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Increased inhibitory action against adenosine 5′-triphosphate in the isolated taenia of the guinea-pig caecum by substitution in the A-ring of 2-phenylisatogen

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1 The ability of a series of 17 isatogen derivatives to relax smooth muscle, inhibit adenosine 5′-diphosphate (ADP)-stimulated respiration in isolated mitochondria and to antagonize the inhibitory effects of adenosine 5′-triphosphate (ATP) on smooth muscle was measured.

2 Substitution in the 4- and 7-positions of the A-ring gave compounds that were strong inhibitors of mitochondrial ATP synthesis and potent, non-specific smooth muscle relaxants. The compounds also possessed ATP-receptor blocking activity.

3 Substitution in the 5- and 6-positions of the A-ring decreased both the relaxant effect on smooth muscle and inhibition of ATP synthesis, whilst enhancing ATP-receptor antagonism.

4 In a series of 6-substituted 2-phenylisatogens, 6-methoxy-2-phenylisatogen was the most effective ATP-receptor antagonist. This compound also showed the greatest separation of the desired pharmacological activity (ATP-receptor blockade) from the other two activities (smooth muscle relaxation and inhibition of mitochondrial ATP synthesis).

Introduction

2–2′-Pyridylisatogen (PIT) has been shown to be a specific antagonist of the inhibitory effects of ATP on the isolated taenia of the guinea-pig caecum, although a direct relaxant action of the derivative is a complicating factor (Hooper, Spedding, Sweetman & Weetman, 1974; Spedding, Sweetman & Weetman, 1975). Therefore, to demonstrate the antagonistic activity of PIT on ATP, a spasmogenic agent must be added to the smooth muscle. In trying to overcome this complication,we prepared and tested a series of isatogen derivatives with substituents in ring B of the molecule (Foster, Hooper, Spedding, Sweetman & Weetman, 1978), with the aim of separating ATP antagonist activity from smooth muscle relaxant activity. All the compounds tested relaxed the guinea-pig taenia caeci, and this activity could be correlated with the ability of the compounds to inhibit ATP synthesis in isolated mitochondria. The antagonism of ATP within the series of compounds was not related to the smooth muscle relaxant activity. However, complete separation of these two actions was not achieved, and none of the compounds had any advantage over PIT in this respect.

The observation that 5-C1-PIT, a derivative substituted in ring A of the molecule, possessed reduced smooth muscle relaxant activity, whilst retaining substantial ATP antagonism suggested that substitution in ring A might yield a derivative with greater separation of the two actions (Foster *et al.*, 1978). This paper therefore examines the effect of substitution in ring A of the isatogen molecule, in an attempt to obtain maximum separation of ATP antagonism from smooth muscle relaxation. Because of problems with the availability of precursors used in the preparation of pyridylisatogens, a series of phenylisatogens have been synthesized. These derivatives have been used to obtain the answer to the following question. What is the chemical nature of and best position for the substituents in ring A in order to produce the greatest separation of ATP antagonism from smooth muscle relaxant activity?

Methods

Smooth muscle

The caecum of a female guinea-pig (200–350 g) was removed and placed in McEwen's solution (McEwen, 1956) of the following composition: (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11.1 and sucrose 13.2 at 30–35°C. Pieces of taenia 2–2.5 cm in length were taken from the dorsal side of the caecum and separated from the circular muscle; three or four pieces being taken from each animal. Taenia preparations were placed in 10 ml isolated organ baths containing McEwen's solution at 35 ± 1°C and gassed with 95% O_2 and 5% CO_2. Responses were recorded isotonically on a smoked drum (magnification 1:5, load 1.5 g).

