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Screening of anti-glioma effects induced by sigma-1 receptor ligands: Potential new use for old anti-psychiatric medicines

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ABSTRACT

The prognosis of glioblastoma (GBM) remains poor. Diffuse invasion of distant brain tissue by migrating cells from the primary tumour mass has already occurred at time of diagnosis. Anti-cancer effects of a selective sigma-1 agonist, 4-(N-benzylpiperidin-4-yl)-4-iodobenzamide (4-IBP), in glioblastoma were shown previously, leading to the present work where the effects on glioblastoma cells of 17 agonists or antagonists of sigma-1 receptors were studied, including currently marketed drugs fluvoxamine, dextromethorphan, donepezil, memantine and haloperidol. We first showed that established GBM cell lines, primary cultures and surgical specimens express sigma-1 receptors. *In vitro* analyses then focused on anti-proliferation and anti-migratory effects on human glioblastoma cell lines using quantitative videomicroscopy analyses. These cell monitoring assays revealed specific impacts on the mitotic cell process. Using an aggressive glioma model orthotopically grafted into the brains of immunocompromised mice, we showed that combining donepezil and temozolomide gave additive benefit in terms of long survivors as compared to temozolomide or donepezil alone. Clinical study is planned if further rodent dose-ranging studies of donepezil with temozolomide continue to show evidence of benefit in this model.

1. Introduction

Glioblastoma (GBM) accounts for 4–5% of solid tumours in adults and 20% of solid tumours in children.[1] Treatment of GBM is multimodal, including primary surgery when possible, radiotherapy and chemotherapy.[1,2] Prognosis remains dismal. GBM is characterised by diffuse invasion of distant brain tissue by a myriad of single or small groups of migrating cells by the time of initial diagnosis. These migrating GBM cells exhibit decreased levels of apoptosis and increased resistance to cytotoxic efforts.[1,3,4] Proautophagic chemotherapy, including the alkylating drug temozolomide, can partly overcome apoptosis resistance in GBM cells, making temozolomide the current standard of care in GBM.[4] Decreasing the levels of migration in various cancer cell types, including GBM cells,[5] commonly restores a certain level of sensitivity to apoptosis and/or cytotoxic drugs.

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Glioma cells tend to display sigma receptors.[6–9] We recently showed that 4-(N-benzylpiperidin-4-yl)-4-iodobenzamide (4-IBP), a selective sigma-1 agonist, was significantly anti-migratory *in vitro* in the different cancer cell lines analysed, including the highly motile human U373-MG GBM cell line.[7] We also showed that combining 4-IBP with temozolomide significantly augmented the therapeutic benefits of temozolomide alone in a U373-MG orthotopic xenograft rodent model.[7]

Several approved and currently marketed drugs with potent sigma-1 agonist activity are already available, including fluvoxamine, dextromethorphan, donepezil and memantine.[10] Haloperidol is a standard anti-psychotic drug that has been used clinically for five decades and has potent sigma-1 antagonist properties. In the present work we analysed the effects of the above-mentioned four marketed sigma-1 agonists and the antagonist haloperidol, with 12 experimental sigma-1 receptor agonists or antagonist, on proliferation and migration of human U373-MG and T98G GBM cell lines.[11,12] We also tested donepezil in combination with temozolomide for synergy *in vitro* and *in vivo* in the very aggressive glioma model of human Hs683 cells.[13–15]

2. Materials and methods

2.1. Cell cultures, surgical specimens and compounds

2.1.1. Established cell lines

The human Hs683 (ATCC code HTB-138), U373 (ATCC code HTB-17), T98G (ATCC code CRL1690), U87 (ATCC code HTB14), SW1783 (ATCC code HTB-13), A172 (ATCC code CRL-1620), SW1088 (ATCC code HTB-12), A138 (ATCC code HTB-16), H4 (ATCC code HTB-148) and U118 (ATCC code HTB-15) glioma cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in our laboratory as detailed previously.[14]

2.1.2. Primocultures

Primary GBM cell cultures (labelled GL-5, GL-16, GL-17 and GL-19 in the present study) were established at Department of Neurosurgery, Wagner Jauregg Hospital, Linz as described previously.[16,17] Briefly, surgical specimens of histologically confirmed primary GBM from individual patients with their informed consent were blended mechanically and transferred to culture flasks containing growth medium (RPMI 1640, 20% foetal calf serum (FCS) v/v, 1% glutamine v/v, 1% penicillin/streptomycin v/v (PAA Laboratories, Linz, Austria). After passage 3, cells were cultured in growth medium with 10% FCS v/v and 1% glutamine v/v but without antibiotics. Three to five culture flasks were set up from each surgical specimen. Cell cultures were then pooled during the first passages. All cell cultures were periodically checked for mycoplasma contamination using Mycoplasma Stain Kit, Sigma, St. Louis, MO and were discarded if positive.

2.1.3. Surgical specimens

The six GBM clinical specimens in this study were histologically diagnosed according to World Health Organisation classification. These samples were kindly provided by Dr. Isabelle Salmon, head of the Department of Pathology at Erasme University Hospital, Université Libre de Bruxelles, Brussels, Belgium.

2.1.4. Compounds

Temozolomide (TMZ) was purchased from Schering Plough, Brussels, Belgium, and PR-084, 4-IBP, 4-IPAB, haloperidol, BD1047, BD1008, eliprodil, BD1063, fluvoxamine, memantine, BMY14802, (+)-SKF10047, SM-21, IPAG and carbetapentane citrate were purchased from Tocris Bioscience, Bristol, UK. Donepezil and dextromethorphan were purchased from the pharmacy. The characteristics of these 17 compounds are summarised in Table 1 in terms of formula, ligand type and sigma-1 receptor affinity. Compound concentrations were determined on the basis of cell viability monitored using computer-assisted phase-contrast videomicroscopy as described below.

2.2. Standard reverse transcription-polymerase chain reaction (RT-PCR) for the determination of sigma-1 receptor variants in human cancer cells

The procedure used was identical to that we described previously.[7] Two pairs of primers were used to evidence the presence or the absence of five sigma-1 receptor variants in human GBM cells from established cell lines, primary cultures or surgical specimens:

- Primer P1: 5′-gccttctctcgtctgatc-3′ (forward) and 5′-cgtgtactaccgtctcc-3′ (reverse).
- Primer P2: 5′-ggagacggtagtacacg-3′ (forward) and 5′-agcataggagcgaagagt-3′ (reverse).

