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Role of spin trapping and P2Y receptor antagonism in the neuroprotective effects of 2,2′-pyridylisatogen tosylate and related compounds

Kevin Menton [a], Michael Spedding [b,*], Pierre Gressens [c], Pascal Villa [d], Toni Williamson [d], Anthony Markham [a]

[a] *Institute of Pharmacy, Chemistry and Biomedical Sciences, School of Sciences, University of Sunderland, Sunderland, SR1 3SD, UK*
[b] *Institut de Recherches Internationales Servier (I.R.I.S.), 192, Avenue Charles de Gaulle, 92200, Neuilly-sur-Seine, France*
[c] *INSERM E 9935 and Service de Neurologie Pédiatrique, Hôpital Robert-Debré, Paris, France*
[d] *Trophos, Parc scientifique de Luminy, Case 931, 13288 Marseilles cedex 9, France*

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Abstract

2,2′-Pyridylisatogen tosylate (PIT) is both an allosteric modulator of P2Y receptors, and an immine oxide, acting as a spin trap for free radicals. PIT (10 mg kg^{-1}, i.p.) was found to be a powerful neuroprotective agent in protecting against the lesions induced by 15 μg *S*-bromo-willardiine injected into the cortex or white matter of 5-day-old mice pups. As the multiple effects of PIT may induce both beneficial and deleterious effects, a reanalysis of the structure–activity relationship was undertaken. PIT (50 μM) and 2,3′-pyridylisatogen were potent antagonists of responses to ATP in the taenia preparation of the guinea-pig caecum, but 2,3′-nitrophenylisatogen was not. The reactive immine oxide group could be substituted by a keto moiety (*N*-(2′-pyridyl)phthalide) while maintaining antagonism of responses to ATP, equivalent to PIT. Thus, antagonism of P2Y receptors was not restricted to the isatogen nucleus. Other spin traps did not antagonise P2Y receptors, although dimethyl-pyrroline-*N*-oxide (DMPO) increased the sensitivity of responses to ATP. Both *N*-(2′-pyridyl)phthalide and 2,3′-nitrophenylisatogen was less neuroprotective than PIT (10 mg kg^{-1}, i.p.) in protecting against the *S*-bromo-willardiine-induced lesions in mice, implying that both antagonism of P2Y receptors and the immine oxide moiety may be important for the neuroprotective effects of PIT. However, the usefulness of the neuroprotection was limited because, in motoneurones obtained from rat embryos, PIT (10–100 μM) exacerbated cell death.

Keywords: 2,2′-Pyridylisatogen; Immine oxide; Spin trap; Motoneuron; ATP antagonist; P2Y receptor; Neuroprotection; AMPA receptor

1. Introduction

The development of P2 receptors antagonists is necessary for full evaluation of the role of ATP as a neurotransmitter, proposed by Burnstock (1972) to explain the phenomenon of nonadrenergic inhibitory transmission. The antagonists that are available, even now, have limited selectivity, and the role of P2 receptors is not clear in either the periphery or the brain.

2,2′-Pyridylisatogen tosylate (PIT) was reported to be a selective antagonist of P2Y responses (Spedding et al., 1975; Spedding and Weetman, 1976) and was used for receptor characterisation. In consequence, the effects of PIT on the responses to nonadrenergic inhibitory transmission were of interest, but PIT was ineffective in blocking these responses under all the conditions tested (Spedding et al., 1975; Spedding, 1977) despite fully blocking responses to ATP. Consequently, it was claimed that ATP may not be the sole transmitter mediating the responses to nonadrenergic inhibitory transmission. The use of PIT as a tool for receptor analysis and for the assessment of the role of purinergic nerves has been reviewed recently (Spedding et al., 2000).

The use of PIT allowed the first clear distinction of receptors for ATP and adenosine, because PIT antagonised responses to ATP but not responses to adenosine in the taenia caeci of the guinea pig. Furthermore, responses to ATP were different from those to adenosine and responses to ATP desensitised whereas responses to adenosine did not (Spedding and Weetman, 1976). This work was a precursor of the P2Y and P2X receptor classification (Burnstock, 1978), and responses to ATP in the taenia caeci are due to

* Corresponding author.

