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Newly identified structurally disparate modulators of osmosensitive taurine efflux inhibit cell cycle progression

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Abstract

FACS analysis and [^{14}C]-taurine efflux were used to determine whether activation of the volume-sensitive organic osmolyte/anion channel plays a role in cell cycle progression. This was achieved by examining the effects of a collection of (i) H_1 antagonists and tricyclic antidepressants with a known inhibitory effect on cell cycle progression, and (ii) antidepressants and oestrogen receptor modulators with molecular structures likely to confer inhibition of the volume-sensitive organic osmolyte/anion channel. Of the 13 compounds examined in this study, the following showed no cytotoxicity following a 48-h exposure, and specifically inhibited osmosensitive taurine efflux (over lactate transport and anion exchange) with IC_{50} values of (in μM): fluoxetine, ~ 14; fluvoxamine, ~ 24; amitriptyline, ~ 32; imipramine, ~ 32; mianserin, ~ 40. A 48-h application of these compounds at these concentrations significantly increased arrest in the G0/1 stage of the cell cycle by ~ 10%. The uniformity and specificity of the response elicited by these compounds strongly reinforces a correlation between cell cycle progression and osmosensitive taurine efflux activation.

Keywords: Taurine efflux, osmosensitive; Cell cycle progression; Antihistamine, second-generation, non-sedating; Tricyclic antidepressant; Volume-sensitive organic osmolyte/anion channel

1. Introduction

During the normal cell cycle, most mammalian cell types need to maintain a constant cell volume when exposed to changes in the osmolarity of the extra- and intracellular environment (Nilius et al., 2000). Under conditions where cells swell, for example, following osmolyte accumulation, a set of mechanisms exists to collectively mediate a process of regulatory volume decrease. Regulatory volume decrease acts to reduce the cell's osmolarity and thereby counteract cell swelling (Emma et al., 1997). Of the several osmosensitive organic and inorganic solute release pathways active during regulatory volume decrease, the volume-sensitive organic osmolyte/anion channel allows efflux of a wide range of physiologically significant organic osmolytes (Jackson et al., 1994). The β-sulphonated amino acid—taurine—is the main organic osmolyte in many mammalian cells and is a principal substrate of the volume-sensitive organic osmolyte/anion channel (Huxtable, 1992).

Taurine transport plays important functions in many cellular processes (Lambert and Sepulveda, 2000; Lima et al., 2001) and is thought to have a key role in cell proliferation, since inhibiting both hypotonically activated taurine efflux (Shen et al., 2001) and hypertonically stimulated taurine accumulation (Alfieri et al., 2002) impacts negatively on this process. However, in the absence of high affinity, high specificity blockers, unequivocal verification of its physiological role in the cell cycle has been hampered. With few exceptions, all volume-activated chloride and taurine effluxes are characterized by their sensitivity to the stilbene disulphonate 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), the anthracilic acid analogue 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and the partial oestrogen antagonist tamoxifen (Jentsch et al., 2002). However, in this study and previous studies, these com-

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pounds were shown not to be selective for inhibiting osmosensitive taurine efflux over anion exchange and lactate transport (Poole and Halestrap, 1991; Branchini et al., 1995; Cranmer et al., 1995; Skelton et al., 1995). Moreover, both DIDS and tamoxifen inhibit the volume-sensitive organic osmolyte/anion channel in different cell types with differing potencies. For example, in some cell types, DIDS exerts only a weak blockade of volume-sensitive organic osmolyte/anion channels (e.g. with an IC_{50} of ~ 900μM in rat supraoptic glial cells; Bres et al., 2000), while tamoxifen has been reported to be ineffective in other cell types (e.g. mouse endothelioma cells and rat sympathetic neurones; Nilius et al., 1994; Leaney et al., 1997). It is therefore questionable whether these compounds can be used to accurately identify the physiological role of the volume-activated organic osmolyte/anion channel, thus highlighting the need for novel, selective and potent blockers of this channel.

In addition to NPPB, DIDS and tamoxifen, there are numerous volume-sensitive organic osmolyte/anion channel blockers with little, if any, structural similarity. These include the selective serotonin reuptake inhibitors, sertraline, paroxetine, fluoxetine, citalopram and fluvoxamine (Maertens et al., 1999, 2002); the lipoxygenase inhibitor, gossypol (Gschwentner et al., 1996); the K^+ channel blocker, quinine (Verdon et al., 1995); the partial oestrogen antagonists, clomiphene and nafoxidine (Maertens et al., 2001); and the ethacrynic-acid derivative, 4-(2-Butyl-6,7-dichlor-2-cyclopentanyl-indan-1-on-5-yl) (DCPIB) (Decher et al., 2001).

