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A novel method for detecting the antiviral activity of flavans in their vapour phase

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This report describes a new method for detecting and measuring the antiviral activity of volatile compounds. This method, the vapour phase test, is a modification of the conventional plaque reduction test, in that the compounds instead of being incorporated in the overlay medium were deposited on the inner surface of the Petri dish lid. During the incubation period, the compounds (if volatile) permeated the overlay medium before exerting their antiviral effect. Compounds which have or may come to clinical trial against rhinovirus infections were compared by normal plaque reduction assays and by means of this new technique. Flavans were shown able to exert their antiviral activity in the vapour phase and thus display an advantage over non-volatile compounds. The report also briefly shows how the technique was used to evaluate the structure–activity relationships in a series of analogous compounds.

rhinovirus; inhibition; flavans; vapour phase.

Several compounds with activity against rhinovirus in vitro have failed to show efficacy when administered intranasally to human volunteers [9]. In 1981 Phillpotts et al. [7] did show a trend to efficacy with enviroxime. However, in a later trial Hayden et al. [4] failed to demonstrate that enviroxime could significantly reduce infection or illness. The compounds which have come to clinical trial, if not soluble in water, have been delivered as suspensions of micronised material or in non-aqueous solution. The nasal mucosa appears to be highly sensitive and frequently displays an adverse response to drugs and their carriers [10]. It is evident that the ability of the upper respiratory tract to maintain an equable environment precludes sufficient contact time for a drug to penetrate and maintain antiviral concentrations in the cells of the nasal mucosa. Reed [9] considers that new methods of administration designed to circumvent the natural clearing mechanisms of the upper respiratory tract should be developed. Compounds which are volatile or lipophilic or both should help to achieve this aim. Volatile compounds will permeate the airway and lipophilic compounds will become cell-associated by binding to cellular lipid components.

We have developed a novel method for detecting the antirhinovirus activity of

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volatile compounds. As a result of this certain substituted flavans have emerged as candidate compounds for clinical administration in inhalers.

The plaque assay technique for rhinovirus was essentially that of Fiala et al. [2] with slight modifications [11]. Activity of antiviral compounds was measured by plaque reduction assays and expressed as an IC_{50} value (that concentration of compound which reduces plaque formation by 50%) [1].

For vapour phase tests the compounds were dissolved in ethanol. Ethanol was the solvent of choice since it did not affect the antiviral activity of any of the compounds, it had no overt action on the plastic of the Petri dish and it did not itself exert antiviral activity in these tests. A maximum volume of 100 μl of the solution was placed at the centre of the inner surface of a 50-mm diameter plastic Petri dish lid and allowed to spread and dry. The volume chosen was not sufficient to spread to the edge of the lid. The lid was then placed on a dish containing a monolayer of M-HeLa cells that had been infected with rhinovirus type 1B (RV1B) and covered with an overlay of nutrient agarose. After incubation for 3 days the monolayer was fixed and stained and examined for the presence of plaques. Control cultures were prepared with an equal volume of ethanol dried on the lid. These controls showed that no inhibitory substances were liberated from the plastic.

Measurement of the activity was achieved by means of plaque reduction assays. The dose of compound (and here we are referring to a specific amount of compound as opposed to a concentration) was varied by adjusting the volume of the solution placed on the lid. Thus, doubling dilutions were obtained by adding volumes of 100, 50, 25 and 12.5 μl. The plaques were counted and expressed as a percentage of the control value and plotted against the logarithm of the compound dose, to yield a dose–response line from which the 50% inhibitory dose (ID_{50}) could be estimated.

The compounds compared in this study were 4′,6-dichloroflavan (BW683C), 2-amino-1-(isopropyl sulphonyl)-6-benzimidazole phenyl ketone oxime (enviroxime), 4-((8-amino-7-chloro-5-methyl-5H-as-triazino(5,6-b)indol-3-yl)amino)-2-methyl-2-butanol (SK&F 40491), 2,6-diphenyl-3-methyl-2,3-dihydroimidazo(2,1-b) thiazole (RP 19326) and 4′-ethoxy-2′-hydroxy-4,6-dimethoxy chalcone (Ro 09-0410).

Table 1 displays the results when the compounds were measured in conventional (IC_{50}) and vapour phase (ID_{50}) plaque reduction assays. It may be seen that the 5 compounds display activity, measured by conventional means, which shows them to

TABLE 1

Comparison of conventional (IC_{50}) and vapour phase (ID_{50}) activities against RV1B

Compound	IC_{50} (μM)	ID_{50} (nmol)
BW683C	0.007	0.6
Enviroxime	0.04	>1000.0
SK&F 40491	1.07	>1000.0
RP 19326	2.60	>1000.0
Ro 09-0410	2.0	>1000.0
Ethanol	Inactive	Inactive

be of clinical interest. Indeed, 3 of the compounds, enviroxime [7], SK&F 40491 and RP 19326 [10] have undergone trials of intranasally administered formulations. 4', 6-Dichloroflavan underwent a clinical trial in an orally administered form and proved to be ineffective [8]. There are no published accounts of clinical trials with Ro 09-0410 but it was reported as active in cell culture at doses as low as 3 ng/ml against 46 of 53 serotypes of rhinovirus [5,6].

