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# Distribution of sigma receptors in EMT-6 cells: preliminary biological evaluation of PB167 and potential for in-vivo PET

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## Abstract

$\sigma_1$ and $\sigma_2$ receptors have been detected in many tissues and are highly expressed in several tumour cell lines from various tissues. The high level of expression observed for $\sigma$ receptors and their involvement in cell proliferation and apoptosis has led to the development of several $\sigma$ ligands in order to obtain a molecular probe for in-vivo diagnostic imaging techniques such as positron emission tomography (PET) and single photon emission computerized tomography (SPECT). The EMT-6 cells implanted in mice were a good model for evaluating the proliferation of solid tumours by in-vivo PET. Moreover, we developed the $\sigma$ ligand PB167, a cyclohexylpiperazine derivative, previously evaluated for $\sigma_2$ receptor affinity and activity in standard protocols. The related results encouraged us to verify if this compound could be developed as a radiotracer for in-vivo PET in order to visualize $\sigma_2$ receptors expressed in EMT-6 cells when implanted in mice. This perspective was thought to be favourable because PB167 bears a methoxy substituent on the tetraline nucleus, an easy point for $^{11}C$ labelling. The aims of this preliminary study were both to assess the relative distribution of $\sigma_1$ and $\sigma_2$ receptors in EMT-6 cells and to verify if PB167 could be developed as a $\sigma_2$ radiotracer for in-vivo PET. The results showed that both $\sigma_1$ and $\sigma_2$ receptors were overexpressed in EMT-6 cells and that the ligand PB167 can be positively considered for radiosynthesis preparation in order to suitably visualize $\sigma_2$ receptors by the in-vivo PET technique and correlate their presence to tumour proliferation.

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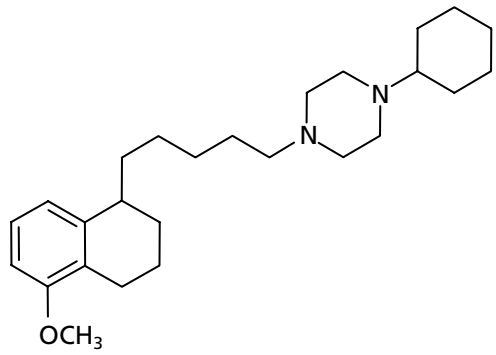
## Introduction

$\sigma_1$ and $\sigma_2$ receptors have been detected in many tissues and are highly expressed in several tumour cell lines from various tissues (Vilner et al 1995; John et al 1996). In particular, the $\sigma_2$ receptors are considered to be potential markers for rapidly proliferating tumour cells (Mach et al 1997; Wheeler et al 2000).

Moreover, both $\sigma_2$ receptor agonists and $\sigma_1$ receptor antagonists display a cytotoxic effect and inhibit cell proliferation in several tumour cell lines, such as neuroblastoma, glioma and sarcoma (Vilner & Bowen 2000; Colabufo et al 2004; Spruce et al 2004). These agents induced apoptosis with p53- and caspase-independent mechanisms in breast cancer cell lines (Crawford & Bowen 2002).

The high level of expression observed for $\sigma$ receptors in tumour cells and their involvement in cell proliferation and apoptosis has led to the development of several $\sigma$ ligands in order to obtain a molecular probe for in-vivo diagnostic imaging techniques such as positron emission tomography (PET) and single photon emission computerized tomography (SPECT) (John et al 1998; Mach et al 2001; Elsinga et al 2002; Kawamura et al 2003a,b; Van Waarde et al 2004). In addition, a new strategy in tumour therapy could be set up based on the use of $\sigma$ agents (Caveliers et al 2001).

Recently, we published the synthesis and biological evaluation of potent $\sigma_2$ ligands. Among them the cyclohexylpiperazine derivative PB167 (Figure 1) displayed high affinity towards $\sigma_1$ ($K_i = 1.52$ nM) and $\sigma_2$ ($K_i = 0.35$ nM) receptors (Berardi et al 2004)




PB167

**Figure 1** The chemical structure of PB167.

and agonist activity at the $\sigma_2$ subtype in an isolated organ bath assay (Colabufo et al 2003). Moreover, in rat C6 glioma PB167 displayed moderate $\sigma_1$ antagonist activity (unpublished results).