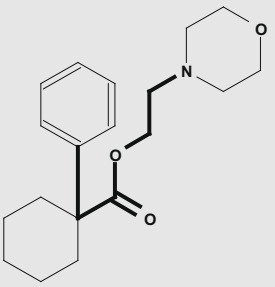
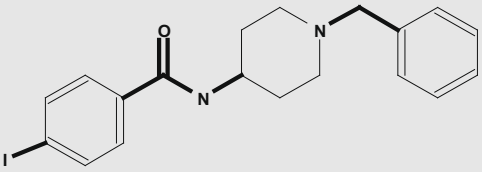
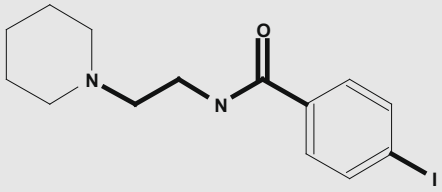
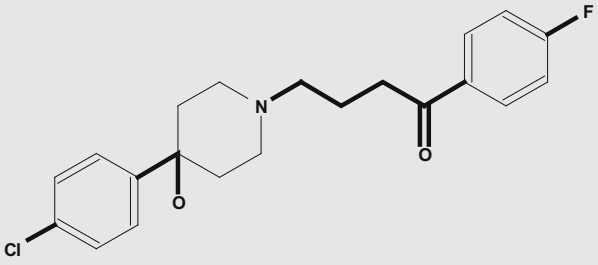
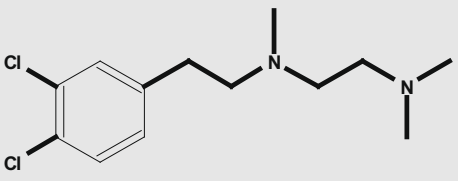
2.3. Computer-assisted phase-contrast videomicroscopy

For human glioma cell lines U373, T98G and Hs683, cell viability, growth, division and cell recolonisation were characterised *in vitro* by the use of computer-assisted phase-contrast videomicroscopy, as described recently.[11] Cells were monitored for 48 h for the scratch wound assays and for 72 h for low cell density cultures used for cell division analyses. Movies were constructed on time-lapse image sequences. This enabled a rapid screening for cell viability. The effects of all 17 compounds on each cell line were first monitored at a 10 μM concentration. The compounds which exhibited cytotoxicity were then analysed at 5 μM and if cytotoxicity remained at 2.5 μM. The final concentrations are indicated in column 'Cell Line' of Table 1 (10 μM if not otherwise specified).

2.3.1. Specific analysis of cell recolonisation

The scratch wound assay was carried out as described previously [7] to monitor GBM cell behaviour in terms of area recolonisation, a process which involves both cell migration and cell replication.[12] The area progressively filled by the cells was identified through a robust method of automatic image segmentation based on the marked watershed transform, as described recently.[11] We measured the recolonised scratch surface across the different frames of the image sequences

Table 1 – Effects of sigma-1 ligands on glioma cell colonisation.

Formula and compound name	Ligand type	Sigma-1 K_i/K_d (nM) (Ref no.)	Cell line	SWAR 2.5%	SWAR 5.0%
PR-084 	Agonist	2 (18)	U373 T98G	1.00 1.02	0.93 0.95
4-IBP 	Agonist	2 (19)	U373 T98G	0.46** 0.75	0.44** 0.41**
4-IPAB 	Selective sigma-1	3 (20)	U373 T98G	1.29 0.69	1.05 1.06
Haloperidol 	Antagonist sigma-1/sigma-2 = 21	2–5 (21)	U373 5 μM T98G 5 μM	0.73 1.02	0.70* 0.72*
BD 1047 	Antagonist	1 (18)	U373 T98G	1.07 1.04	0.93 0.84

(continued on next page)

Table 1 – (continued)