The objective of this study was to determine whether activation of the volume-sensitive organic osmolyte/anion channel plays a role in cell cycle progression. This was achieved by examining: (i) the effects of a collection of antihistamines and tricyclic antidepressants with a known inhibitory effect on cell cycle progression attributed to blockade of other ion channels (Gavrilova-Ruch et al., 2002; Ouadid-Ahidouch et al., 2001) and (ii) a collection of other antidepressants and oestrogen receptor modulators with molecular structures likely to confer volume-sensitive organic osmolyte/anion channel inhibition (Maertens et al., 1999; Mulvaney et al., 2000). In order to achieve this aim, we designed an experimental program that culminated with an assessment of ability to inhibit cell cycle progression. Prior to this test, the effects of these compounds on osmosensitive taurine efflux inhibition was examined, followed by an assessment of their specificity for osmosensitive taurine efflux inhibition over other anion transport pathways. This was done by analysing their ability to block lactate transport and anion exchange: pathways known to possess a similar pharmacology (Poole and Halestrap, 1991; Branchini et al., 1995; Cranmer et al., 1995; Skelton et al., 1995). An assessment of cytotoxicity was also made. The purpose of this sieving process was to try to identify compounds that could be used unambiguously to define a link between activation of the volume-sensitive organic osmolyte/anion channel and cell cycle progression. HeLa cells were chosen for the proposed experiments since they represent a well-characterized model for studying osmosensitive taurine efflux (Lambert and Sepulveda, 2000). The specificity of each inhibitor was examined by screening against the lactate transporter and anion exchanger (Band 3) in human erythrocytes. Cytotoxicity of selected compounds was assessed using trypan blue exclusion and cell counting. Finally, cells were analysed by FACS to determine whether the selected compounds showed any effect on cell cycle progression.

2. Materials and methods

2.1. Cell culture

The adhesive human cervical carcinoma cell-line, HeLa, was cultured as a monolayer in Dulbecco's modified essential medium (DMEM), supplemented with 10% (v/v) foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air.

2.2. Reagents

Cell culture reagents were supplied by Gibco Life Technologies (Paisley, UK). [^{14}C]-taurine, [^{14}C]-lactate and $^{35}SO_4^{2-}$ were all obtained from NEN Life sciences (Hounslow, Middlesex, UK). Amitriptyline, astemizole, β-estradiol, imipramine, mianserin, nafoxidine, nomifensine, tamoxifen and terfenadine were obtained from Sigma (Poole, Dorset, UK). ICI 182,780 and fluvoxamine were obtained from Tocris Cookson (Bristol, UK). These compounds were made up as stock solutions (50 mM) in dimethyl sulfoxide (DMSO).

2.3. Osmosensitive [^{14}C]-taurine efflux measurements

HeLa cells were seeded onto 35-mm culture plates and incubated until at least 70% confluent. The cells were preincubated with 0.1 μCi/plate [^{14}C]-taurine at 37 °C for 1 h. The cell monolayer was washed five times with 1 ml of ice-cold isotonic solution as used by Kirk and Kirk (1994) (150 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), pH adjusted to 7.4 with an osmolarity of 281 ± 2 mosM/kg H_2O). After washing, 1 ml isotonic solution was added to the plate and was replaced with fresh solution at 2-min intervals. Effluxed [^{14}C]-taurine in each fraction was determined by β-scintillation spectroscopy. After 8 min, the isotonic medium was replaced with the hypotonic KCl solution (95 mM KCl substituted for 150 mM KCl with an osmolarity of 180 ± 2 mosM/kg H_2O) in the absence or presence of the test compound. Following a 16-min test period, the

amount of [^{14}C]-taurine remaining inside the cells was determined by lysing the cells with 0.1 M NaOH and quantifying [^{14}C]-taurine by β-scintillation spectroscopy. All results were calculated as the rate constant (k, min^{-1}) for osmosensitive [^{14}C]-taurine efflux [ln $(C_\infty - C_s/C_\infty - C_0)/t$] where C_∞, C_s and C_0 are the summation of the extracellular radio-activities effluxed up to *time infinity, time t* and *time zero*, respectively. Unidirectional rate constant k is given by the slope of the line [ln $((C_\infty - C_s)/(C_\infty - C_0))/t$]. All compounds exhibiting $\geq 50\%$ inhibition at 100 μM were tested at a range of concentrations and concentration–response curves were constructed using SigmaPlot (Chicago, IL, USA), using data that was fitted to the Hill equation:

$$\%\ \text{inhibition} = \frac{100}{1 + (\text{IC}_{50}/C)n_H}$$

where C is the drug concentration, n_H is the Hill coefficient and IC_{50} the drug concentration required for half maximal inhibition.