In the vapour phase tests enviroxime, SK&F 40491, RP 19326 and Ro 09-0410 were inactive at 1.0 μmol. The ID_{50} of BW683C was 0.6 nmol, indicating that it is sufficiently volatile to be active under laboratory conditions. Furthermore, it has a partition coefficient, expressed as log *P* (defined as the logarithm of the ratio of the concentration in the non-aqueous phase to the concentration in the aqueous phase) which may be calculated by means of the fragmental method of Hansch et al. [3] to be 5.7. This is significant in that the higher the number the more lipid soluble the compound is. The log *P* values of enviroxime, SK&F 40491, RP 19326 and Ro 09-0410 were similarly calculated to be 1.0, 0.4, 3.7 and 3.0, respectively. RP 19326 and Ro 09-0410 are highly lipophilic but are some 200-fold less active than BW683C in the conventional assay. The clinical efficacy of Ro 09-0410 remains to be proved. RP 19326 did display a slight trend towards efficacy in reducing and delaying the severity of the symptoms in human volunteers [10]. SK&F 40491 was more effective in vitro than RP 19326 but did not significantly alter the course of the infection in volunteers [10]. This may reflect its log *P* of 0.4 which shows the compound to be soluble in aqueous solution. After nasal administration it may not achieve an effective antiviral concentration due to dilution by the nasal mucus. Hayden et al. [4] in a study of intranasally administered enviroxime considered that it was an inadequate drug concentration in the cells of the nasal mucosa which prevented efficacy. A log *P* of 1.0 would suggest a lack of lipid partitioning ability. It has been shown by Margaret Tisdale (pers. commun.) that 4', 6-dichloroflavan is able to associate itself with cells to the extent of 100–150 fold the concentration in the medium. The compound is therefore highly lipophilic and would have affinity for cells in the nasal mucosa. The vapour phase experiment shows that despite its lipophilicity the compound is able to pass through an aqueous proteinaceous overlay and inhibit virus replication in infected cells.

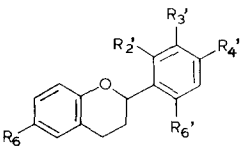
It would seem that whatever means is selected for intranasal administration, drops, spray or vapour inhalation, the properties of volatility and lipophilicity might enable 4', 6-dichloroflavan to overcome the natural barriers faced by intranasally administered compounds in the past.

Table 2 compares the IC_{50} and ID_{50} values of 22 flavan analogues obtained by conventional and vapour phase plaque reduction experiments. The IC_{50} values are similar and it would be difficult to select one compound for development based on the results of that single test. However, inspection of the ID_{50} values shows that there is a greater spread of activity which would make the selection of a compound that much simpler.

We feel that this technique is an important extension to the test systems currently in use for the detection and measurement of the activity of compounds designed for the treatment of respiratory infections. It remains to be seen whether volatile lipophilic

TABLE 2

Activity of substituted flavans in the conventional and vapour phase plaque reduction tests



Compound	Substituents	IC_{50} (μM)	ID_{50} (nmol)
(1)	None	0.05	5.7
(2)	4'-Cl	0.04	1.7
(3)	4'-F	0.02	5.6
(4)	4'-Br	0.04	2.6
(5)	4'-Me	0.03	8.0
(6)	4'-MeO	0.07	6.8
(7)	4'-NH_2	0.05	8.3
(8)	4'-OH	0.06	114.0
(9)	6-F	0.02	5.0
(10)	6-Cl	0.05	0.9
(11)	6-Br	0.02	2.3
(12)	6-MeO	0.01	7.5
(13)	2', 4'-diCl	0.06	5.2
(14)	2', 6'-diCl	0.05	45.0
(15)	3', 4'-diCl	0.04	5.9
(16)	4', 6-diCl	0.007	0.6
(17)	4'-Cl, 6-Me	0.02	1.0
(18)	4'-Cl, 6-Br	0.01	0.7
(19)	4'-Br, 6-Cl	0.02	0.7
(20)	4'-Me, 6-Br	0.02	2.3
(21)	4', 6-diMe	0.04	5.0
(22)	4'-MeO, 6-Cl	0.01	2.6

compounds will prove efficacious in clinical trials. These properties, on the experimental evidence to date, may give an advantage to antiviral compounds possessing them.

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