Although different compounds were studied displaying high selectivity for the $\sigma_1$ receptor or binding $\sigma_1$ and $\sigma_2$ receptors equally (John et al 1999; Kawamura et al 2000, 2003a; Mach et al 2001), it is uncertain whether or not a high selectivity for the $\sigma_2$ receptor is a strict requirement for developing tumour-imaging agents based on $\sigma$ receptors. Among human and murine tumour cell lines overexpressing $\sigma$ receptors, we selected EMT-6 mammary sarcoma murine cells because (i) their aggressiveness allows a rapid evaluation of tumour xenograft and (ii) they have already been used for the in-vivo assessment of other biological pathways of potential interest in diagnostic imaging (Dearling et al 2002; Barthel et al 2004). Although these cells recently were used for the in-vivo assessment of a potential PET $\sigma_2$ ligand (Rowland et al 2003), the density of $\sigma_1$ and $\sigma_2$ receptors has not yet been described. The aims of this preliminary study were (i) to assess the relative distribution of $\sigma_1$ and $\sigma_2$ receptors in EMT-6 cells by saturation and kinetic analysis with radioligands; (ii) to determine the affinities of PB167 towards both $\sigma$ receptor subtypes expressed in cell lines; and (iii) to verify if PB167 displayed agonist activity at the $\sigma_2$ receptor as proved in an isolated organ bath assay. This study was an important preliminary step to ascertain if PB167, having a methoxy group in the tetrahydronaphthalen-1-yl moiety as an easy point for $^{11}C$ labelling, was a potential radiotracer suitable for the in-vivo PET technique in a mice-implanted EMT-6 model.

## Materials and Methods

Waymouth's medium was purchased from Invitrogen s.r.l. San Giuliano Milanese (Milan, Italy). Trypsin-EDTA, penicillin (10 000 U $mL^{-1}$), streptomycin (10 mg $mL^{-1}$), L-glutamine solution (100 ×) and fetal calf serum were purchased from Celbio s.r.l. (Milan, Italy). Disposable culture flasks and petri dishes were from Corning Glassworks (Corning, NY). The radioligands [$^3$H]-1,3-di-2-tolylguanidine (DTG; 2.15 TBq $mmol^{-1}$) and (+)-[$^3$H]-pentazocine (1291.3 GBq $mmol^{-1}$) were purchased from PerkinElmer Life Sciences (Zavantem, Belgium). (+)-Pentazocine was obtained from Sigma-Aldrich s.r.l. (Milan, Italy). DTG was purchased from Tocris Cookson Ltd, UK.

### Cell culture

The EMT-6 cells were grown in Waymouth's medium with 15% heat-inactivated fetal calf serum, 100 U $mL^{-1}$ penicillin, 100 $\mu$g $mL^{-1}$ streptomycin and 2 mM L-glutamine in a humidified atmosphere of 5% $CO_2$ at 37°C.

### Membrane preparation

The membrane preparations from tumour cell lines were carried out as described by Vilner et al (1995) with minor modifications. Briefly, EMT-6 cells were cultured to 80% confluence, the medium was removed and the cells were rinsed in phosphate saline buffer (PBS). After detaching, the cells were suspended in ice-cold 10 mM Tris-HCl (pH 7.4), containing 0.32 M sucrose and homogenized in a Potter–Elvehjem homogenizer (teflon pestle). The homogenate was centrifuged at 31 000 × $g$ for 15 min at 4°C and the supernatant was discarded. The final pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 7.4) and stored at −80°C until use. The protein content was determined by the Lowry method (Lowry et al 1951).