2.4. Anion exchange assay

$^{35}SO_4^{2-}$ influx was measured using methods previously described (Skelton et al., 1995). Briefly, freshly drawn erythrocytes from healthy, consenting adults were washed twice in phosphate-buffered saline, then washed three times in Cl^- free solution (130 mM Na gluconate, 5 mM K gluconate, 10 mM glucose and 10 mM HEPES, pH adjusted to 7.4). The red blood cells were then resuspended in the Cl^- free medium at approximately 5% haematocrit. Aliquots of the red blood cell suspension were pipetted into 1.5 ml microcentrifuge tubes. The inhibitors (50 mM stock in DMSO) were added to give a final concentration of 100 μM, mixed and incubated for 10 min at 37 °C before starting the flux by addition of $^{35}SO_4^{2-}$ (final concentration of 0.2 μCi/ml) in the Cl^- free buffer. Samples were withdrawn after 15 s and cells were pelleted by centrifugation through dibutyl phthalate for 15 s at 13,000 × g, giving a determination of the 'trapped' radioisotope. Further samples were taken in triplicate at 10 min and again spun through dibutyl phthalate oil. The spun samples covered by the layer of dibutyl phthalate oil were washed four times by submersion in water followed by aspiration. The dibutyl phthalate oil layer was removed by aspiration on the final wash. The remaining blood cell pellet was lysed with 0.5 ml Triton-X-100 (0.5% (v/v)) and the protein was precipitated by the addition of 0.5 ml trichloroacetic acid (5% (w/v)) followed by centrifugation at 13,000 × g for 5 min. The supernatant was transferred to a scintillation vial and counted by β-scintillation spectroscopy. The percentage inhibition/activation was calculated by comparing the counts for each inhibitor with the control (DMSO only) after subtraction of the 'trapped' counts.

2.5. Lactate transport assay

[^{14}C]-lactate influx was measured using methods previously described (Poole et al., 1991) and this protocol was similar to that described for the anion exchange assay. [^{14}C]-lactate is transported across the cell membrane by three difference mechanisms: (i) the lactate transporter, (ii) the anion exchanger, and (iii) by simple diffusion of the protonated acid across the cell membrane (Skelton et al., 1995). To ensure that lactate uptake was mediated by the lactate transporter alone, the second and third mechanisms were eliminated by preincubating the erythrocytes with DIDS and by keeping the flux time short to eliminate lactate diffusion across the membrane, respectively. Erythrocytes were washed three times in a phosphate-buffered saline, then incubated for 1 h at 37 °C to deplete the cells of intracellular lactate since intracellular lactate is known to modulate the rate of lactate influx (Cranmer et al., 1995). The cells were then washed twice in a Cl^- free solution (130 mM Na gluconate, 5 mM K gluconate, 10 mM glucose and 10 mM HEPES, pH adjusted to 7.4). The red blood cells were then resuspended in the Cl^- free medium at approximately 5% haematocrit and incubated in the presence of 10 μM DIDS for 30 min at 37 °C. Aliquots of the red blood cell suspension were pipetted into 1.5 ml microcentrifuge tubes. The inhibitors (50 mM stock in DMSO) were added to give a final concentration of 100 μM, mixed and incubated for 10 min at 37 °C before starting the flux by addition of [^{14}C]-lactate (final concentration of 0.2 μCi/ml) in the Cl^- free buffer. Samples were withdrawn after 15 s and cells pelleted by centrifugation through dibutyl phthalate for 15 s at 13,000 × g, giving a determination of the 'trapped' radioisotope. Further samples were taken in triplicate at 1 min and again pelleted by centrifugation through dibutyl phthalate oil. The tubes were treated in the same way as for the anion exchange assay.

2.6. Determination of cell viability

Following a 48-h exposure of each test compound at its IC_{50} concentration for osmosensitive taurine efflux inhibition on HeLa cells, cell viability was determined using the trypan blue exclusion assay, using 1:1 (v/v) with 0.4% trypan blue dye. Cytotoxicity was assessed by measuring the percentage of dead cells, which were trypan stained.

2.7. Determination of cell cycle stage by FACS

HeLa cells were synchronised with exposure to DMEM medium containing 0.1% fetal bovine serum for 24 h and subsequently plated into 75-cm^3 flasks with DMEM medium containing 10% fetal bovine serum. After 6 h, the cells were incubated with the test compound at its IC_{50} concentration for osmosensitive taurine efflux inhibition, for 48 h. The cells were then harvested by trypsinisation, washed three times in ice-cold PBS and fixed in cool 70% ethanol

for at least 48 h. Subsequently, the fixed cells were incubated for 5 min in 1 mg/ml RNAse A, after which, 20 mg/ml propidium iodide was added, and incubation with both RNAse A and propidium iodide continued for a further 30 min. For each cell population, 30,000 cells were analysed by FACS (FACScan, Becton-Dickinson, Rutherford, NJ, USA) and the proportion of cells in G0/G1, G2/M and S phases was estimated using Modfit cell cycle analysis program (v. 2.0, Verity Software House). The percentage of cells in G0/G1, G2/M or S phase was determined, depending on the intensity of the fluorescent peaks (MacFarlane and Sontheimer, 2000). FACS measurements were performed in three independent experiments.