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activation of P2Y receptors. The taenia preparation is useful in being spontaneously active and thus inducing a tone, or spontaneous contracture, which allowed the relaxant effects of ATP, or adenosine, to be quantified. The experimental conditions were optimised to allow preparations to develop good tone (Spedding et al., 1975). Cumulative concentration–response curves to ATP were obtained which were stable over time, allowing drug screening (Foster et al., 1978), and this methodology was used to define structure–activity in the present report.

However, the use of PIT has several drawbacks. PIT is an irreversible antagonist at P2Y receptors, is chemically reactive and interacts with sulphydryl groups (Hooper and Robertson, 1971), and inhibits mitochondrial oxidative phosphorylation in high concentrations (Spedding and Weetman, 1978a) and modulates the mitochondrial permeability transition (Menton et al., 1997a,b). Furthermore, PIT was not an effective antagonist of ATP responses in all smooth muscles (Spedding and Weetman, 1978a), suggesting its use as an antagonist might be limited to certain subtypes of P_2 purinoceptors.

The isatogen nucleus is chemically reactive and relaxes the smooth muscle of the taenia (Spedding and Weetman, 1978a,b). This effect complicates analysis of antagonism of responses to ATP and necessitates the use of spasmogens (histamine, carbachol) to recontract the tissue before ATP can be retested in the presence of the antagonists. The direct relaxant effects of the isatogen derivatives were correlated with inhibition of mitochondrial metabolism, but this effect was independent of the antagonism of the responses to ATP within the series of isatogen derivatives (Foster et al., 1978).

King et al. (1996) examined the effects of PIT on recombinant P2Y1 purinoceptors expressed in *Xenopus* oocytes. PIT was found to be an allosteric modulator of the responses to ATP, causing potentiation of responses to ATP at low concentrations (0.1–10 μM) and antagonism at higher concentrations (>10 μM).

Other P2 receptors antagonists have been reported. Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; Lambrecht et al., 1992) is perhaps the most useful compound available, with selectivity for some P2X receptors and for endothelial P2Y receptors (Brown et al., 1995; Ralevic and Burnstock, 1996). Reactive blue 2 has low selectivity between P2Y and P2X receptors (Choo, 1981; Bultmann and Starke, 1994; Zhong et al., 1998). Suramin is nonselective at P2 receptors (Hoyle et al., 1990; Zhong et al., 1998).

PIT (1–20 μM), but not PPADS (20–300 μM), has been shown to prevent glutamate (100 μM)-induced cell death in cerebellar granule nerve cell cultures prepared from 8-day-old rats (Volonté et al., 1999). Volonté et al. (1999) considered on the basis of their study and previous work that antagonism of P2Y receptors rather than P2X receptors may be important for neuroprotective effects. We therefore wished to determine whether P2Y receptor antagonism could yield robust neuroprotective effects.

However, PIT is an immine oxide (immine oxides are also termed nitrones or *N*-oxides) and has been shown to form highly stable adducts with free radicals via this mechanism (Nepveu et al., 1998). PIT was more potent than the reference compound dimethyl-pyrroline-*N*-oxide (DMPO) (Nepveu et al., 1998), although the potency of PIT as a spin trap is dependent on the experimental conditions (Rosen et al., in press). Immine oxides, such as α-phenyl-*tert*-butyl-nitrone (*t*-BPN), have been shown to be neuroprotective, both in vitro and in vivo, by trapping free radicals (Phillis and Clough-Helfman, 1990; Cao and Phillis, 1994; Zhao et al., 1994; Thomas, 1997). However, immine oxides have not been studied as P2Y receptors antagonists.

We have therefore further tested PIT, two reference immine oxides, and some new analogues of PIT (Fig. 1), with and without the immine oxide function, as P2Y receptors antagonists. The most interesting compounds were then tested as neuroprotective agents. For neuroprotective effects, a well-characterised animal model of excitotoxic lesions in