Tissues were allowed 30 min to develop tone, and only those preparations that contracted to at least a quarter of their fully relaxed length were used. Cumulative concentration-response curves to ATP were produced at 30 min intervals, each dose of ATP being allowed to exert its full effect (3–15 s contact) before the concentration in the bath was increased (Van Rossum, 1963). The first two curves were frequently followed by an enhancement of tone, so the results of these were routinely discarded. The third curve was used to calculate the EC_{50} value for ATP (i.e. the concentration of ATP producing 50% maximal relaxation of the tissue). After the third curve to ATP, an isatogen derivative (50 μM) was allowed to react with the tissue for 30 min. This treatment usually relaxed the muscle, so carbachol (5 nM–2 μM) was used to contract the tissue again to within 10% of the original level of tone. A measure of the potency of the isatogen in relaxing the tissue was obtained by expressing the degree of relaxation as a percentage of the maximal relaxation induced by ATP in the third concentration-response curve. Following incubation with isatogen and subsequent contraction with carbachol, a fourth cumulative concentration-response curve to ATP was obtained. If the tone could not be restored to within 10% of the control level with carbachol, a lower concentration of the isatogen derivative (10 or 5 μM) was used. Dose ratios (DR) were calculated as the ratios of the EC_{50} values for ATP, in the presence (fourth curve) and absence (third curve) of isatogen.

Isolated mitochondria

The conditions employed to prepare mitochondria from guinea-pig liver were those previously used for the rat (Chappell & Hansford, 1969). The mitochondrial suspension was adjusted to a final concentration of 50 mg mitochondrial protein/ml, using a medium containing 250 mM sucrose and 3.4 mM Tris-HCl, pH 7.4. The protein content of the mitochondrial fraction was determined by the Biuret method (Gornall, Bardawill & David, 1949).

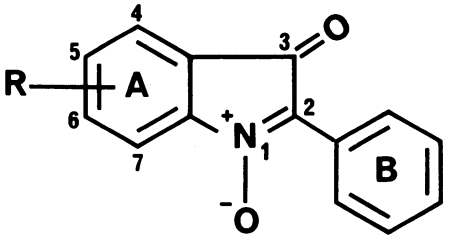
Mitochondrial respiration was measured polarographically with a Clark-type oxygen electrode, the reaction volume being 3 ml and the temperature 30 ± 1°C. The reaction medium contained (mM): sucrose 217, Tris-HCl 2.8, sodium succinate 3.3, phosphate buffer 3.3; pH was 7.4. Mitochondria (5 mg protein) were added at zero time and the oxygen consumption was followed for 2 min (control rate); then a test drug was added and the oxygen consumption followed for a further 3 min (test rate). Any stimulation of respiration due to the drug (uncoupling action) was determined by subtracting the control rate from the test rate. If a test drug failed to stimulate respiration, it was examined for its ability to inhibit ADP-stimulated respiration. Mitochondrial respiration was stimulated by the addition of 0.5 μmol ADP, to establish a control rate. An isatogen derivative was added to the reaction chamber 2 min before the addition of ADP and the degree of inhibition was determined. An IC_{50} value was obtained for each isatogen. This was expressed either as uncoupling activity (IC_{50} = concentration of isatogen required to stimulate respiration by 50% of the maximal stimulation), or the ability to inhibit ADP-stimulated respiration (IC_{50} = concentration of isatogen required to inhibit ADP-stimulated respiration by 50%), depending upon which type of activity was observed. Note that both types of drug action will result in an inhibition of mitochondrial ATP synthesis. Uncoupling dissipates the mitochondrial proton gradient and, in the case of isatogens, inhibition of ADP-stimulated respiration is caused by inhibition of ADP uptake into the mitochondrial matrix (Lovett & Sweetman, 1981).

Analysis of results

Values in the text refer to the mean ± s.e.mean. Differences in means were determined by Student's *t* test, after checking the homogeneity of the variances (Snedecor & Cochran, 1967). Correlations of the results were made on an ICL 2950 computer.