2.8. Statistics

Results are presented, where appropriate, as the mean ± standard error of the mean (S.E.M.). Significance (P) was determined using the unpaired Student's t-test.

3. Results

The structures of the compounds used in this study are shown in Fig. 1.

3.1. Osmosensitive [^{14}C]-taurine efflux in HeLa cells

In this study, osmosensitive [^{14}C]-taurine efflux was used to assay volume-sensitive organic osmolyte/anion channel activation in HeLa cells. Fig. 2 illustrates concentration-dependent block of osmosensitive taurine efflux by terfenadine from which unidirectional efflux rate constants were estimated. The basal osmosensitive [^{14}C]-taurine efflux rate constant was 0.007 ± 0.001 min^{-1} ($n=6$), increasing to 0.155 ± 0.021 min^{-1} ($n=6$) under hypotonic conditions: an approximately 21-fold increase. A lag period of 2–3 min following exposure of HeLa cells to a hypotonic medium was observed before taurine efflux reached peak. Shown in Fig. 3 are the effects of the compounds tested on osmosensitive [^{14}C]-taurine efflux inhibition at 100 μM. Consistent with the previous reports where NPPB blocks osmosensitive taurine efflux between 80% and 90% (Jackson and Strange, 1993), osmosensitive taurine efflux was inhibited by $81.8 \pm 0.8\%$ ($n=3$) by 100 μM NPPB. Maximal volume-sensitive organic osmolyte/anion channel inhibition was therefore deemed to be achieved if osmosensitive taurine efflux was inhibited by ~ 80%, since 10–20% of taurine efflux is non-osmosensitive. The IC_{50} obtained for inhibition of osmosensitive taurine efflux by NPPB was 21.8 μM.

