viruses: Semliki forest, vesicular stomatitis, Mengo, herpes simplex, influenza 1/Prague/1/56 (H7 N7), influenza A/Jap/305/57 (H2N2) and influenza B(Massachusetts) viruses (Andrews *et al.*, 1974). Tilorone induces IFN both after oral and s.c. applications, its serum levels preserving for 18 hr (Stringfellow and Glasgow, 1972). Then follows a sudden drop in IFN titres to almost undetectable levels. The second dose of tilorone administered in an interval shorter than 7—8 days is not followed by an increase in IFN titres due to the so-called tolerance or hyporeactivity phenomenon to IFN induction.

The disadvantage of tilorone in therapy of human virus infections is its toxicity. Repeated administration of tilorone in doses of 1,000 mg per day

were accompanied by adverse effects on central nervous system and by gastrointestinal complaints. To obtain a compound inducing high levels of IFN, but at the same time being of a low toxicity, the tilorone structure was widely modified. The whole spectrum of bis-basic esters of 2,7-dicarboxylic acids derived from fluorene (Sill *et al.*, 1973) 9-fluorenol and 9-fluorenone (Albrecht *et al.*, 1974; Andrews *et al.*, 1974) was investigated and in each group effective antiviral compounds were found.

By analogy with IFN inducers, compounds with the structure of benzo(c)-fluorene were synthesized on the basis of fluorenone derivatives substituted in different positions of aromatic skeleton. Some of them exerted certain effects against vaccinia, EMC and influenza A Sing/57 (H2N2) (Křepelka *et al.*, 1982*a*) and antitumour activity in animals (Křepelka *et al.*, 1982*b*; Pujman and Černochová, 1981). To obtain a compound with higher antiviral activity, derivatives of benzo(c)fluorenone were synthesized containing in their molecules two basic substituents bound etherically in the position 5 and esterically in the position 6 (Vančurová *et al.*, 1982).

The purpose of this study was to investigate antiviral effects of 3 chosen benzo(c)fluorenone derivatives on the infection with EMC and vaccinia viruses in mice, to compare them with those of THCl, to find out their IFN-inducing ability as well as the kinetics of IFN production and IFN hyporeactivity phenomenon.

Materials and Methods

Viruses. EMC virus from the virus collection of the Institute of Virology, Slovak Academy of Sciences in Bratislava was kindly provided by Dr. V. Lackovič. For experiments in mice, the virus originally passaged in human embryo lung cells was adapted for growth in mouse brain. Vaccinia virus was purchased as a lyophilized vaccine from SEVAC Prague (5×10^7 virus infectious units per ml).

Mice. In experiments with EMC and vaccinia viruses SPF white outbred mise from the breed VELAZ (farm Černý Vůl) weighing 12 and 15 g, respectively, were employed. IFN production and hyporeactivity were investigated on females weighing 18—20 g.

Compounds and their application. The derivatives of benzo(c)-fluorenone were as follows: VÚFB 13371 — 3,9-diethyl-5-[2-(diethylamino)ethoxy]-6-[2-diethylamino)ethoxycarbonyl-7-oxo--7H-benzo(c)fluorene dihydrochloride; VÚFB 13431 — 5-[2-(diethylamino)ethoxy]-6-[2-(diethylamino)ethoxycarbonyl]-7-oxo-7H-benzo(c)fluorene dihydrochloride; VÚFB 14162 — 5-[2-(dimethalamino)ethoxy]-6-[2-(dimethylamino)ethoxycarbonyl]-7-oxo-7H-benzo(c)fluorene. VÚFB 14162 — 5-[2-dimethylamino)ethoxy]-6-(2-)dimethylamino(ethoxycarbonyl)-7-oxo-benzo(c)fluorenone. The antiviral preparation tilorone VÚFB — 2,7-bis(2-diethyl-aminoethoxy)-9-fluorenone dihydrochloride was used as positive control. All compounds were dissolved in distilled water or suspended by means of Tween-80. The compounds were applied either orally using a cannula or by s.c. injection into the back skin fold in 0.2 ml volumes. The treatment doses were calculated from their molecular masses and related to the molecular mass of VÚFB tilorone which served as positive control. At oral treatment a single dose of the compound was applied 24 hr before virus infection; s.c. treatment was performed in 4 doses by 28, 22 and 2 hr before and at 2 hr after virus infection.