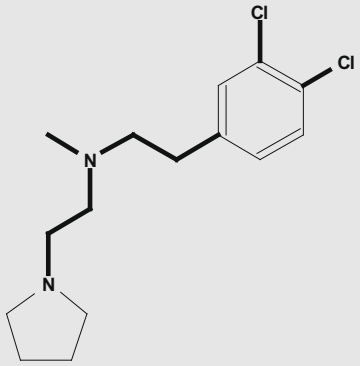
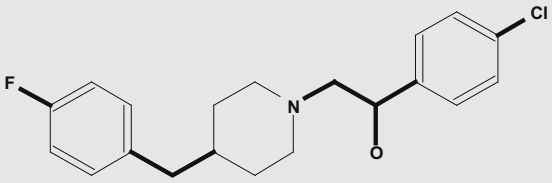
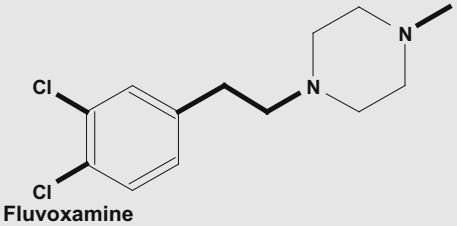
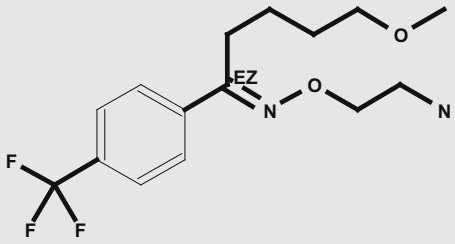
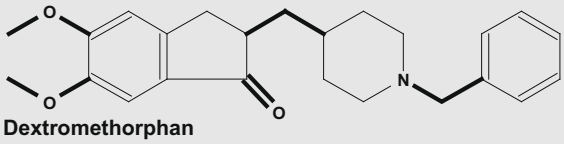
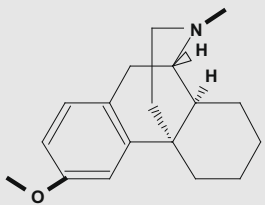
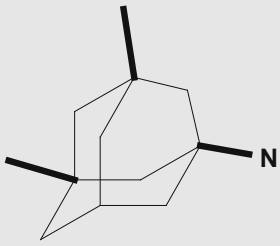
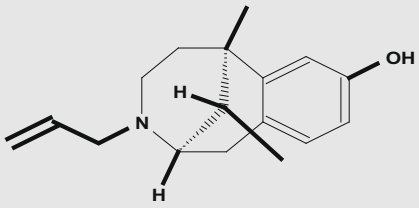
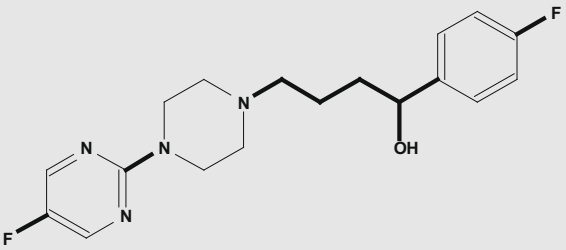
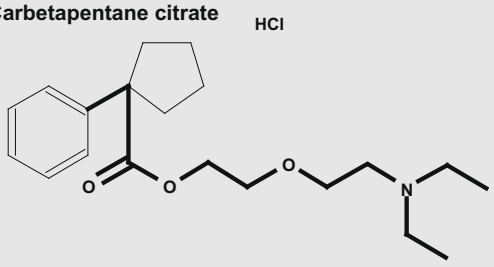
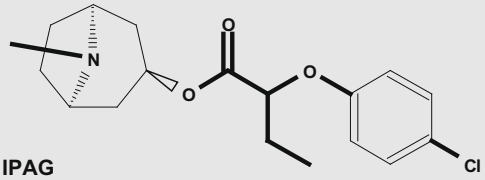
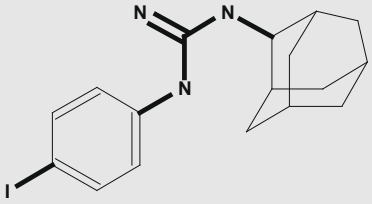
Formula and compound name	Ligand type	Sigma-1 K_i/K_d (nM) (Ref no.)	Cell line	SWAR	
				2.5%	5.0%
BD 1008  **Eliprodil**	Selective sigma-1	2 (22)	U373 T98G	0.72 0.90	0.71 0.71*
 **BD 1063**	Agonist	– (23)	U373 5 μM T98G 5 μM	0.78 0.80	0.71 0.91
 **Fluvoxamine**	Antagonist	6 (24)	U373 T98G	0.83 0.85	1.01 1.12
 **Donepezil**	Agonist	36 (25)	U373 T98G	0.76 0.94	0.78 1.08
 **Dextromethorphan**	Agonist	15 (26)	U373 2.5 μM T98G 2.5 μM	0.66 0.65*	0.34** 0.26**
	Agonist	10 (27)	U373 T98G	0.71 0.65*	1.06 0.93

Table 1 – (continued)

Formula and compound name	Ligand type	Sigma-1 K_i/K_d (nM) (Ref no.)	Cell line	SWAR 2.5%	SWAR 5.0%
Memantine 	Agonist	20,000 (28)	U373 T98G	1.03 1.14	0.87 1.10
(+)-SKF 10047 hydrochloride 	Agonist	0.5–5 (18)	U373 T98G	1.03 0.96	0.95 0.99
BMY 14802 hydrochloride 	Antagonist	112 (29)	U373 T98G	0.86 0.98	0.90 1.01
Carbetapentane citrate 	Selective sigma-1	3 (30)	U373 T98G	0.86 0.87	1.03 0.88
SM-21 	Sigma-2 antagonist	1050 (31)	U373 T98G	0.80 0.78	0.88 0.99
IPAG 	Antagonist	– (32)	U373 2.5 μM T98G 2.5 μM	0.47** 0.47**	0.65** 0.43**

U373 and T98G were monitored during 48 h and 24 h, respectively. The compound concentration used in the experiments was 10 μM if not otherwise specified in column cell line. SWAR (Scratch Wound Area Ratio) is the ratio of the means of the recolonised area percentages between the treated and control conditions in the case of culture media supplemented with 2.5% and 5% of serum. The significance levels (*: $p < 0.05$; **: $p < 0.01$) were evaluated as detailed in Section 2.

by subtracting the cell-free surface measured in the current frame (at time *t*) from that measured in the first frame (at time *t*0). As variations were observed between the initial scratch surfaces measured at time *t*0, we divided all the measurements by the smallest scratch surface measured at *t*0 across all the experiments under comparison. This means that the percentage of wound recolonisation reached 100% when a surface equal to the smallest wound surface was recolonised by cells.

2.3.2. *Cell count-based determination of global growth ratio (GGR)*

In each condition – control or treated –, cell growth level was evaluated by the ratio between the numbers of cells counted in the last and first frames of the image sequences acquired from low cell density cultures. The global growth ratio (GGR) was defined by the ratio between the two growth levels obtained in the treated and control conditions. All the cell counts were performed in triplicate using an interactive computer tool.

2.3.3. *Specific analysis of cell mitotic process*

Cells undergoing division exhibit very bright patterns compared to non-dividing cells. Based on this observation, we developed a custom division detector capable of identifying cells undergoing division in time-lapse sequences. This detector method is based on an automatic event detection completed by an interactive validation/correction procedure, as described previously.[11] Briefly, the candidate dividing cells on each frame were first identified by an adaptive threshold-based segmentation in which only sufficiently large and bright image areas were considered as cell division candidates. In a second stage, the candidates were linked from one frame to the next in order to establish the cell divisions in time. At the end of the sequence analysis, all events were linked into different cell divisions, making the number of cell divisions as well as their durations available. To summarise the compound-induced effects on cell division, we computed two ratios. From the cell division numbers obtained after a given monitoring period, we computed the cell division number ratio (CDNR) which is the ratio between the treated and untreated conditions of the cell division numbers normalised by the number of cells counted in the first frame. Based on the cell division durations, we computed the division time ratio (DTR) which is the ratio of the medians of the cells division durations computed in the treated condition versus control condition.