### Saturation binding assay

The saturation experiments were carried out as described by Vilner et al (1995) with minor modifications. $\sigma_2$ receptor membranes in EMT-6 cells were radiolabelled using [$^3$H]-DTG concentrations ranging from 1.0 to 150 nM. Samples containing 400 $\mu$g of membrane protein, radioligand and 1 $\mu$M (+)-pentazocine to mask $\sigma_1$ receptors were equilibrated in a final volume of 500 $\mu$L of 50 mM Tris-HCl pH 8.0 for 120 min at 25°C. The non-specific binding was determined in the presence of 10 $\mu$M DTG. Incubations were stopped by addition of 5 mL of ice-cold buffer (50 mM Tris-HCl, pH 7.4) and the suspension was then filtered through GF/C pre-soaked in polyethylenimine 0.5% for at least 30 min prior to use. The filters were washed twice with 5 mL of ice-cold buffer. $\sigma_1$ receptors were radiolabelled using (+)-[$^3$H]-pentazocine concentrations ranging from 0.1 to 50 nM. Samples contained 400 $\mu$g of membrane protein and radioligand. The non-specific binding was determined in the presence of 10 $\mu$M (+)-pentazocine. Samples were incubated in a final volume of 500 $\mu$L of 50 mM Tris-HCl pH 8.0 for 120 min at 25°C and the following manipulations were as described above for $\sigma_2$ receptors.

### Competition binding assay

$\sigma_2$ receptors expressed in EMT-6 cells were radiolabelled with 12 nM [$^3$H]-DTG. Samples containing 400 $\mu$g of

membrane protein, 1 $\mu$M (+)-pentazocine to mask $\sigma_1$ receptors, different PB167 concentrations ranging from 0.1 nM to 10 $\mu$M and 10 $\mu$M DTG to determine non-specific binding were incubated in a final volume of 500 $\mu$L of 50 mM Tris-HCl pH 8.0 for 120 min at 25°C and the following manipulations were as described above for saturation $\sigma_2$ receptor assays.

### Kinetic experiments

Association and dissociation experiments were performed as reported by Colabufo et al (2001) with minor modifications. The association kinetic was initiated by addition of EMT-6 membranes in a sample containing incubation buffer (50 mM Tris-HCl, pH 8.0) and 4.4 nM (+)-[$^3$H]-pentazocine or 7.02 nM [$^3$H]-DTG in a total volume of 500 $\mu$L in $\sigma_1$ and $\sigma_2$ association experiments, respectively. The incubation was performed at 25°C for various lengths of time (0–125 min) before filtration. Samples were filtered and washed as reported above in competition binding assays. The specific binding was determined in the presence of 10 $\mu$M (+)-pentazocine or 10 $\mu$M DTG for $\sigma_1$ and $\sigma_2$ association experiments, respectively. In $\sigma_2$ kinetic experiments, 1 $\mu$M (+)-pentazocine was added to mask $\sigma_1$ receptors.

The dissociation kinetic was performed by equilibrating for 30 min at 25°C the samples containing 4.4 nM [$^3$H]-(+)-pentazocine for the $\sigma_1$ kinetic experiment and 7.02 nM [$^3$H]-DTG for the $\sigma_2$ kinetic experiment and 400 $\mu$g of EMT-6 cell membranes in 50 mM Tris-HCl pH 8.0 in a total volume of 500 $\mu$L. In the $\sigma_2$ kinetic experiments, 1 $\mu$M (+)-pentazocine was added to mask $\sigma_1$ receptors. At equilibrium an excess of 10 $\mu$M (+)-pentazocine or 10 $\mu$M DTG was added for the $\sigma_1$ and $\sigma_2$ dissociation experiments, respectively. Samples were filtered and washed as described above.

### Antiproliferative assay

The antiproliferative effect was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported by Mossman (1983). The EMT-6 cells were seeded to 96-well plates in the absence and presence of known concentrations of test compound for 48 h. The medium was removed and replaced with 5 mg mL$^{-1}$ of sterilized MTT solution freshly prepared. The plates containing MTT solution were wrapped in aluminium foil and placed in a 5% $CO_2$ incubator for 4 h at 37°C. Then 100 $\mu$L containing 10% SDS and 50% DMF was added to each well to dissolve the blue formazan crystals. The optical density was measured at 595 nm using an ELISA spectrophotometer (Spectra Shell). Assays were performed in duplicate.