Virus infection of animals and evaluation of antiviral effectiveness of the compounds. EMC virus was applied s.c. into the back skin in 0.2 ml volumes. The LD_{50} value was determined in a preliminary experiment and calculated according to Reed and Muench (1938). The first signs of disease were tremor, then pareses of hind legs and later also of front extremities appeared. Mice died from day 3 to 7 post infection (p.i.), depending on the virus dose applied. Vaccinia virus was administered intravenously according to the method of Boyle *et al.* (1966). In 0.1 ml volumes were given 2.5×10^4 or 1.25×10^5 PFU causing 30 and 100 necrotic lesions on the mouse tail on days 6—8 p.i. To facilitate the counting of lesions, the tails were stained with a solut ion

Table 1. Effect of a single-dose (250 mg/kg) oral treatment with 3 benzo(c)fluorenone derivatives in EMC virus-infected mice

VÚFB derivative	Virus dose		
	5 LD_{50}	50 LD_{50}	500 LD_{50}
13371	89*	91	70
13431	100	100	59
14162	100	107	103

* Mouse survival expressed in per cent antiviral activity of THCl

containing 1% fluorescein and 0.5% methylene blue. In untreated control animals the vaccinal skin lesions healed spontaneously and disappeared within 14 days p. i. The development of disease did not lead to death of animals. The antiviral effectiveness of each compound was tested on a group of 10 mice; each experiment was repeated three times. The antiviral effect in EMC virus infection was expressed as the survival of treated and untreated mice (in days), while in vaccinia virus infection in per cents of inhibition of necrotic lesions on the mouse tail. In either case the effect was related to antiviral activity of tilorone.

IFN induction, assay, and hyporeactivity investigation. Groups of 8 mice each were given orally 0.2 ml volumes of solution or suspension of the tested compound in an amount of a 250 mg/kg body mass. After 24 hr the mice were bled and sterile serum was tested for IFN activity on L929 cells kindly supplied by Dr. Šnejdar from the Military Institute of Hygiene, Epidemiology and Microbiology (Prague). The cells were grown in minimal essential medium (MEM)-1959 supplemented with 5% calf serum (ÚSOL Prague) containing 100 IU of penicillin and 100 μg of streptomycin per ml. Double-fold dilutions from 1 : 25 to 1 : 800 of sera in MEM were added to the tube cultures of L-929 cells, which were then incubated overnight at 37 °C. Thereafter the inoculum was removed, the cells were washed and exposed to 1 ml of MEM containing 10—100 $TCID_{50}$ of EMC virus. Cytopathic effect (CPE) was read at 24 and 48 hr after virus infection. IFN titre was expressed as the reciprocal of serum dilution inhibiting the CPE of EMC virus by 50%. IFN hyporeactivity was investigated in the groups of 8 mice each, which were given daily for 10 consecutive days 250 mg/kg body mass of the compound tested and from which the serum was collected at 24 hr intervals. The values of IFN obtained were graphically depicted.

Table 2. Effect of repeated s.c. application of 3 benzo(c)fluorenone derivatives on EMC virus infection in mice

VÚFB derivatives	Virus dose		
	10 LD_{50}	100 LD_{50}	100 LD_{50}
13371	100*	100*	256**
13431	82	90	toxic
14162	100	100	266

* Expressed in per cents as related to the values in THCl-treated mice.
** Per cents related to untreated (control) mice.

Table 3. Effect of oral and s.c. treatment with 3 benzo(c)fluorenone derivatives on vaccinia virus-infected mice

VÚFB derivative	Virus dose 1.25×10^5 PFU				
	Oral treatment			S.c. treatment	
	50	100	250	25	50
13371	42*	57	80	59	74
13431	54	80	96	13	73
14162	80	96	97	70	96
tilorone HCl	80	94	99	70	97

* Per cent inhibition of vaccinia virus-induced necrotic lesions

Results

Effectiveness of benzo(c)fluorenone derivatives against EMC virus after single-dose oral treatment

As shown in Table 1, all benzo(c)fluorenone derivatives under study were effective against EMC virus when applied orally in a single dose of 250 mg/kg body mass. The most effective was VÚFB 14162 derivative, the antiviral activity of which against 5, 50 and 500 LD_{50} of EMC virus was as high as that of control tilorone preparation. The antiviral effectiveness of VÚFB 13431 derivative was lower. It corresponded to the antiviral effect of tilorone against 5 and 50 LD_{50} of EMC virus, but it was lower by 41% against 500 LD_{50} of the virus. The least effective was the VÚFB 13371 derivative; its antiviral activity compared to that of tilorone was lower by 11%, 9% and 30% against 5, 20 and 500 LD_{50} of EMC virus, respectively.