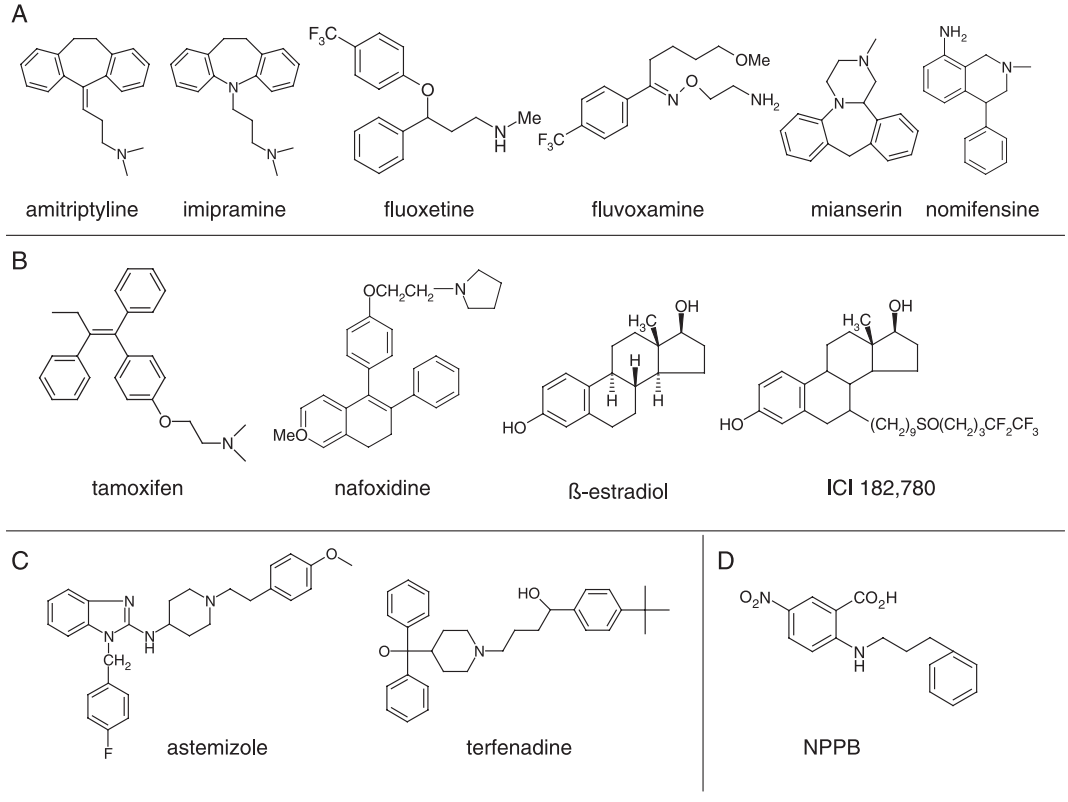

Fig. 1. Structures of all compounds tested in this study: (A) antidepressant compounds, including the tricyclic antidepressants amitriptyline and imipramine, the selective serotonin reuptake inhibitors fluoxetine and fluvoxamine, the tetracyclic antidepressant mianserin and the atypical antidepressant nomifensine; (B) oestrogen receptor targeting compounds, including the partial oestrogen antagonists tamoxifen and nafoxidine, the oestrogen derivative β-estradiol and the pure anti-oestrogen ICI 182,780; (C) the second-generation non-sedating antihistamines astemizole and terfenadine; and (D) the anthracilic acid analogue NPPB.

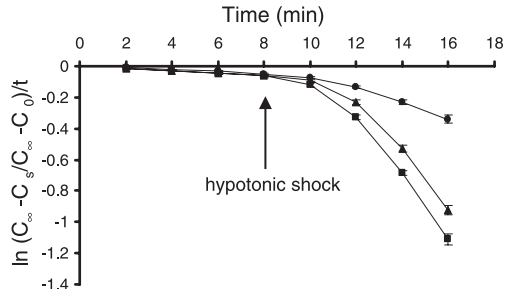

Fig. 2. Efflux of [^{14}C]-taurine in isotonic saline (0–8 min) followed by (10–16 min): hypotonic saline (■), hypotonic saline + terfenadine (3.3 μM) (▲) and hypotonic saline + terfenadine (33 μM) (●). The arrow indicates the exposure of cells to hypotonic solution. Error bars indicate S.E.M. ($n = 3$).

All the antidepressants tested (amitriptyline, imipramine, fluoxetine, fluvoxamine, mianserin and nomifensine) with the exception of nomifensine inhibited osmosensitive taurine efflux at 100 μM by ≥ 50% (as shown in Fig. 3). The effects of these compounds were therefore examined in more detail by studying their concentration–response relationships (Fig. 4A, B and C). These experiments revealed IC_{50} values for osmosensitive taurine efflux inhibition of (in μM): fluoxetine, 13.8 ± 5.4 μM ($n = 3$); fluvoxamine, 24.2 ± 7.5 μM ($n = 3$); amitriptyline, 32.2 ± 4.6 μM ($n = 3$); imipramine, 32.3 ± 1.6 μM ($n = 3$); and mianserin, 39.6 ± 9.2 μM ($n = 3$). Fluoxetine was the most potent and its potency was approximately twice that of fluvoxamine: a finding consistent with the of these compounds on $I_{Cl,vol}$ blockade in cultured endothelial cells (Maertens et al., 2002).

We also examined the effects of compounds targeting oestrogen receptors on osmosensitive taurine efflux, which included the partial oestrogen antagonists tamoxifen and nafoxidine, the oestrogen derivative β-estradiol, and the pure anti-oestrogen ICI 182,780 (Maertens et al., 2001). Only the well-established volume-sensitive organic osmolyte/anion channel blocker tamoxifen reduced osmosensitive taurine efflux by ≥ 50% at 100 μM, although nafoxidine did show a small inhibitory effect (Fig. 3), inhibiting osmosensitive taurine efflux by 36.3 ± 4.1% ($n = 3$) at 100 μM. Fig. 4C shows the concentration–response curve for inhibition of osmosensitive taurine efflux by tamoxifen, from which an IC_{50} value of 9.4 ± 4.0 μM ($n = 3$) was derived.

The effects of the second-generation non-sedating antihistamines, astemizole and terfenadine, were also examined. These compounds blocked osmosensitive taurine efflux with IC_{50} values of 14.0 ± 2.1 μM ($n = 3$) and 12.7 ± 4.0 μM ($n = 3$), respectively. The concentration–response curves for inhibition of osmosensitive taurine efflux by astemizole and terfenadine are shown in Fig 4D.

3.2. Anion exchange and lactate transport in human erythrocytes

To determine the specificity of compounds used in the present study, their effects on the anion exchange pathway (Band 3) and the lactate transport pathway were examined. At 100 μM, out of the compounds tested, only tamoxifen and terfenadine inhibited these pathways by more than 50%, therefore these compounds were deemed nonselective for osmosensitive taurine efflux inhibition over other anion transport pathways. However, most compounds exerted a slight inhibitory effect (see Fig. 5A and B). Control experiments were performed using 100 μM NPPB which, consistent with the previous reports (Branchini et al., 1995; Cranmer et al., 1995), inhibited lactate transport and anion exchange by 96.9 ± 1.6% ($n = 3$) and 15.7 ± 5.2% ($n = 3$), respectively.