### Cytotoxicity assay

The assay was performed using the CytoTox-One kit from the Promega Corporation (Madison, WI) as reported by Crawford & Bowen (2002) with minor modifications. Cell death was determined as the release of lactate dehydrogenase (LDH) into the culture medium. The percentage of cytotoxicity was calculated relative to the LDH release from total lysis of cells in untreated control. It is assumed here that the drug-treated wells and the control wells contained the same total number of cells (dead plus alive cells) at the end of the treatment period (Crawford & Bowen 2002). Therefore, the cytotoxic effect of tested compounds was unaffected by any underestimation of cytotoxicity that could occur because of the decreased total number of cells in the treated samples compared to the untreated control. Cells were seeded into 96-well plates for optical performance in the fluorescent cell-based assay in 100 $\mu$L of complete medium in the presence or absence of different concentrations of test compounds. The plate was incubated for 24 h in a humidified atmosphere of 5% $CO_2$ at 37°C and 100 $\mu$L of substrate mix in assay buffer was added. Ten microlitres of lysis solution was added to untreated wells in order to estimate total LDH. Plates were kept protected from light for 10 min at room temperature and 50 $\mu$L of stop solution was added to all wells. The fluorescence was recorded using a LS55 PerkinElmer luminescence spectrometer with a 560 nm excitation wavelength and a 590 nm emission wavelength. The cytotoxicity percentage was estimated as follows:

100 × (LDH in medium of treated cells – culture medium background)/(total LDH in untreated cells – culture medium background).

### Statistical method

The saturation results ($K_d$ and $B_{max}$) and kinetic constants ($K_{obs}$, $K_{on}$, $K_{off}$) reported in Table 1 were analysed by non-linear curve fitting utilizing the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, USA). The p$K_i$ values reported in Table 2 were determined by subsequent conversion of $IC_{50}$ to $K_i$ values using the Cheng & Prusoff (1973) equation. The values are means ± s.e.m. from three experiments in triplicate. The $pEC_{50}$ values were obtained from non-linear iterative curve fitting by GraphPad Prism. One-way Anova analysis of variance was used to estimate the significance of difference. Statistical differences in Table 1 were determined by the Mann–Whitney U test. A difference with $P < 0.05$ was considered statistically significant.

## Results

The saturation analysis displayed comparable receptor density expression for both $\sigma$ receptor subtypes ($B_{max} = 826 \pm 50$ and $740 \pm 30$ fmol mg$^{-1}$ of protein for $\sigma_1$ and $\sigma_2$ receptors, respectively). The inhibition constant values were $4.17 \pm 0.30$ nM ($P < 0.0001$, $r = 0.95$) for $\sigma_1$ radioligand (+)-[$^3$H]-pentazocine and $6.41 \pm 0.50$ nM ($P < 0.001$, $r = 0.90$) for $\sigma_2$ radioligand [$^3$H]-DTG as depicted in Figures 2A and 2B, respectively. Kinetic and all binding data are listed in Table 1.

The kinetic $K_d$ values confirm the corresponding results obtained in saturation analysis for each receptor. The $K_d$