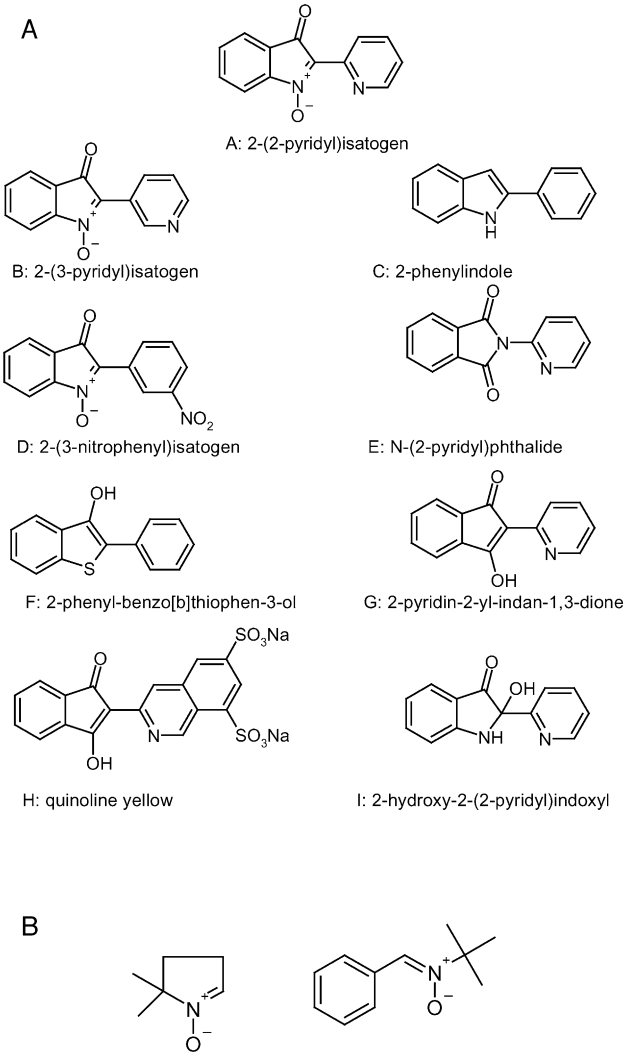

Fig. 1. (A) Structure of PIT and related compounds. (B) Structure of the spin trap immine oxides, DMPO and tBPN.

newborn mice has been used (Marret et al., 1995, 1996; Dommergues et al., 1998, 2000, submitted for publication; Gressens et al., 1999a,b; Redecker et al., 1998a,b; Tahraoui et al., 2000). Brain damage is induced with intracerebral administration of *S*-bromo-willardiine acting on α-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors. When *S*-bromo-willardiine is injected on postnatal day 5, neuronal death induces cortical brain lesions which resemble the lesions observed in full-term human infants. Furthermore, injections into periventricular white matter induces cystic lesions that mimicked several aspects of human cystic periventricular leukomalacia which is observed in very premature human infants. The *S*-bromo-willardiine-induced lesion of the white matter involves both NMDA and AMPA/kainate receptors, while the cortical plate lesion observed in this model is purely mediated by AMPA/kainate receptors (Tahraoui et al., in press) and these lesions can be reduced by antioxidants (Largeron et al., 2001). For comparison, an in vitro model of domoate-induced cell death of motoneurones was studied, using highly purified motoneurones from rats. Preliminary reports of this work have been published (Menton et al., 1997a,b).

2. Methods

2.1. Smooth muscle assays: P2Y receptors

Taenia caeci preparations were taken from female guinea pigs (Hartley, weight range: 180–300 g). Isolated preparations were suspended in 10 ml organ baths filled with McEwen's solution (McEwen, 1956), which was maintained at 36 ± 1 °C and gassed with 95% O_2 and 5% CO_2. After an equilibration period of 30 min, contractile responses were recorded isotonically on a Grass 3F recorder (load, 1.5 g; Spedding et al., 1975).

Cumulative concentration–response curves (Van Rossum, 1963) for the relaxant effects of ATP (1–1000 μM) relaxing taenia caeci strips were determined at 20 min intervals. Each dose of drug was allowed to produce its full effect (5–40 s contact) before the concentration of the drug in the bath was increased. Preparations with low tone (i.e., preparations that failed to contract in the organ bath or were less than 25% of their fully relaxed length) were discarded. The initial concentration–response curve for each agonist was disregarded.

All test compounds (concentrations 50 μM) were incubated for 30 min prior to ATP responses. Any tissues which relaxed following incubation of test compound were recontracted to an equivalent initial tone using carbachol (0.2 μM). The t_{50} is the time taken for the tissue to relax 50% of its initial tone.