The relationships between the different biological variables were examined using Spearman rank correlation coefficients, r_s, (Siegel, 1956). Rank order correlations were investigated between the biological variables for ATP antagonist activity (DR) and smooth muscle relaxant activity (MR) and a wide variety of physicochemical parameters. These were: lipophilicity, π (Martin, 1978) and f (Rekker, 1977); steric effects, E_s (Martin, 1978) and Verloop constants (Verloop, Hoogenstraaten & Tipker, 1976); molar refractivity, (Martin, 1978); electronic effects,

Table 1 Effect of substitution in the A ring of 2-phenylisatogen



Derivative (R)	*Conc* (μM)	*Antagonism of ATP-receptors* (DR ± s.e.mean)	*Relaxant activity* (% maximal ATP-induced relaxation ± s.e.mean)	*Inhibition of ATP synthesis* (IC_{50}, μM ± s.e.mean)
4-Methyl	10	72.7 ± 57.5(4)	84.3 ± 11(3)	43 ± 2.5(3)
5-Methyl	50	129 ± 85(4)	63.5 ± 9.5(4)	37 ± 7.6(3)
6-Methyl	50	1.9 ± 0.7(4)	45 ± 8.7(4)	79.3 ± 15.2(3)
7-Methyl	5	2.3 ± 2(5)	84.6 ± 13(5)	4.3 ± 1.2(3)
5-Methoxy	10	51.9 ± 14.7(4)	42 ± 7(5)	30.2 ± 5.1(3)
6-Methoxy	10	150 ± 75.8(9)	30.7 ± 8(9)	263 ± 48(3)
7-Methoxy	5	17.6 ± 14.3(3)	79 ± 16(3)	9.4 ± 1.4(3)

DR is the ratio of the concentrations of ATP giving 50% relaxation of the taenia, in the presence and absence of isatogen. Smooth muscle relaxant activity is described by expressing the relaxation of the tissue obtained in the presence of the isatogen as a percentage of the maximal ATP-induced relaxation. IC_{50} is the concentration of isatogen that produced 50% inhibition of mitochondrial ATP synthesis (all the compounds in this table inhibited ADP-stimulated respiration: no compound was an uncoupling agent). Results are presented as the mean ± s.e.mean, with the number of experiments shown in parentheses. The parent compound, 2-phenylisatogen, possesses potent relaxant activity and inhibits ATP synthesis in isolated mitochondria, but does not antagonize ATP-induced relaxations of the taenia (Foster, Hooper, Spedding, Sweetman & Weetman, 1978).

σ_p, σ_m, σ^+, σ^- (Martin, 1978). In the case of inhibition of mitochondrial respiration, the small number of compounds possessing either uncoupling activity, or the ability to inhibit ADP-stimulated respiration only allowed single parameter equations to be developed, since it was considered important to distinguish between the two effects. This was done using log IC_{50} and the above physicochemical parameters in the usual Hansch analysis (Martin, 1978).

Drugs

The isatogen derivatives were synthesized in our laboratories by a variety of routes (Ruggli & Cuenin, 1944; Bond & Hooper, 1969; Sonogashira, Tohda & Nagihara, 1975; Foster *et al.*, 1978; Swain, 1980).

Other drugs used were: carbachol chloride, adenosine 5'-diphosphate disodium salt, adenosine 5'-triphosphate trisodium salt, Tris-HCl and dimethylformamide (BDH). The isatogen derivatives were dissolved in dimethylformamide. All drug concentrations are expressed as molarities.

Results

Effect of varying the position of the substituent in ring A

Table 1 shows the pharmacological and biochemical activity of a series of monomethyl isatogens, substituted in each of the four available positions in the A ring, and a series of monomethoxy derivatives. With respect to antagonism of ATP-induced relaxation of the taenia, both methyl and methoxy derivatives were active, although the greater activity was found with the methoxy derivatives; the most active compound was 6-methoxy-2-phenylisatogen.