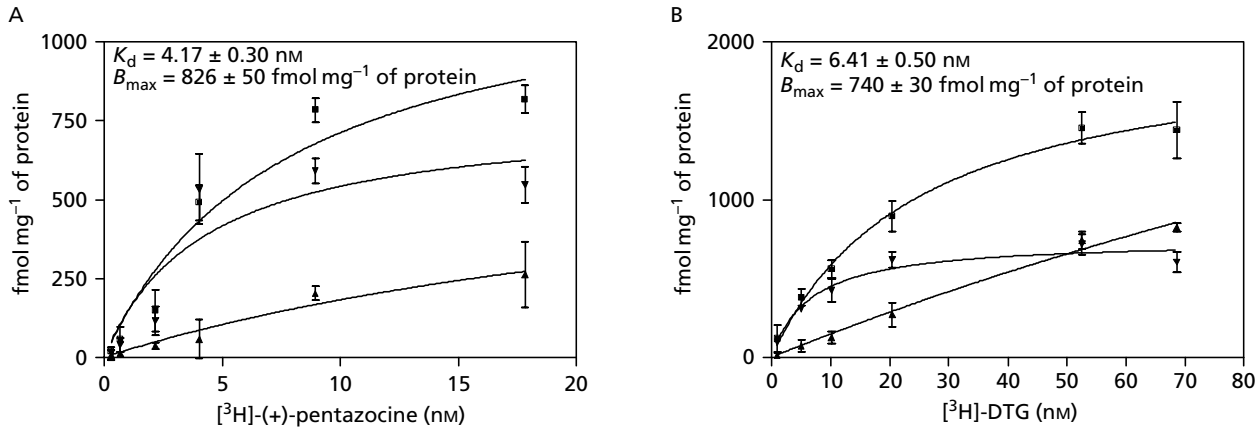



**Figure 2** Representative saturation curves for (+)-[$^3$H]-pentazocine (A) and [$^3$H]-DTG (B) binding at EMT-6 cell membranes. $K_d$ and $B_{max}$ were obtained from specific binding curves ($P < 0.0001$). Values are means ± s.e.m. from three experiments in triplicate. Groups of data were compared with analysis of variance followed by Tukey's multiple comparison tests (total binding vs specific binding, total binding vs non-specific binding, non-specific vs specific binding $P < 0.001$); ■, total binding; ▼, specific binding; ▲, non-specific binding.

**Table 1** Kinetic and saturation binding data of (+)-[$^3$H]-pentazocine and [$^3$H]-DTG at $\sigma_1$ and $\sigma_2$ receptors, respectively

| Receptor | $B_{max}$ (fmol mg$^{-1}$ of protein) | $K_d$ (by saturation) (nM) | $K_d$ ($K_{off}/K_{on}$) (nM) | $K_{off}$ ($s^{-1}$) | $K_{on}$ ($s^{-1}$ $M^{-1}$) | $K_{obs}$ ($s^{-1}$) |
|---|---|---|---|---|---|---|
| $\sigma_1$ | 826 ± 50 | 4.17 ± 0.30 | 4.42 ± 0.55 | 1.35 ± 0.09 | 0.30 ± 0.01 | 2.69 ± 0.72 |
| $\sigma_2$ | 740 ± 30 | 6.41 ± 0.50 | 7.08 ± 0.40 | 4.42 ± 0.10 | 0.62 ± 0.01 | 8.81 ± 0.15 |

The values are means ± s.e.m. from three experiments in triplicate ($P < 0.0001$). The differences in the $\sigma_1$ and $\sigma_2$ receptors with respect to kinetic and saturation binding data were examined using the Mann–Whitney U test ($P = 0.2403$, Mann–Whitney U = 10.50).

values were calculated as the $K_{off}/K_{on}$ ratio by kinetic experiment and were 4.42 ± 0.55 and 7.08 ± 0.40 nM for the $\sigma_1$ and $\sigma_2$ receptors, respectively. The $K_{off}$ constant values obtained in dissociation experiments were 1.35 and 4.42 $s^{-1}$ for $\sigma_1$ and $\sigma_2$, respectively. The association constant values $K_{on}$ were calculated by the formula $K_{obs} - K_{off}$/[radioligand] and were 0.30 and 0.62 $s^{-1} \cdot M^{-1}$ for the $\sigma_1$ and $\sigma_2$ receptors, respectively (Figure 3). In association experiments the $K_{obs}$ constant values were 2.69 and 8.81 $s^{-1}$ for the $\sigma_1$ and $\sigma_2$ receptors, respectively.

The $\sigma_1$ and $\sigma_2$ kinetic and affinity receptor properties were compared by the Mann–Whitney U test. The differences were not statistically significant ($P = 0.2403$) considering that $\sigma_1$ and $\sigma_2$ are receptor subtypes of the sigma receptor family.