2.4. *In vivo orthotopic xenografts*

We orthotopically implanted Hs683 cells into the brains of four groups of 11 immunocompromised mice each (6-week-old female nu/nu mice 21 to 23 g; Iffa Credo, Charles Rivers, Arbresle, France), as described previously.[5,7,33] The first group was left untreated as control group. The second group was treated with donepezil 2 mg/kg per os thrice a week (Monday – Wednesday – Friday) during three consecutive weeks, with the treatment starting on the 5th day post-tumour graft. The third group was given temozolomide 40 mg/kg per os according to a protocol identical to the one used for donepezil. The fourth group was treated with combined donepezil and temozolomide as in groups 2 and 3.

2.5. *Statistical analysis*

Control and treated conditions were compared using the non-parametric Mann–Whitney test, except in the case of the scratch wound assay because only three measurements were available per condition. In the latter case, we modelled the distribution of the ratios corresponding to the absence of drug effect (null hypothesis) in a given cell line (U373 or T98G) by computing ratios between pairs of CT experiments for a given compound, i.e. 6 pairs extracted from the 3 CT experiments, giving a total of 102 CT ratios for the 17 compounds per cell line. For each analysed cell line the logarithm transformation of these ratios strongly fits a Gaussian distribution – centred on zero – from which we extracted percentiles 2.5% and 97.5% as thresholds for rejecting the null hypothesis with a significance level of 5%, and percentiles 0.5% and 99.5% as thresholds for rejecting the null hypothesis with a significance level of 1%.

3. Results

3.1. *Identification of sigma-1 receptors in human GBM cells*

Kekuda and colleagues[34] originally cloned functional sigma-1 receptor cDNA from a human placental choriocarcinoma cell (JAR) library using a guinea pig-specific mRNA RT-PCR product.[35] Prasad and colleagues[36] then detailed the structure and organisation of the human gene coding for this sigma-1 receptor. The gene contains four exons interrupted by three introns. A number of researchers then submitted various transcription variants of this mRNA directly to the Gene Bank Data Base (without further scientific publication). These variants are numbered from 1 to 5 and referenced as NM_005866, NM_147157, NM_147158, NM_147159 and NM_147160, respectively. As sigma-1 receptor agonists and antagonists may bind differentially to these five sigma-1 receptor variants,[37] an RT-PCR approach was used to investigate their whole expression pattern in the glioma cell lines, primary explant cultures and surgical specimens.

As shown in Fig. 1, all GBM samples under study whatever be the origin-established cell line, primary culture or surgical specimens express sigma-1 receptors at different levels.

3.2. *Characterisation of sigma-1 receptor agonist- and antagonist-mediated effects on GBM cell colonisation*

Cancer cell colonisation *in vitro*, results from a combination of cell growth and migration processes.[12] The drug-induced effects on GBM cell colonisation were observed through time-lapse imaging of scratch wound models, as illustrated in Fig. 2A and B in the case of 4-IBP administered to U373-MG cells. In our experiments two culture conditions were assayed (in triplicate), i.e. 2.5% and 5% of serum. Fig. 2C and D shows the progression (reported every 4 h) of the percentages of wound recolonisation in the absence (CT) by black dots and in the presence of 4-IBP by open dots. To allow comparisons,

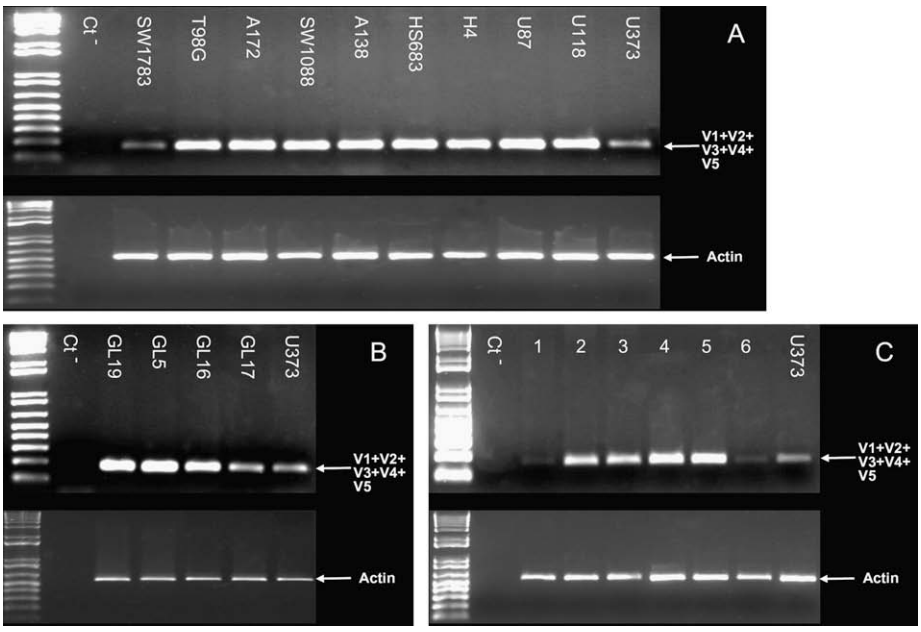


Fig. 1 – Expression of the splice variants of the σ_1 receptor mRNA (V1–V5) assessed by means of RT-PCR analyses in (A) 10 different human glioma cell lines, (B) 4 glioma primocultures and (C) 6 human glioma tissue samples. First lane: 1-kb plus DNA size ladder; Ct: the non-template control (H_2O). In (B) and (C) U373-MG cells are used as control.