On the basis of their biological activity, the isatogens could be separated into two distinct groups. First, substitution in the 4 and 7 positions gave compounds that were potent relaxants of the taenia; and in the case of the 7 position, potent inhibitors of mitochondrial ATP synthesis. Secondly, a greater separation of activities was found with the 5 and 6 derivatives. All four compounds had a reduced

Table 2 Structure-activity relationships in a series of 6-substituted 2-phenylisatogens

6-Position substituent	*Conc* (μM)	*Antagonism of ATP-receptors* (DR ± s.e.mean)	*Relaxant activity* (% maximal ATP-induced relaxation ± s.e.mean)	*Inhibition of ATP synthesis* (IC_{50}, μM ± s.e.mean)
Acetamido-	10	61.5 ±45(4)	52± 5.8(4)	39± 6.3(3)*
Amino-	10	23±10(5)	65.6±18.7(5)	168±23(3)
Carboxamido-	50	16±14.5(4)	47± 6.8(4)	69± 7.3(3)*
Carboxyl-	50	1.18±0.43(3)	42±16(3)	99.7±18(3)*
Chloro-	50	9± 6.6(3)	42.3±12.7(3)	16.8± 3.1(3)
Ethoxycarbonyl-	50	1.47±1(3)	43±11(3)	55.3±8(3)*
Hydrogen	10	11.1 ±7(5)	51.6±13(5)	2.4± 0.3(5)
Methoxy-	10	150±75.8(9)	30.7±8(9)	263±48(3)
Methyl-	50	1.9 ± 0.7(4)	45± 8.7(4)	79.3±15.2(3)
Nitro-	50	0.05±0.02(3)	68.3±17.4(3)	61.3±12(3)*
Phenyl-	10	2.7 ± 0.8(4)	44.6±14.4(5)	37±10.2(3)
Trifluoromethyl-	50	1± 0.5(4)	70±23(3)	33.5± 5.2(3)*

Results are expressed in the same way as described in the legend to Table 1. In the case of inhibition of mitochondrial ATP synthesis, compounds marked (*) were uncoupling agents; the remainder were inhibitors of ADP-stimulated respiration. Results are presented as the mean ± s.e.mean, with the number of experiments shown in parentheses.

smooth muscle relaxant activity, compared with 4 and 7 substituted compounds, whilst retaining the ability to antagonize ATP-induced relaxations. This latter effect was especially noticeable when the substituent was a methoxy group. The 6-methyl and 6-methoxy compounds had low activity against mitochondrial ATP synthesis, but the 5-methyl and 5-methoxy derivatives retained the ability to inhibit this process.

Effect of 6-substitution

A series of 6-substituted 2-phenylisatogens was prepared and examined for activity in the test systems described above (Table 2). The most potent ATP-receptor antagonist was 6-methoxy-2-phenylisatogen (the dose-ratio for ATP was found to be 150 ± 75.8; $n = 9$).

Relationship between the inhibitory actions of the isatogens

The relationships among the different biological variables (ATP antagonist activity, smooth muscle relaxant activity, uncoupling of mitochondrial ATP synthesis and inhibition of ADP-stimulated respiration) were examined using the information presented in Table 2. Each biological variable was compared with each other using non-parametric rank order correlations, because both dose-ratio measurements and values for smooth muscle relaxation could not be presumed to be normally distributed. When the biological activities were compared, there were no significant correlations at the $P = < 0.05$ level.

There was only one significant correlation detected when each of the biological variables was compared with each of the physicochemical parameters listed in the Methods section. This took the form of a negative correlation between ATP antagonism and the electronic effect, σ_p for the 6-substituted derivatives given in Table 2 ($r_s = -0.77$; $P = < 0.05$; $n = 12$).

Discussion

A major aim of the present work was to improve the selectivity of isatogen derivatives, with respect to ATP-receptor antagonism. In particular, we wished to separate ATP-receptor blockade from smooth muscle relaxation and inhibition of ATP synthesis, because one disadvantage of currently available antagonists is their lack of specificity (Spedding *et al.*, 1975; Spedding & Weetman, 1978). The approach we used was based on the observation that 5-chloro-2-2′-pyridylisatogen was a derivative that had a decreased smooth muscle relaxant action, whilst retaining ATP-receptor antagonism (Foster *et al.*, 1978). We therefore examined the effect of substitution in the A-ring of 2-phenylisatogen on the biological activity of the molecule.