Considering the high expression of both $\sigma$ receptors, PB167 was tested to evaluate its affinity at $\sigma_1$ and $\sigma_2$ receptors by competition assays (Table 2). In EMT-6 cells, PB167 displayed high $\sigma_2$ receptor affinity ($K_i = 0.30 \pm 0.10$ nM, $P < 0.0001$) and moderate $\sigma_1$ receptor affinity ($K_i = 16.5 \pm 0.40$ nM, $P < 0.0001$). The $\sigma_2$ receptor affinity was consistent with the corresponding affinity found in the standard binding assay, while the $\sigma_1$ receptor affinity value differed by about one order rank from the corresponding $\sigma_1$ receptor affinity determined in the standard binding protocol (Table 2). Moreover, we studied PB167 for antiproliferative effect at 48 h and cytotoxic effect at 24 h in EMT-6 cells because was it reported that the $\sigma_2$ agonist induced these effects dose dependently. In these assays PB167 displayed both antiproliferative and cytotoxic effects ($EC_{50} = 15.9 \pm 2.60$ μM, $P < 0.0001$, and 56.5 ± 2.80 μM, $P < 0.0001$, respectively) as reported in Table 2. The corresponding dose–response curves are depicted in Figure 4A (antiproliferative effect) and 4B (cytotoxic effect). These results are consistent with the agonist activity of PB167 at the $\sigma_2$ receptor measured in isolated guinea-pig bladder assay, as a functional method to evaluate $\sigma_2$ receptor activity (Colabufo et al 2003).

## Discussion

As stated above, the aims of this study were to ascertain the $\sigma$ receptor distribution in the EMT-6 cells and verify the receptor affinity and activity profile of PB167, a cyclohexylpiperazine derivative synthesized in our laboratories.

The results displayed a large presence of $\sigma_1$ and $\sigma_2$ receptors in the EMT-6 cell line so these cells could be not considered a specific tool for $\sigma_2$ receptor visualization as conversely reported (Rowland et al 2003). Moreover, kinetic experiments displayed an appreciable difference in the association constant ($K_{on}$) and dissociation constant ($K_{off}$) between the two $\sigma$ receptor subtypes. In the association experiments $K_{on}$ was higher for the $\sigma_2$ receptor than the $\sigma_1$ receptor, while in the dissociation study $K_{off}$ was higher for the $\sigma_1$ receptor than the $\sigma_2$ receptor. These findings represent an important aspect to