3.3. Cell cycle progression analysis

The chloride channel blocker NPPB and the partial oestrogen antagonist tamoxifen were used as positive controls since they have previously been shown to inhibit cell proliferation by arresting cells in the G0/G1 stage of the cell cycle (Shen et al., 2000, 2001). Compounds shown to inhibit osmosensitive taurine efflux by ≥ 50% at 100 μM and which showed specificity over lactate and anion transport were examined following a 48-h exposure, firstly for cytotoxicity, then, provided they were non-cytotoxic, for their effect on cell cycle progression.

Application of amitriptyline, fluoxetine, fluvoxamine, imipramine and mianserin at their IC_{50} concentration for osmosensitive taurine efflux inhibition had no detectable effect on the ratio of stained cells versus the total number of cells over 48 h as determined by trypan blue exclusion, suggesting that these compounds were not cytotoxic at these concentrations (data not shown). However, the second-generation non-sedating antihistamines, terfenadine and astemizole, caused profound cytotoxicity over 48 h at their IC_{50} concentration for osmosensitive taurine efflux, and >90% of the cells present were trypan stained at ≥ 4 and ≥ 10 μM, respectively (data not shown). The effects of compounds which showed selectivity for osmosensitive taurine efflux and no cytotoxicity at 48 h at their IC_{50} for osmosensitive taurine efflux, were examined on cell cycle progression, with NPPB and tamoxifen also included as

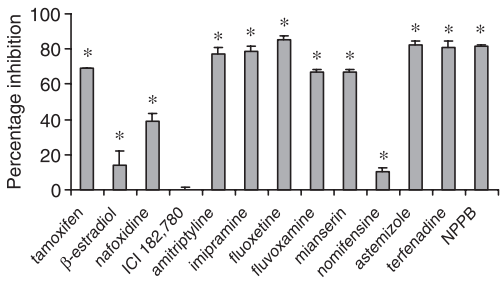

Fig. 3. Effect of test compounds on time-averaged volume-sensitive [^{14}C]-taurine efflux in HeLa cells at 100 μM. Results are presented as percentage of control. *$P \leq 0.05$, as compared to control. Error bars indicate S.E.M. ($n = 3$).

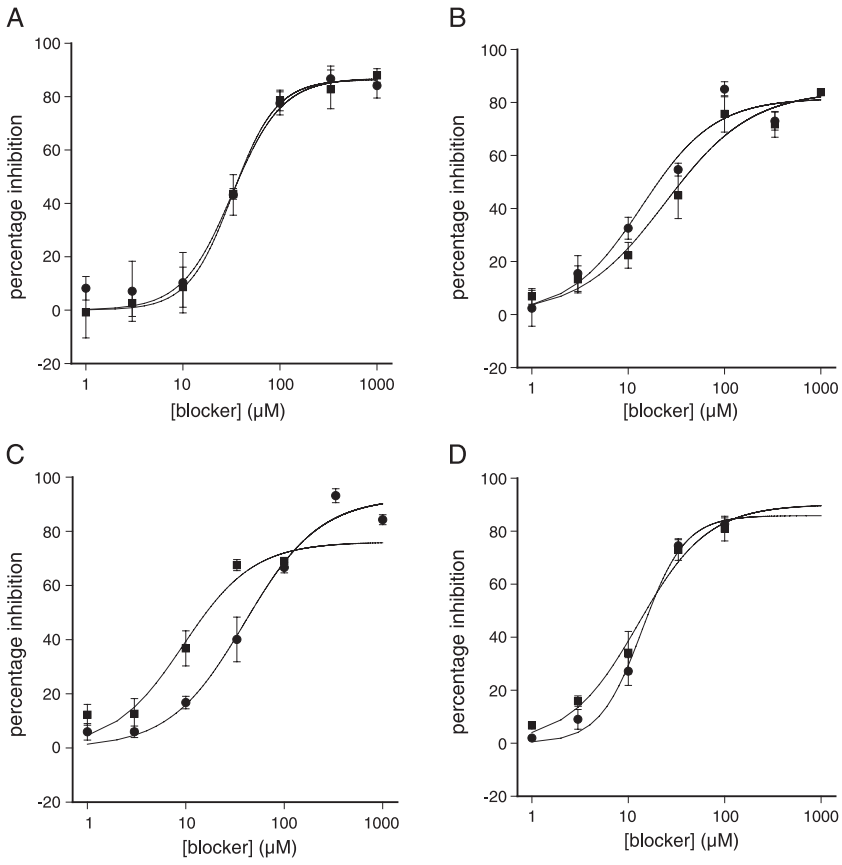

Fig. 4. Concentration–response curves showing the blockade of volume-sensitive [^{14}C]-taurine efflux in HeLa cells by (A) the tricyclic antidepressants amitriptyline (●) and imipramine (■); (B) the selective serotonin reuptake inhibitor antidepressants fluoxetine (●) and fluvoxamine (■); (C) the tetracyclic antidepressant mianserin (●) and the partial oestrogen antagonist tamoxifen (■) and (D) the second-generation non-sedating antihistamines astemizole (■) and terfenadine (●). Error bars indicate S.E.M. ($n = 3$).