2.2. Motoneurones: purification and culture

Motoneurones were purified using a combination of density gradient centrifugation and immuno-purification, as described previously (Henderson et al., 1995; Raoul et al., 1999). Spinal cords were dissected from day E14.5 Sprague–Dawley rat embryos (Elevage Janvier, France). The largest cells were isolated by centrifugation on a 6.5% (w/v) metrizamide (Serva) density gradient. The immunoaffinity purification step performed previously by immunopanning (Henderson et al., 1995) was replaced by a cell-sorting step using microbeads (Arce et al., 1999). Cells were incubated with a mouse antibody (anti-rat p75 antibody [Ig192]). Subsequently motoneurones were incubated with magnetic microbeads conjugated to anti-mouse secondary antibodies, thus allowing the purification of motoneurones on separating columns (Miltenyi Biotech).

The cells were then centrifuged through a bovine serum albumin cushion and resuspended in complete medium (neurobasal medium supplemented with B27 (Life Technologies), 2% horse serum, 25 μM 2-mercaptoethanol). Cells were plated onto 384-well dishes coated with polyornithine and laminin in complete medium using a fully automatised system (Trophos). The final volume was 100 μl/well in the presence of 1 ng/ml brain-derived neurotrophic factor (BDNF, RandD Systems) (Sendtner et al., 1992; Yan et al., 1992).

2.2.1. Assay of glutamate toxicity

After 4 days of culture (time for the upregulation of AMPA/kainate glutamate receptors as the neurones mature), half of the medium was removed and replaced by a freshly made medium containing compounds. One hour later, the cells were treated with domoic acid (Tocris) (10 μM final). Domoic acid, an AMPA/kainate receptor agonist, was used instead of glutamate as it elicits non-desensitizing responses at AMPA receptors (Debonnel et al., 1990). The concentration of domoic acid was optimised from an extensive series of preliminary experiments. Three controls were included (all with BDNF): no treatment; domoic acid treatment (10 μM final); domoic acid plus 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulphonamide disodium (NBQX, Tocris) (10 μM final concentration).

NBQX, a specific AMPA/kainate receptor antagonist (Zeman and Lodge, 1992; Sheardown, 1993), was used as an internal control. PIT was tested at four different concentrations (between 0.1 and 100 μM), with eight replicates for each concentration and for all controls. Each experiment was carried out twice.

Fresh solutions were made from powdered stocks before each experiment. The compounds were weighed and dissolved, diluted to twice their final concentration, then 50 μl was added per well. An equal volume of the appropriate diluent was added to the controls.

2.2.2. Assay of motoneuron survival

The number of surviving motoneurones was counted 2 days later. Live cells were counted by an automated image analyzer (Trophos) after labelling with a vital dye, calcein