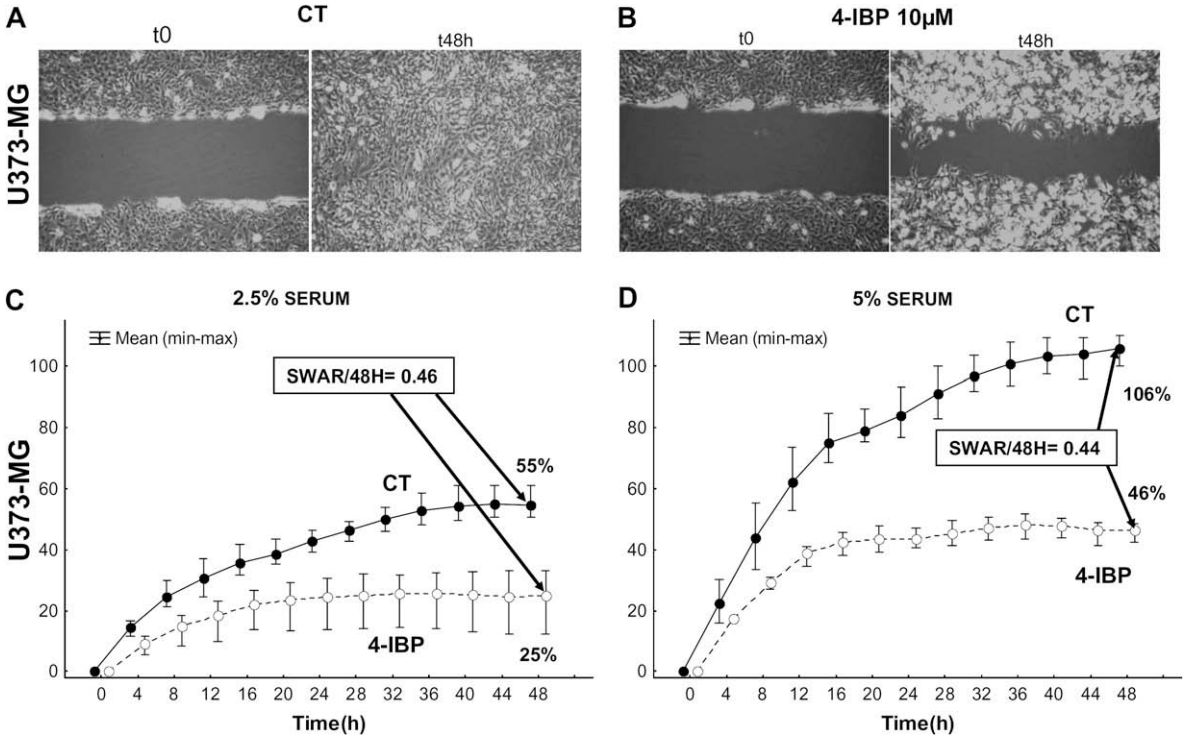


Fig. 2 – (A, B) Illustration of the cell recolonisation process operated during 48 h by CT and 4-IBP-treated U373-MG cells, respectively. (C, D) Cell recolonisation was quantitatively assessed through the percentages of the areas progressively recovered by CT and 4-IBP-treated U373-MG cells in scratch wound models (100% = area of the smallest scratch surface across the compared experiments) with culture media supplemented with 2.5% and 5% of serum, respectively. Each condition was run in three independent experiments. The data are expressed in terms of means and min–max interval values (bars). SWAR (Scratch Wound Area Ratio) is the ratio of the means of the recolonised area percentages.

these percentages were normalised with respect to the smallest initial wound areas and were then represented by the triplicate means (dots) and min–max interval values (bars). Fig. 2 shows that 4-IBP caused an inhibition of the cell colonisation

process in both cell culture conditions and resulted in Scratch Wound Area Ratio, i.e. SWAR, which is the ratio of the average percentages of wound recolonisation between the treated and the untreated conditions, of less than 50% after 48 h of monitoring. This means that 4-IBP induced an inhibition of more than 50% in the colonisation ability of U373 GBM cells.

Table 1 reports the SWAR results similarly evaluated for the 17 compounds on the U373 and T98G GBM cell lines. The significance levels were established by modelling the distribution of ratios characterising the absence of drug effect as detailed in Section 2. Table 1 indicated significant inhibition induced by different compounds, including 4-IBP, donepezil, IPAG and haloperidol. These effects were observed on the two analysed cell lines in the 5%-serum condition.

3.3. *Characterisation of sigma-1 receptor agonist- and antagonist-mediated effects on GBM cell proliferation*

Distinguishing between the contributions of cell migration and cell proliferation in compound-induced effects *in vitro* requires appropriate cell models and correct interpretation of the extracted measurements.[12] This is why we used a new approach, which was recently developed by our group, to extract specific information related to cell division.[11] Fig. 3A and B display two sequences of thumbnails, each centred on a U373 cell detected in division by our method in the control and the 4-IBP-treated conditions, respectively. The green squares indicate the part of each sequence identified as a cell division event from the beginning when the cell exhibits a bright round shape to the end after the separation of the two daughter cells. Fig. 3C reveals that 4-IBP induces a marked decrease of the cell division rate (black columns) as compared to CT (grey columns). After 48 h of monitoring, the cell division number ratio (CDNR) reveals that the cell division rate computed under 4-IBP corresponds to 39% of the control division rate and decreases to 24% after 72 h. Fig. 3D shows that this decrease is associated with a marked increase in the cell division duration characterised by a division time ratio, DTR, of 2 computed between the 4-IBP condition and CT after 72 h.

The same analysis was carried out on the five compounds showing significant SWAR results in Table 1, i.e. 4-IBP, haloperidol, BD 1008, donepezil and IPAG. We also included eliprodil in this analysis because we observed effects on cell