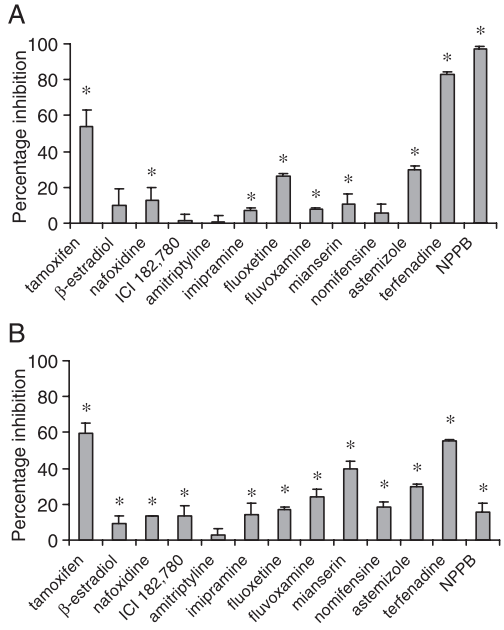

Fig. 5. Effect of test compounds at 100 μM on (A) $^{35}SO_4^{2-}$ transport via the anion exchanger and (B) [^{14}C]-lactate transport via the lactate transporter in human erythrocytes. Results are presented as percentage of control. *$P \leq 0.05$, as compared to control. Error bars indicate S.E.M. ($n = 3$).

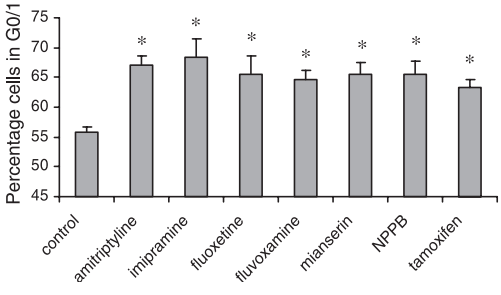

Fig. 6. Effect of the antidepressants amitriptyline, imipramine, fluoxetine, fluvoxamine and mianserin and the two positive control compounds (NPPB and tamoxifen) on cell cycle progression, measured using FACS, showing the percentage of cells in G0/1 stage of the cell cycle after 48-h incubation with the test compound. Each compound was tested at its IC_{50} concentration of osmosensitive taurine efflux inhibition, as derived from Fig. 4. *$P \leq 0.05$, as compared to control. Error bars indicate S.E.M. ($n = 3–4$).

positive controls. Amitriptyline, imipramine, fluoxetine, fluvoxamine, and mianserin significantly increased the percentage of cells arrested in the G0/1 stage of the cell cycle by 8.8–12.4%, as shown in Fig. 6. These values are similar to those obtained for NPPB and tamoxifen at their IC_{50} for osmosensitive taurine efflux (7.4–9.6%), as shown in Fig. 6.

4. Discussion

We have shown for the first time that a group of structurally disparate antidepressant molecules (amitriptyline, imipramine, fluoxetine, fluvoxamine and mianserin) selectively inhibit osmosensitive taurine efflux with IC_{50} concentrations of between 14 and 40 μM. At these IC_{50} concentrations, these compounds inhibit cell cycle progression by increasing arrest in the G0/1 stage of the cell cycle to approximately the same degree as each other (~ 10%). Our data suggest that this occurs through a mechanism associated with inhibition of osmosensitive taurine efflux, but which is independent of cytotoxicity and effects on other anion transport pathways. Based on the similarity of the concentrations required to mediate these effects, our results provide strong evidence for the involvement of volume-sensitive/organic osmolyte ion channel activity in progression through the G0/1 stage of the cell cycle. Due to the structural disparity of the compounds which both inhibited osmosensitive taurine efflux and cell cycle progression, this study also demonstrates the pharmacological promiscuity of the volume-sensitive organic osmolyte/anion channel.