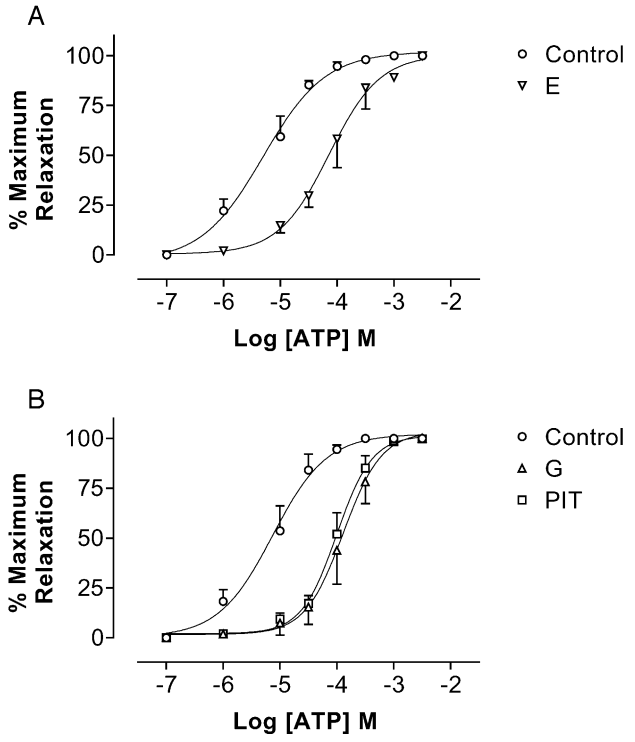

Fig. 2. Concentration–response curves showing the effect of PIT and analogues (E and G) on ATP responses in the isolated taenia of the guinea-pig caecum. The effects of each drug (50 μM) were determined after an initial cumulative concentration–response to ATP (0.1–300 μM). Drug incubation time was 30 min during which time there was a steady relaxation of the taenia for PIT and both analogues. The tone of the tissue was restored by the addition of carbachol (0.2 μM), which was followed by a second cumulative concentration–response curve to ATP (0.1–300 μM). 'A' shows the responses in the absence (○) and presence of 50 μM *N*-(2′-pyridyl)phthalide (E; ▽) and 'B' shows the control response in the absence of drug (○), in the presence of 50 μM PIT (A; □) and 50 μM 2-pyridin-2-yl-indan-1,3-done (G; △). Data are represented as the mean ± S.E.M. for eight (PIT; A) and four (E and G) separate experiments.

(Fluka). Results were analysed using Student's *t*-test (two-tailed, unpaired).

2.3. Neuroprotection in newborn mice

At postnatal day 5, Swiss mouse pups were anesthetized for intracerebral (i.c.) and i.p. injections. I.c. injections were performed using a 26-gauge needle mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere 2 mm from the midline in the lateral–medial plane and 3 mm (in the rostro–caudal plane) from the junction between sagittal and lambdoid sutures. Two 1-μl boluses were injected at a 30-s interval.

Fifteen micrograms of *S*-bromo-willardiine (Tocris), diluted in phosphate-buffered saline (PBS), was injected i.c. *S*-bromo-willardiine is a glutamatergic agonist acting on AMPA and kainate receptors. Immediately following i.c. injection, 10 mg/kg PIT ($n = 10$), 10 mg/kg compound D ($n = 8$) or 10 mg/kg compound E ($n = 12$), diluted in PBS, were administered i.p. Controls received i.p. PBS alone ($n = 19$).

Five days later, the surviving pups were sacrificed and brains fixed in formalin. Coronal serial sections, 15 μm thick, were cut and every third section was stained with cresyl violet. Brains were completely and serially sectioned from the frontal pole to the occipital lobes permitting an accurate and reproducible determination of the maximal sagittal fronto-occipital diameter of both the cortical plate and white matter lesions. This diameter was used as an index of the lesion size. Statistical analyses were performed with Student *t*-test. Results were expressed as means ± S.E.M.

2.4. Drugs

All drugs were purchased from Sigma, except for 2-MeSATP, which was purchased from Semat (RBI). PIT was synthesised by Dr. G. Dorey and Dr. P. Casara, Institut de Recherches Servier, France. All drugs were dissolved in saline solution, except PIT (in 0.1 N HCl, then titrated to pH 7.4).

3. Results

3.1. Smooth muscle

ATP rapidly relaxed taenia caeci preparations as previously reported and cumulative concentration–response curves did not change with time. PIT (compound A; 50 μM) caused a direct smooth muscle relaxation as previously

Table 1
Summary of dose ratios (DR) and t_{50} values for pit, analogues of pit and spin trapping agents on the isolated taenia of the guinea-pig caecum

Drug	Dose ratio	t_{50} (min)
PIT (A)	20 ± 6[a]	6 ± 1
B	10 ± 4[a]	5 ± 1
C	2 ± 0.1	5 ± 1
D	2 ± 0.2	13 ± 2
E	53 ± 10[a]	6 ± 1
F	5 ± 2	12 ± 1
G	25 ± 7[a]	4 ± 1
H	2 ± 1	>30
I	7 ± 2[a]	9 ± 2
tBPN	1 ± 0.1	>30
DMPO	0.8 ± 0.1[a]	>30

DR represents the ratio of the concentration of ATP producing a 50% maximal relaxation of the taenia after and before the addition of drug. The t_{50} values represent the time taken for the tissue to relax by 50% following exposure to drug (50 μM), each value represents the mean of four to eight separate experiments.