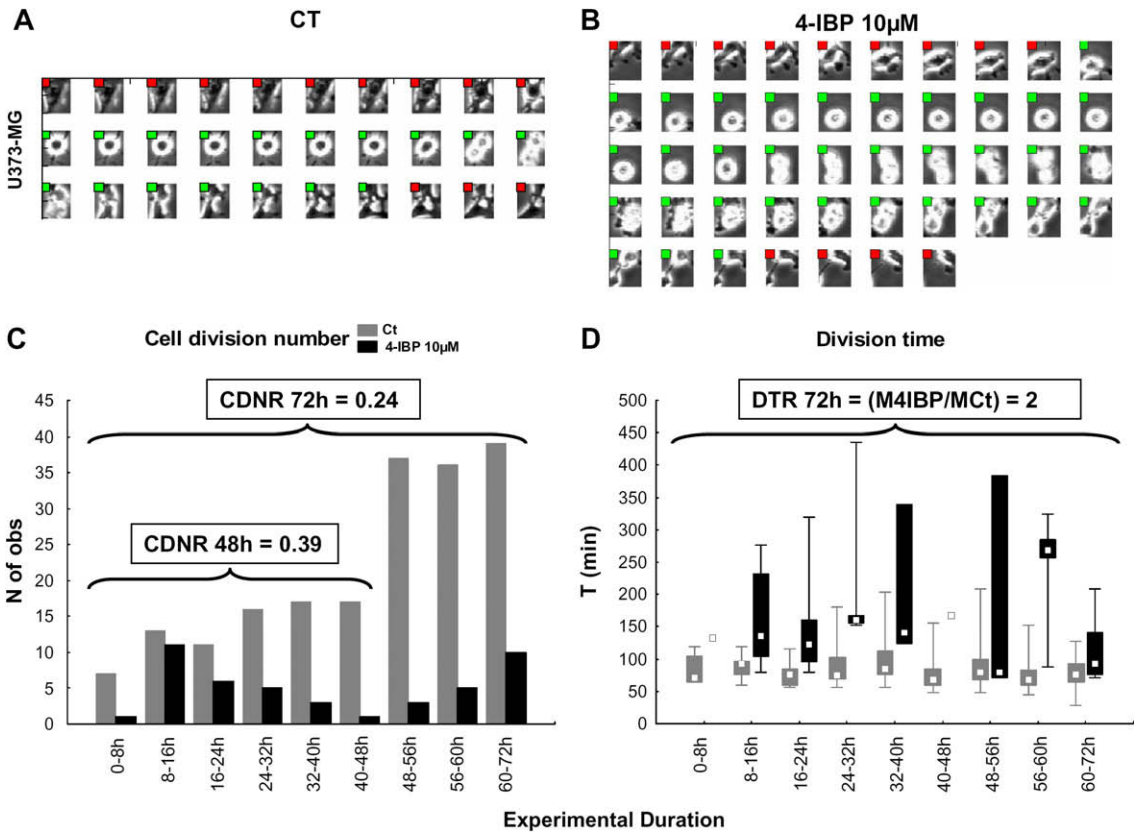
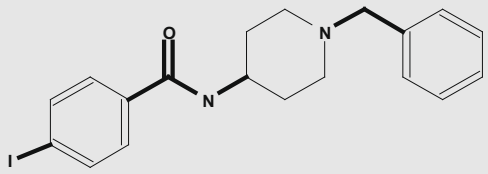
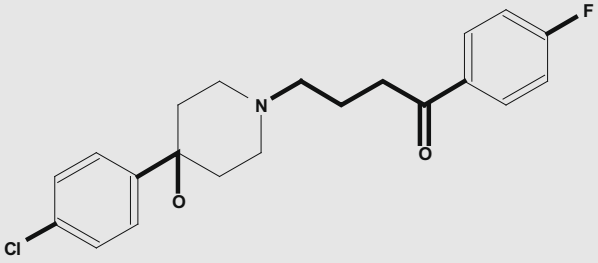
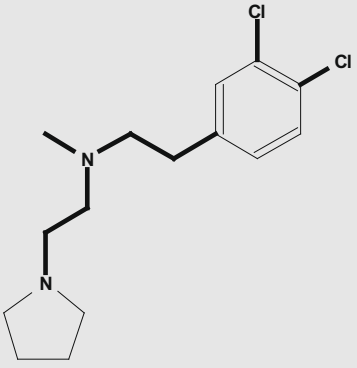
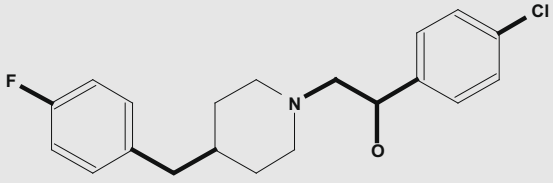
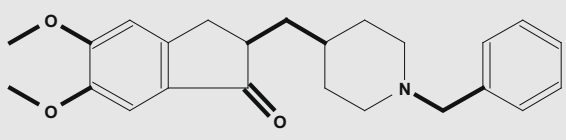


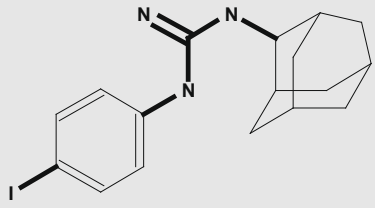
Fig. 3 – (A, B) Two illustrative sequences of thumbnails showing complete cell division events in CT and 4-IBP-treated U373-MG cells, respectively. The green squares indicate the part of the sequences identified as cell division events. (C) Numbers of cell division events detected every 8 h until 72 h for CT (grey columns) and 4-IBP-treated (black columns) U373-MG cells. CDNR (cell division number ratio) is the ratio of the cell division counts (normalised by the number of cells counted in the first frame) computed in treated (%4IBP) condition versus Control (% CT) condition. This ratio was computed after 48 h and 72 h. (D) Distribution of the cell division durations measured every 8 h until 72 h for CT (grey boxes) and 4-IBP-treated (black boxes) U373-MG cells. DTR (division time ratio) is the ratio of the medians of the cells division durations computed in treated condition (M4IBP) versus control condition (MCT).

Table 2 – Effects of sigma-1 ligands on glioma cell division.

Formula and compound name	Cell line	GGR		CDNR		DTR (72 h)
		48 h	72 h	48 h	72 h	
4-IBP 	U373	0.70	0.46	0.39	0.24	2.00***
	T98G	0.58	0.42	0.41	0.33	1.55***
Haloperidol 	U373 5 μM	0.73	0.80	0.55	0.75	1 47***
	T98G 5 μM	0.86	0.80	0.79	0. 76	1.42***
BD 1008 	U373	0.84	0.73	0.86	0.68	1.44***
	T98G	0.67	0.51	0.60	0.50	1.75***
Eliprodil 	U373 5 μM	TBD				
	T98G 5 μM	0.99	0.57	0.62	0.36	1.67***
Donepezil 	U373 2.5 μM	TBD				
	T98G 2.5 μM	0.82	0.40	0.56	0.27	2.71***

(continued on next page)

Table 2 – (continued)

Formula and compound name	Cell line	GGR		CDNR		DTR (72 h)
		48 h	72 h	48 h	72 h	
IPAG 	U373 2.5 μM	TBD				
	T98G 2.5 μM	0.30	0.21	0.20	0.20	1.36***

TBD means 'total blocked division'. GGR (global growth ratio) is the ratio between the two growth levels (which are the ratios between the numbers of cells counted in the last and first frames of the image sequences) obtained in the treated and control conditions. CDNR (cell division number ratio) is the ratio of the cell division numbers (normalised by the number of cells counted in the first frame) computed in the treated versus control condition. DTR (division time ratio) is the ratio of the medians of the cells division durations computed in the treated versus control condition. The significance levels (***: $p < 0.001$) were evaluated by means of the Mann–Whitney test.

division during the prescreening step performed to determine the compound concentrations. All these compounds either significantly increased cell mitosis duration or induced cell mitotic arrests, labelled TBD in Table 2. Cells in mitotic arrest remained in the bright round pattern exhibited at the division beginning and finished by dying after long time periods. Except haloperidol and BD 1008, the compounds more strongly impacted U373-MG cell division than T98G.