The identification of selective, non-cytotoxic compounds that exert their inhibitory effects on both osmosensitive taurine efflux and cell cycle progression at similar concentrations is an important step in understanding the link between the two processes. Until now, volume-activated chloride and taurine effluxes and their physiological roles have been characterized by their sensitivity to DIDS, NPPB, or tamoxifen (Jentsch et al., 2002). However, in both this and previous studies, these compounds are shown to be highly nonselective and inhibit a number of anion transport pathways (Poole and Halestrap, 1991; Branchini et al., 1995; Cranmer et al., 1995; Skelton et al., 1995). Moreover, their effects are often cell-specific and therefore do not represent reliable pharmacological tools for specific identification and/or modulation of volume-sensitive organic osmolyte/anion channel activity and its physiological role in cell cycle progression. For example, in some cell types, DIDS exerts only a weak blockade of volume-sensitive organic osmolyte/anion channels (Bres et al., 2000), while tamoxifen has been reported to be ineffective in other cell types (Leaney et al., 1997; Nilius et al., 1994). Interestingly, we also show a similar disparity of effects with another partial oestrogen antagonist, nafoxidine, which was a weak inhibitor of osmosensitive taurine efflux in our study, yet Maertens et al. (2001) report potent inhibition using electrophysiological methods in a pulmonary artery endothelial cell-line.

Structure–activity relationship studies have in the past focussed on identifying functional groups that confer specificity and selectivity for blockade of the volume-sensitive organic osmolyte/anion channel, although structural characteristics recognised as important differ between studies. For example, in an extensive structure–activity relationship study using phenol derivatives, hydrophobic non-polar phenols were described as more sensitive than both anionic and hydrophilic molecules (Roy et al., 1998). This is in contrast to Wangemann et al. (1986), who determined that high affinity blockers should be anionic. More recently, it has been suggested that the $-CF_3$ functional group present in fluoxetine confers volume-sensitive organic osmolyte/anion channel blockade (Roy et al., 1998; Mulvaney et al., 2000). Fluvoxamine also contains the trifluoromethyl group, although it is not present in any other compounds in this study, which indicates that the $-CF_3$ group is not the only functional group responsible for conferring inhibition. In the light of this observation, it was important to identify structural elements common to the test compounds that could represent potential functional groups responsible for conferring volume-sensitive organic osmolyte/anion channel inhibition. Examples of such structures include the para-substituted piperidine ring joined to a phenyl ring through a saturated chain, found in astemizole and terfenadine, and the dimethylamine group found in tamoxifen, amitriptyline and imipramine.

This study demonstrates a link between osmosensitive taurine efflux and cell cycle progression which builds on the hypothesis proposed by Shen et al. (2001) that cells in the G0/1 stage of the cell cycle do not progress to the G1/S checkpoint when osmosensitive taurine efflux is inhibited. It is likely that in addition to the volume-sensitive organic osmolyte/anion channel, other ion transport pathways may be involved in modulating cell cycle progression (Day et al., 1993; Guo et al., 1998, Gavrilova-Ruch et al., 2002). Interestingly, cell cycle arrest in the G0/1 stage of the cell cycle has also been attributed to inhibiting the human ether-a-go-go K^+ channel (hEAG), using either antisense oligonucleotides (Pardo et al., 1999) or TEA and astemizole (Ouadid-Ahidouch et al., 2001), providing evidence for the involvement of hEAG in this process. However, in attempting to determine whether inhibition of cell cycle progression by imipramine (a hEAG blocker) is mediated via blockade of the volume-sensitive organic osmolyte/anion channels or hEAG, Gavrilova-Ruch et al. (2002) attributed the inhibition of cell cycle progression to hEAG blockade in the human melanoma cell-line, IGR1. This was because the volume-sensitive organic osmolyte/anion channel blockers DIDS and pamoic acid did not inhibit cell proliferation in this cell-line. Given that the pharmacological profile of the volume-sensitive organic osmolyte/anion channel is different between non-excitable cells such as HeLa and IGR1

(Nilius et al., 1994), it is possible that the anti-proliferative effects of compounds previously attributed to hEAG blockade (such as imipramine and astemizole) may also be due to inhibition of osmosensitive taurine efflux. This highlights the importance of screening cell cycle progression inhibitors on the volume-sensitive organic osmolyte/anion channel before attributing their inhibitory effect to the blockade of other ion channels.

In conclusion, we have used a number of techniques to sieve a collection of compounds which had either an anti-proliferative effect or blocked the volume-sensitive organic osmolyte/anion channel. Our methods revealed that the antihistamines tested showed greater potency of osmosensitive taurine efflux inhibition than NPPB, yet terfenadine was nonspecific for osmosensitive taurine efflux over other anion transport pathways and both were highly cytotoxic. From the compounds targeting oestrogen receptors, only tamoxifen blocked osmosensitive taurine efflux potently. Although tamoxifen was not cytotoxic, it did not show selectivity for osmosensitive taurine efflux over other anion transport pathways. However, the tricyclic, tetracyclic and selective serotonin reuptake inhibitor antidepressants showed selective osmosensitive taurine efflux inhibition, were not cytotoxic and increased the percentage of cells arrested in the G0/1 stage of the cell cycle. These compounds may therefore provide a useful template for designing more potent, selective volume-sensitive organic osmolyte/anion channel blockers for use in clarifying its role in the cell cycle and perhaps other physiological processes.

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