[a] $P < 0.05$ compared to control incubations.

reported, necessitating the use of spasmogens to recontract the smooth muscle (Fig. 2). PIT (50 μM) was a potent antagonist when tested against ATP-induced relaxations in the taenia preparation from the guinea-pig caecum. Concentration–response curves for ATP in the presence or absence of the antagonists (50 μM for 30 min, structures as shown in Fig. 1) are shown in Fig. 4. The other compounds were then tested at 50 μM for antagonism of responses to ATP. Compounds B, E and G were active whereas compounds C, D, F, H and I showed little or no significant activity as antagonists of the responses to ATP (Table 1). The spin traps tBPN and DMPO did not antagonise responses to ATP, although DMPO caused a small but significant potentiation of the response to ATP, indicated by a leftward shift of the concentration–response curve (Table 1).

Low concentrations of PIT (0.1–25 μM) did not cause any rapid relaxations of the smooth muscle, indicating that the compound was not a partial agonist: only slow relaxations were observed. Quinslav yellow failed to relax any tissue preparation during the period of drug incubation. All other analogues relaxed smooth muscle with various t_{50} values which were not dependent upon the ability of the drug to antagonise ATP-induced relaxations (Table 1). Both compounds C and D, ineffective ATP antagonists, relaxed smooth muscle directly although the t_{50} for D was significantly greater than that for C which was one of the most potent relaxants of the smooth muscle preparation. The spin traps DMPO and tBPN did not relax the smooth muscle directly, but DMPO caused a small but significant leftward shift of the cumulative concentration–response curves to ATP (Table 1).

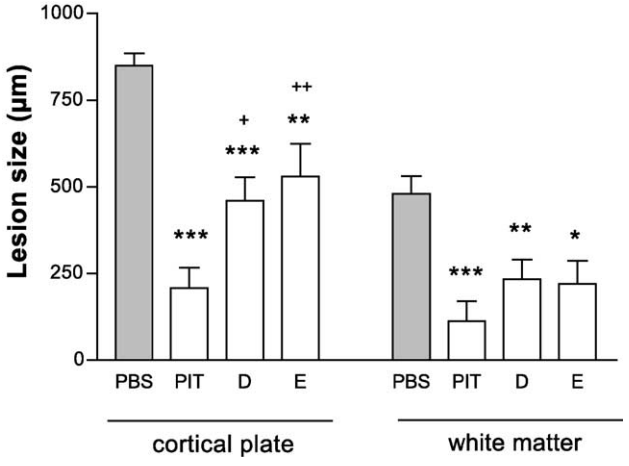

Fig. 3. PIT-induced neuroprotection in a newborn model of excitotoxic brain lesions produced by focal i.c. injection of *S*-bromo-willardiine. Bar represents the mean length of the neocortical lesion in the sagittal fronto-occipital axis ± S.E.M. Asterisks indicate difference from control (***$p < 0.0001$ in a Student's *t*-test). PBS, 19 control animals co-injected with i.c. *S*-bromo-willardiine and i.p. PBS; PIT, 10 pups co-treated with i.c. *S*-bromo-willardiine and PIT (10 mg kg^{-1}, i.p.); D, 12 pups co-treated with i.c. *S*-bromo-willardiine and compound D (2-(3-nitrophenyl)isatogen, 10 mg kg^{-1}, i.p.); E, 8 pups co-treated with i.c. *S*-bromo-willardiine and compound E (*N*-(2′-pyridyl)phthalide, 10 mg kg^{-1}, i.p.), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control; +$p < 0.05$, ++$p < 0.01$ versus PIT.

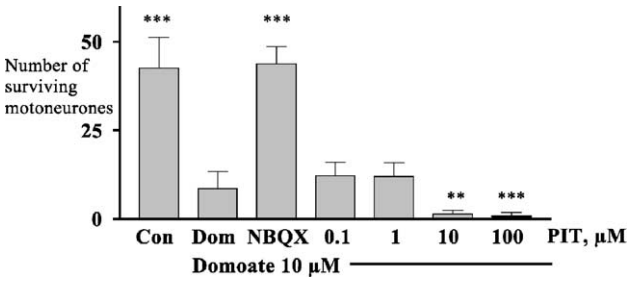

Fig. 4. Survival of motoneurones in culture, in the presence of BDNF, in the absence (Con, control) or in the presence of domoic acid (Dom, 10 μM). Domoic acid was also co-incubated with NBQX (10 μM) or with PIT. Values shown are the number of motoneurones surviving: error bars represent standard deviation from 8 wells.