These results strongly suggest cytostatic abilities for these compounds, resulting in cell death in the case of completely blocked cell division.

3.4. *Characterisation of in vivo anti-tumour effects of temozolomide and/or donepezil on Hs683 glioma xenografts orthotopically grafted into the brains of immunocompromised mice*

The *in vitro* results motivated us to test donepezil effects *in vivo* using a very aggressive model based on Hs683 glioma cells, as described previously.[33] Fig. 4A illustrates the effects of 2.5 μM donepezil during 72 h on the wound healing processes of Hs683 glioma cells. The resulting data shown in Fig. 4B for Hs683 glioma cells confirm those obtained with T98G and U373 cells in Table 1. In the same manner, Fig. 4C shows that a treatment of Hs683 cells with 2.5 μM donepezil for 72 h blocked a large majority of Hs683 cells in division, as also observed for T98G and U373 cells shown in Table 2. In fact, our cell monitoring revealed that Hs683 cell division was affected after 40 h of this treatment (data not shown).

Before *in vivo* experiments, we analysed the ability of donepezil to modify the *in vitro* sensitivity of Hs683 cells to temozolomide. For this purpose, we compared Hs683 cell behaviour in 4 conditions consisting in Hs683 cells left untreated or pretreated with 2.5 μM donepezil during 40 h, followed by culture medium renewal to which 10 μM temozolomide was added or not added. Fig. 4D describes the cell recolonisation process observed in scratch wounds (carried out after the pretreatment) and evidences a very weak benefit of the combined treatment (see black dots) as compared to donepezil pretreatment alone (see open triangles). In contrast, Fig. 4E evidences increased temozolomide effects on Hs683 cell division after donepezil pretreatment, as compared to all the other conditions. These results also show that temozolomide, alone or in combination with donepezil, affected the cell cultures more in sparse conditions (used for cell division monitoring) than the confluent cultures used in the scratch wound assay. We next tested whether this increased sensitisation to temozolomide is sufficient to intensify its therapeutic benefit *in vivo*.

After having orthotopically implanted Hs683 cells into the brains of immunocompromised mice, we analysed the therapeutic effects of donepezil (open dots in Fig. 4F), temozolomide (open squares in Fig. 4F) and their combination (stars in Fig. 4F). Donepezil alone failed to provide significant therapeutic benefits in contrast to temozolomide which significantly increased the survival of Hs683-grafted mice as compared to controls. Combined treatment donepezil + temozolomide gave additive benefit – an additional 3/11 long survivors as compared to temozolomide or donepezil alone (see Fig. 4F).

4. Discussion

In their review, Aydar and colleagues[38] report that the sigma receptors and their agonists have been implicated in a myriad of cellular functions, biological processes and diseases. These authors state that whereas the precise molecular mechanism(s) of sigma receptors and their involvement in cancer cell biology have not been elucidated, recent work has started to shed some light on these issues. Sigma receptors have been found to be frequently up-regulated in human cancer cells and tissues.[38,39] Proliferation and a variety of other migration-related cellular behaviours such as adhesion, motility and secretion may also be affected by sigma receptor agonists and/or antagonist.[38] As mentioned in the Introduction, we recently showed that 4-(N-benzylpiperidin-4-yl)-4-iodobenzamide (4-IBP), a selective sigma-1 agonist, was significantly