Effectiveness of s.c. treatment with benzo(c)fluorenone derivatives against EMC virus

When treating mice s.c. with four 50 mg/kg doses of benzo(c)fluorenone derivatives at intervals of 28, 22 and 2 hr before and 2 hr after virus infection, the effectiveness of VÚFB 13371 and 14162 derivatives against 10 and 100 LD_{50} of EMC virus was the same as antiviral activity of tilorone (Table 2). At the same time, VÚFB 13431 derivative was less effective: its antiviral activity against 10 and 100 LD_{50} of EMC virus reached 82% and 90.6%, respectively, of that of the control tilorone preparation. To find out the maximum effectiveness of compounds tested as well as of the control tilorone preparation, groups of mice were treated s.c. 4-times with the 100 mg/kg dose of each drug. Tilorone and VÚFB 13431 derivative were found toxic in this concentration, causing deaths of all mice. By contrast, all mice survived following the treatment with the same concentrations of VÚFB 13371 and 14162 derivatives, proving their non-toxicity. Because the results of mice

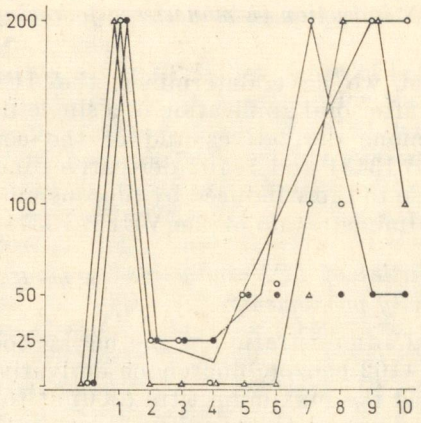

Fig. 1.
IFN production, period of tolerance and reaction to further IFN stimulus in mouse sera after administration of benzo(c)-fluorenone derivates
Compounds designation:
○———○ VÚFB Tilorone HCl
●———● VÚFB 13431 derivative
△———△ VÚFB 14162 derivative
The compounds were applied orally in a dose of 250 mg/kg daily in the course of 10 consecutive days starting from 0 hr.
Abscissa: number of days.
Ordinate: IFN titre.

survival could not be related to the group of tilorone-treated mice, they had to be expressed as related to survival in the control group of untreated but virus-infected mice. Thus, 4-times repeated s.c. treatment with 100 mg/kg body mass of VÚFB 14162 derivative prolonged mean survival time of mice (in days) by 266% and that of VÚFB 13371 derivative by 256%.

Effectiveness of benzo(c)fluorenone derivatives against vaccinia virus following oral treatment

The most effective against vaccinia virus following a single-dose oral treatment was the VÚFB 14162 derivative, which used in concentrations of 50, 100 and 250 mg/kg body mass inhibited the proportion of necrotic tail lesions at a rate similar to that of the tilorone preparation, so that its antiviral activity against vaccinia virus corresponded to that of tilorone (Table 3). Though the second most effective VÚFB 13431 derivative inhibited 80% of vaccinal tail lesions following a single-dose treatment with 100 mg/kg drug, its antiviral activity reached only one half of that of tilorone inhibiting 80% of vaccinal lesions after the treatment with a 50 mg/kg dose. Least effective was the VÚFB 13 371 derivative, which inhibited 80% of vaccinal lesions in the highest used concentration of 250 mg/kg body mass, so that its antiviral activity was 5-fold lower than that of tilorone.

Effectiveness of s.c. treatment with benzo(c)fluorenone against vaccinia virus

As follows from Table 3, the most effective derivative against vaccinia virus after s.c. treatment as by oral application was VÚFB 14162, which in the concentration of 25 mg/kg inhibited the same proportion (70%) of vaccinal lesions as tilorone did. VÚFB 13371 and 13431 derivatives were 2-fold less effective, since they inhibited 70% of vaccinal lesions only at the dose of 50 mg/kg.

IFN induction in mouse sera following oral administration of the compounds tested

First we have determined the IFN levels in mouse sera collected at 24 hr after oral application of a single-dose of 250 mg/kg of all three bento(c)-fluorenone derivatives and of the control tilorone preparation. Whereas VÚFB 13431 and 14162 derivatives induced in mouse sera IFN levels comparable to those induced by tilorone, no IFN could be demonstrated following the administration of the VÚFB 13371 derivative.