ATP-receptor antagonism

The ability of methyl and methoxy derivatives of 2-phenylisatogen to affect the sensitivity of the taenia to ATP depended upon the position of the substituent in the A-ring. The 6-position was considered to be the most promising, since substitution here gave

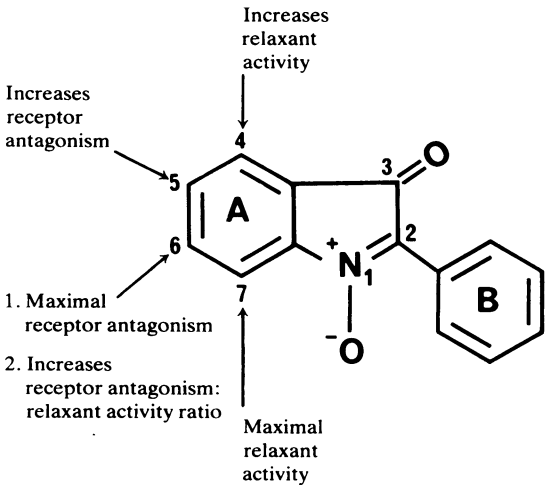


Figure 1 Summary of the main structure-activity relationships in substituted 2-phenylisatogens. The structure shown is 2-phenylisatogen. From the derivatives considered in this paper: (i) substitution in positions 5 and 6 increased ATP-receptor antagonism; the maximal effect being obtained when electron releasing moieties were introduced at position 6; (ii) substitution in positions 4 and 7 increased smooth muscle relaxant activity, which was maximal with 7-substituted derivatives; (iii) any substitution in the A-ring reduced activity against mitochondrial ATP synthesis, and the introduction of electron withdrawing groups to position 6 resulted in the emergence of an uncoupling action against mitochondrial oxidative phosphorylation; (iv) substitution in position 6 gave an increased separation of ATP-receptor antagonism from both relaxant activity and inhibition of ATP synthesis. This effect was most marked when the substituent was a methoxy group.

a high potency against ATP-receptors, together with the desired reduced effectiveness in relaxing the tissue and blocking mitochondrial ATP synthesis.

In a series of 6-substituted 2-phenylisatogens, activity against ATP-induced relaxations depended upon the nature of the substituent; the range of activity varying from enhancement to antagonism (dose-ratios from 0.05–150). The only significant physicochemical parameter related to this activity, which was found by a Spearman rank order correlation analysis, was σ_p ($r_s = -0.77$; $P = < 0.05$). The negative value of r_s indicates that electron release by the substituent is a major factor in increasing ATP-receptor blocking activity. The correlation is not of a very high order for this type of analysis of structure-activity relationships (Martin, 1978), suggesting that other factors, not identified in this study, may also play an important role in determining activity. The observation that 6-methoxy-2-phenylisatogen was the most potent ATP-receptor blocking agent, although the 6-amino group has a greater negative σ_p value is consistent with this reasoning.

Overall, the electronic nature of the 6-substituent has been identified as the single most important factor controlling both the potency and the selectivity of action of the compounds listed in Table 2.

Smooth muscle relaxation

The non-specific smooth muscle relaxant activity has not been lost in this series of compounds. Potency depended upon the position of the substituent in ring A of the isatogen molecule; the order of activity being $7 > 4 > 5 > 6$.

The major effects of substitution in ring A of 2-phenylisatogen are summarized in Figure 1. It is concluded that 6-methoxy-2-phenylisatogen represents an improvement over 2–2′-pyridylisatogen as a selective ATP-receptor antagonist. In support of this statement is the observation that 6-methoxy-2-phenylisatogen is approximately 100 times more potent than 2-2′-pyridylisatogen as an ATP-receptor antagonist. Furthermore, 6-methoxy-2-phenylisatogen has 60 times less activity as a smooth muscle relaxant, and 2800 times less activity against mitochondrial ATP synthesis, than 2-2′-pyridylisatogen. One disadvantage is that 6-methoxy-2-phenylisatogen is not soluble in water; nevertheless it should prove useful in investigations on the nature of purine receptors and the possible involvement of ATP in physiological processes.

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