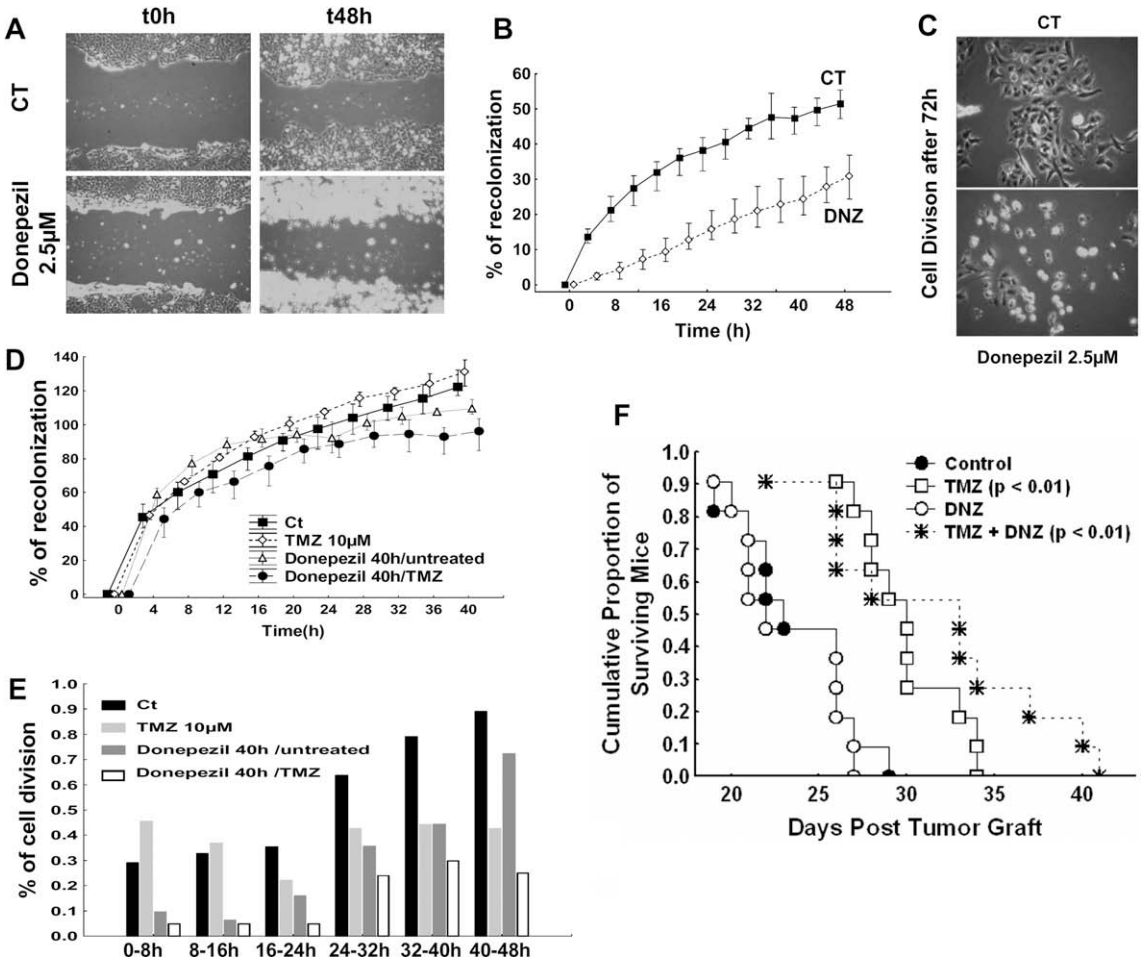


Fig. 4 – (A, B) Cell recolonisation process operated during 48 h by CT and donepezil-treated (DNZ) Hs683 cells and quantitatively assessed by the percentages of the areas progressively recovered as detailed in Fig. 2. (C) As compared to control, donepezil blocked the cell division process of Hs683 cells as evidenced by their bright round patterns. (D) Cell recolonisation process operated during 40 h by Hs683 cells submitted to temozolomide treatment (TMZ 10 μm) or left untreated after donepezil pretreatment (Donepezil 40 h), as compared to control (CT). (E) Percentage of cell division events (i.e. the cell division numbers normalised by the number of cells counted in the first frame) detected every 8 h until 48 h for CT (black columns), 40 h of donepezil pretreatment alone (dark grey columns), and temozolomide-treated Hs683 cells without or with 40 h of donepezil pretreatment (light grey and white columns, respectively). (F) Survival curves of 4 groups of immunocompromised mice orthotopically grafted with Hs683 cells. The mice were either left untreated (control, black dots), or treated with temozolomide (open squares) or donepezil (open dots) or both (stars). Differences between control and each treated condition were evaluated using the log-rank test (only significant results are indicated).

anti-migratory in different cancer cell lines.[7] This may result from modifications to the actin cytoskeleton at least in U373-MG cells.[7] 4-IBP modified the sensitivity of U373-MG cells *in vitro* to enhance the effects of lomustin and temozolomide. 4-IBP markedly decreased expression of two proteins involved in drug resistance: glucosylceramide synthase and Rho guanine nucleotide dissociation inhibitor.[7] *In vivo*, 4-IBP increased anti-tumour effects of temozolomide and irinotecan in immunodeficient mice that were orthotopically grafted with invasive cancer cells[7] in accord with the enhanced cytotoxicity of temozolomide in the presence of donepezil shown here.

In the present study we analysed the anti-glioma effects of several approved and marketed drugs with potent sigma-1 agonist activity – the antidepressant fluvoxamine, the antitussive dextromethorphan, Alzheimer's disease cholinesterase inhibitor donepezil and the amantadine derivative used to treat Alzheimer's disease, memantine.

Haloperidol and donepezil exhibited significant effects on GBM cell colonisation. By using a new approach that is able to extract specific information related to cell division rate and duration *in vitro*,[11] we additionally showed that these two drugs affect GBM cell mitotic process. By monitoring GBM cells over 72 h, we observed mitotic arrest followed by cell death, similarly to microtubule-stabilising drugs which kill cells by inducing a prolonged mitotic block.[40]

Haloperidol, an antagonist of dopaminergic receptors, is one of the oldest drugs still in wide use as an anti-psychotic medicine and is also used commonly to treat delirium, including post-operative delirium. In low and commonly used doses haloperidol shows high affinity antagonism of the sig-

ma-1 receptor.[41] Recently, Wei and colleagues[42] showed that haloperidol causes apoptosis in primary neuronal cultures with an IC50 value of 57 ± 16 μM. The present study reveals that colonisation and proliferation abilities of GBM cells can be inhibited using a lower non-cytotoxic haloperidol concentration (5 μM). Decades of haloperidol use results in increased risk of neuronal damage,[42] as evidenced by tardive dyskinesia (whereas most people do not show any damage signs). Interestingly, our data support haloperidol use at lower doses as a potential anti-glioma therapeutic.

Donepezil is a cholinesterase inhibitor used for over a decade in the treatment of Alzheimer's disease that has coincidentally high affinity and agonist activity at the sigma-1 receptor. A pharmacological activity of donepezil on memory capacities was demonstrated as involving the sigma-1 receptor, and not N-methyl-D-aspartate receptor.[26] Donepezil has also been recently demonstrated to significantly improve cognitive functioning, mood and health-related quality of life in irradiated brain tumour patients.[43] We then analysed the *in vivo* consequences of the strong donepezil-induced effects on glioma cell division evidenced *in vitro*. Using a very aggressive glioma model orthotopically grafted in immunodeficient mice we observed survival benefits for mice treated with temozolomide and donepezil as compared to temozolomide alone. These benefits could be due to donepezil-induced sensitisation of isolated infiltrating glioma cells to temozolomide effects, as suggested by the results obtained *in vitro* on low density cell cultures. These results also agree with recent data showing that prolonged mitosis renders cells hypersensitive to additional prodeath cues.[40]

If further rodent dose-ranging studies of donepezil with temozolomide continue to show evidence of benefit, human study of this combination should be done. Inducing potent cytostatic effects with mitotic arrest as our data show donepezil may be capable of doing in GBM cells, may be an advance in GBM treatment.

Conflict of interest statement

None declared.

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