Kinetics of IFN induction in mouse sera and demonstration of the hyporeactivity phenomenon

Oral administration of 250 mg/kg body mass of tilorone and VÚFB 13431 and 14162 benzo(c)fluorenone derivatives induced IFN in mouse sera which reached the maximum titre (200) at the 24 hr interval (Fig. 1). At 48 hr its levels dropped to the minimal values (titres up to 25). Further application of the drugs at consecutive days did not markedly increase the IFN titres which varied from 0 to 50. The hyporeactivity observed corresponded to data known of IFN induction by tilorone. Significant increase in IFN titres (comparable to original values of 200 at 24 hr interval) was noticed on day 9 in the case of tilorone and on days 7—8 in the case of VÚFB 13431 and 14162 benzo(c)fluorenone derivatives, respectively.

Discussion

Krueger and Mayer (1970) found that dihydrochloride bis-(3-dibutyl-aminopropyl)-9-fluorenone-2,7-dicarboxylate prevented mice from the lethal infection with EMC virus. Changing the substituents on 9-fluorenone core led to the discovery of tilorone.

Marked antiviral activity and IFN-inducing ability was also found with anthraquinone and pyrazoloquinoline derivatives, which of the most effective being 1,5-(bis)-morpholinopropyl(amino)9,10-anthraquinone (Stringfellow *et al.*, 1979). In the laboratories of Bristol Co., 4-substituted 1H-pyrazolo (3,4-b)quinolines were evaluated as IFN inducers. One of them, dichloride 1,3-dimethyl-4-(3-dimethylaminopropylamino)-1H-pyrazolo(3,4-b)quinoline, designated as BL-20803, was found as a good IFN inducer in mice both after oral and extraoral applications (Siminoff *et al.*, 1973, Siminoff, 1975).

A'common property of all these compounds is the so-called tolerance or hyporeactivity phenomenon, i.e. the inability of IFN production by repeated administrations of IFN inducers. The hyporeactivity was first demonstrated in cell cultures (Cantell and Paukner, 1963), later on in animals (Ho *et al.*, 1965).

Practical importance of the hyporeactivity phenomenon consists in the fact that derivatives of fluorenone, anthraquinone, pyrazoloquinoline, pyrimidine and other compounds, in which this phenomenon was also observed, must be repeatedly administered when used for the therapy of viral infections. From this follows the necessity to search for such IFN in-

ducers which will not inhibit IFN production after repeated application. The requirement was also the purpose of our study.

The compounds investigated were chosen out of the spectrum of benzo (c)-fluorene derivatives synthesized in our Institute, and studied for their antiviral and IFN-inducing abilities. The most effective VÚFB 14162 derivative displayed antiviral activity against EMC and vaccinia viruses similar to the THCl preparation used as positive control regardless whether administered by oral or s.c. routes. VÚFB 13371 and 13431 derivatives were less effective. When comparing their activity against EMC and vaccinia viruses after different routes of application, by oral treatment was more effective the VÚFB 13431 derivative, but after s.c. administration the VÚFB 13371 derivative. This can be explained by higher s.c. toxicity of the former compound, because s.c. administration of 100 mg/kg of VÚFB 13431derivative as well as of tilorone caused deaths of all mice inoculated. By contrast, survival of all mice treated s.c. with the same concentrations of VÚFB 14162 and 13371 derivatives indicates the lower toxicity of these compounds.

IFN-inducing ability was followed with all 3 compounds under study and with the control tilorone preparation. Mouse sera collected following oral administration of tilorone and benzo(c)fluorenone VÚFB 13431 and 14162 derivatives contained IFN as evidenced by the inhibition of CPE induced in L-929 cell cultures by EMC virus, whereas VÚFB 13371 derivative was not effective in inducing IFN activity. When the kinetics of IFN production and hyporeactivity phenomenon were examined, it was found that tilorone as well as VÚFB 13431 and 14162 benzo(c)fluorenone derivatives induced the highest IFN levels at 24 hr interval, which was followed by their sudden drop at 48 hr. Hyporeactivity of IFN production lasted in case of control tilorone preparation for 9 days; in case of benzo(c)fluorenone derivatives it was shorter by 24—28 hr.

As follows from the results obtained, we characterized antiviral and IFN-inducing ability of a new as yet not described group of compounds, thus extending present knowledge on synthetic IFN inducers. Based on the studies on dependence of biological activity on the chemical structure, according to the nature of substituents in different positions of the benzo(c)fluorenone skeleton, the compounds with antitumour (Mělka *et al.*, 1982), antibacterial (Křepelka *et al.*, 1983) or antiviral activities can be prepared, the presence of bisbasic substitution being the requirement for their IFN-inducing ability as in the case of fluorenone derivatives and tilorone, respectively. From this standpoint, taking into account toxicity relations, the group of benzo(c)-fluorenone derivatives can be considered as perspective for the purposeful preparation of compounds with properties of an IFN inducer.

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