3.2. Neuroprotection in newborn mice pups

S-bromo-willardiine injection induced tonic and tonico–clonic seizures in most of the pups; these epileptic manifestations were observed during the first 8 h following i.c. injection and were not modified in terms of frequency, severity or phenotype by co-treatment with PIT, compound D or E (data not shown). None of the pups died.

At the neuropathological level, neopallial injection of *S*-bromo-willardiine induced a focal neuronal death affecting all cortical layers and a periventricular white matter cystic lesion, as reported in previous studies. Co-treatment with PIT (10 mg kg^{-1}, i.p., at day 5, at the same time as administration of *S*-bromo-willardiine) significantly protected both the white matter and the cortical plate lesions against the insult (Fig. 3). However, both *N*-(2′-pyridyl)phthalide (E) and 2,3′-pyridylisatogen (D), although possessing weak protective effects, were less neuroprotective than PIT (10 mg kg^{-1}, i.p.), in protecting against the *S*-bromo-willardiine-induced lesions in mice.

3.3. Motoneurones

Motoneurones survived in 384-well plates for 6 days in the media containing BDNF. However, domoic acid (10 μM) caused death of almost all the motoneurones within 2 days (Fig. 4). Co-administration of the AMPA receptor antagonist NBQX (10 μM) prevented the deleterious effects of domoic acid (Fig. 4): these concentrations were chosen from full concentration–response curves used to validate the technique. However, PIT (0.1 and 1 μM) had no effect on the deleterious effects of domoic acid (Fig. 4), and at higher concentrations (10 and 100 μM), deleterious effects were observed. In further experiments, 1 10 and 100 μM PIT were found to be deleterious (not shown). Thus, PIT is not protective in all experimental conditions.

4. Discussion

2,2′-Pyridylisatogen tosylate (PIT) was not only an antagonist of ATP-induced relaxations in the taenia preparation from the guinea-pig caecum, but also a neuroprotective agent in protecting against the lesions induced by 15 μg *S*-bromo-willardiine injected into the cortex or white matter of 5-day-old mice pups. *S*-Bromo-willardiine is a potent agonist at AMPA receptors which causes marked neurodegeneration in this model: the effects are antagonised by AMPA receptors antagonists (Tahraoui et al., in press; Gressens and Spedding, in preparation). In this model, the cortical damage mimics microgyria in the developing infant and the white matter damage mimics leukomalacia (Marret et al., 1995, 1996; Dommergues et al., 1998, 2000, submitted for publication; Gressens et al., 1999a,b; Redecker et al., 1998a,b; Tahraoui et al., in press). Neuroprotection may be afforded by glutamate antagonists, neurotrophic agents, such as brain-derived neurotrophic factor (BDNF) or antioxidants (Marret et al., 1995, 1996; Dommergues et al., 1998, 2000, submitted for publication; Gressens et al., 1999a; Redecker et al., 1998b; Tahraoui et al., in press). PIT (10 mg/kg, i.p.) was as active as the AMPA receptors antagonists tested in this model (1-(4-amino-phenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466), 3 mg/kg, i.p.; Gressens, personal communication) and thus shows a powerful neuroprotective effect in the model. The neuroprotective effects of PIT may be due to modulation of responses to ATP, as suggested by Volonté et al. (1999), or by the powerful ability of PIT to act as a stable spin trap because of the immine oxide moiety (Nepveu et al., 1998).

In order to identify which effect was important, we compared structural analogues of PIT, with and without the immine oxide spin-trapping function, with the reference spin traps, DMPO and tBPN, as ATP antagonists using the taenia caeci preparation from the guinea pig, which is a well-characterised model of P2Y receptor antagonism. First, PIT did not have direct partial agonist effects at ATP receptors, and thus did not behave in the taenia as an allosteric modulator of P2Y receptors, with agonist effects at low concentrations and antagonist effects at high concentrations, as occurs in oocytes transfected with P2Y1 receptors (King et al., 1996). Slow relaxations caused by PIT have been shown to be related to inhibition of mitochondrial metabolism and are not consequent to partial agonist effects on ATP receptors (Spedding and Weetman, 1978a). Several of the other analogues directly relaxed the smooth muscle, in a similar manner to PIT, indicating that effects on mitochondrial metabolism, as reflected by relaxant effects, are independent of ATP antagonism, as suggested by the first structure–activity studies with this type of molecule (Foster et al., 1978). The spin traps DMPO and tPBN did not relax the smooth muscle, but DMPO caused a leftward shift in the concentration–response curve to ATP. This effect may be due to allosteric modulation of P2Y receptors, as occurs in some circumstances with PIT (King et al., 1996), or to inhibitory effects on ATP metabolism.

From the structure–activity data, it is apparent that a substituent in the 2′ position of the phenyl ring is important; otherwise, with the exception of the less effective 2,3′ -PIT, inactive antagonists are produced. Nevertheless, for the first time, ATP antagonism was found to be associated with compounds (E, G) which did not have the immine oxide group and which will not function as spin traps. Both compounds had the 2′ pyridyl function which had been optimised in previous studies (Foster et al., 1978) and confirmed to be important in this study. The 2′ pyridyl moiety may be important in maintaining the pyridyl ring at right angles to the planar isatogen structure. The pyridyl ring may also stabilise free radical adducts (Nepveu et al., 1998). Nevertheless, compounds E and G relaxed smooth muscle and may also have effects on mitochondrial function.

Immine oxides such as tBPN function as spin traps and may trap free radicals. The production of free radicals from complex III of mitochondria may be a critical factor in setting the life span of different species (Harman, 1956, 1972; Harman et al., 1976; Barja et al., 1994), and free radical production may be increased in neurodegenerative disease (Phillis, 1994; Benzi and Moretti, 1995; Gerlach et al., 1995; Cowley et al., 1996; Wolz and Krieglstein, 1996). Antioxidants may be useful agents in the treatment of Parkinson's disease, Alzheimer's disease and cerebral ageing, amyotrophic lateral sclerosis (ALS) and in reperfusion-driven damage after ischaemia (Harman et al., 1976; Phillis, 1994; Benzi and Moretti, 1995; Gerlach et al., 1995; Mattson, 1995; Cowley et al., 1996; Wolz and Krieglstein, 1996). Immine oxides by trapping free radicals have marked neuroprotective effects in a range of models of stroke and neurodegeneration (Phillis and Clough-Helfman, 1990; Cao and Phillis, 1994; Zhao et al., 1994; Thomas, 1997) and immine oxides are in clinical development for the therapy of stroke. Immine oxides are not necessarily P2Y receptors antagonists, at least as assessed in the taenia preparation, differentiating this class of compounds from PIT.

Are the neuroprotective effects of PIT due to P2Y receptor antagonism or to the ability of the compound to act as spin trap? We therefore tested a compound which was a equivalent P2Y receptor antagonist to PIT, but was without the immine oxide function (E), and a compound (D) which was an immine oxide without P2Y receptor antagonism. *N*-(2′-pyridyl)phthalide (E), which will not function as a spin trap, was less active than PIT in inhibiting cortical lesions in newborn mice, despite being a potent ATP antagonist, and being a structural isostere of PIT. *N*-(2′-pyridyl)phthalide does not have the immine oxide function and therefore should not function as a spin trap. It is possible that the weaker effects of *N*-(2′-pyridyl)phthalide may be due to antagonism of ATP receptors. This is supported by the fact that 2-3′-nitrophenyl isatogen (D), the immine oxide devoid of effects on P2Y receptors, was as potent as compound E,

but less potent than PIT. The ability of PIT to antagonise P2Y receptors and to act as a spin trap may therefore be the reason for the potency as a neuroprotective agent in this model, although some of the differences may be secondary to differences in metabolism in vivo.

We wished to confirm the PIT was neuroprotective in an in vitro model of neurodegeneration: domoate-induced death of motoneurones. The AMPA receptor antagonist, NBQX, was fully effective in preventing motor neurone cell death induced by domoic acid, indicating a potential role of AMPA receptors. In two separate series of experiments, however, PIT (at 10 and 100 μM) exacerbated motor neurone cell death, and in one of the experiments, using a different lot of motoneurones, PIT at 1 μM induced cell death. Thus, it is probable that the multiple effects of PIT (ATP antagonism, inhibition of mitochondrial respiration, efficacy as a spin trap for free radicals, Spedding et al., 2000) may predominate to different extents in different experimental circumstances, conferring advantageous or deleterious effects. Useful neuroprotective agents should be active in a wide range of experimental models.

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