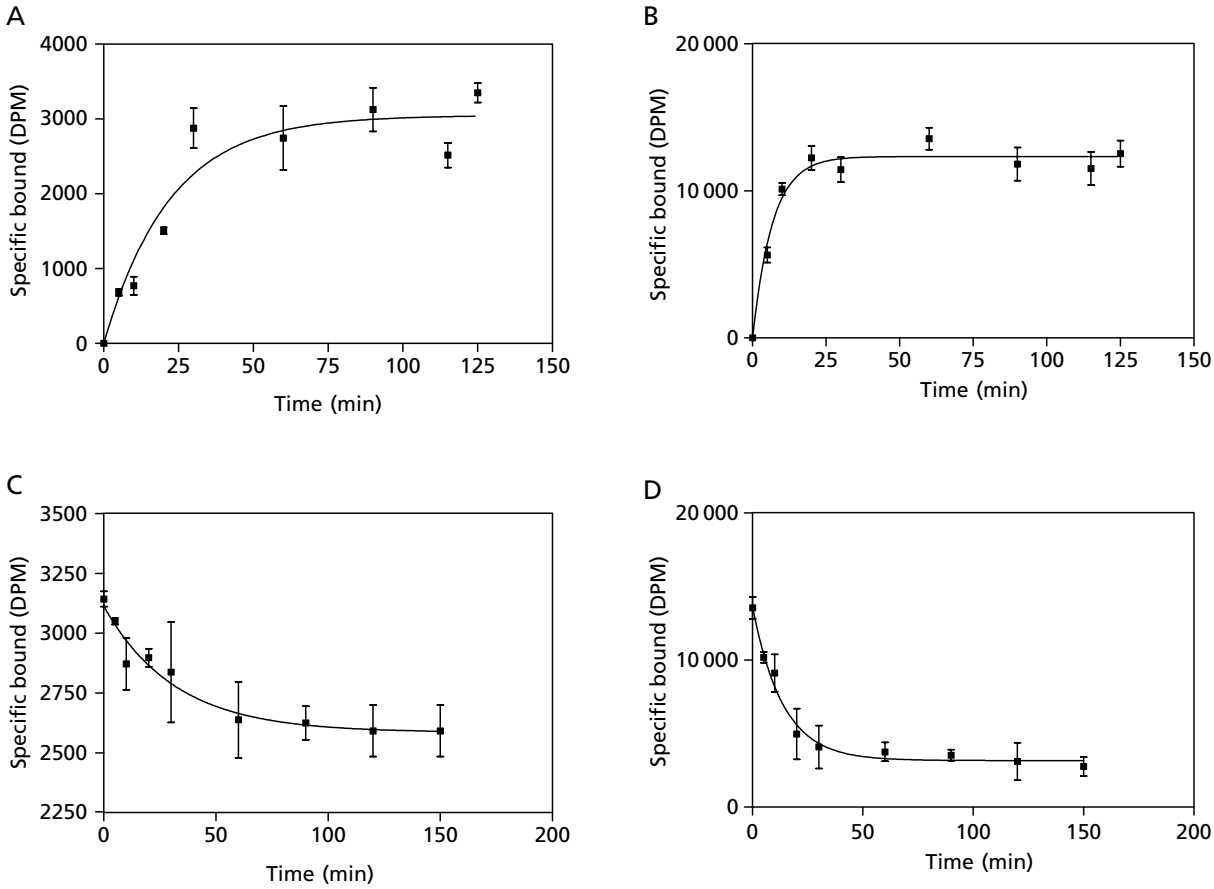



**Figure 3** Representative kinetic curves from three experiments in triplicate of (+)-[$^3$H]-pentazocine and [$^3$H]-DTG binding at EMT-6 cell membranes: (+)-[$^3$H]-pentazocine (4.4 nM) association (A) and dissociation (C) experiments at the $\sigma_1$ receptor; [$^3$H]-DTG (7.02 nM) association (B) and dissociation (D) experiments at the $\sigma_2$ receptor.

**Table 2** Affinity towards $\sigma$ receptors and activity of PB167

| **Affinity** | | **Activity** | |
|---|---|---|---|
| Receptor membranes | $K_i$ (nM)[a] | Functional assay | $EC_{50}$ ($\mu$M)[a] |
| $\sigma_1$ (guinea-pig brain) | $1.52 \pm 0.63$[b] | Inhibition of electrical stimulation (guinea-pig bladder) | $4.90 \pm 0.20$[b] |
| $\sigma_2$ (rat liver) | $0.35 \pm 0.09$[b] | Antiproliferative effect (EMT-6 cells) | $15.9 \pm 2.6$ |
| $\sigma_1$ (EMT-6 cells) | $16.5 \pm 0.4$ | Cytotoxic effect (EMT-6 cells) | $56.5 \pm 2.8$ |
| $\sigma_2$ (EMT-6 cells) | $0.30 \pm 0.10$ | | |

[a]The values are means $\pm$ s.e.m. from three experiments in triplicate. [b]See Berardi et al 2004. The $K_i$ values of the cell subsets were statistically analysed using the Kruskal–Wallis test ($P < 0.0001$, $H = 25.90$). Individual differences between the groups were examined using the Bonferroni post hoc test ($P < 0.005$).

consider for in-vivo PET studies because the specific binding of radiotracer towards $\sigma_1$ or $\sigma_2$ receptors could be discriminated on the basis of the different association and dissociation kinetics of the receptor–radiotracer complex. $K_d$ values by saturation analyses at $\sigma_1$ and $\sigma_2$ receptors for (+)-[$^3$H]-pentazocine and [$^3$H]-DTG were consistent with the corresponding $K_d$ values from the kinetic constants ratio. Nevertheless, [$^3$H]-DTG displayed a higher affinity than was expected ($K_d = 6.41$ nM) at the $\sigma_2$ receptors. In fact to date in several tissues and various cell lines $K_d$ values ranging from 15 to 60 nM have been reported for [$^3$H]-DTG. This discrepancy could be explained by supposing that $\sigma_2$ receptors are in a high affinity state in this cell line and this hypothesis was corroborated by a high velocity association of [$^3$H]-DTG at the $\sigma_2$ receptor.

As regards the ligand PB167, it has been evaluated for $\sigma_2$ receptor affinity and activity in standard protocols (Matsumoto et al 1995; Colabufo et al 2003). The related

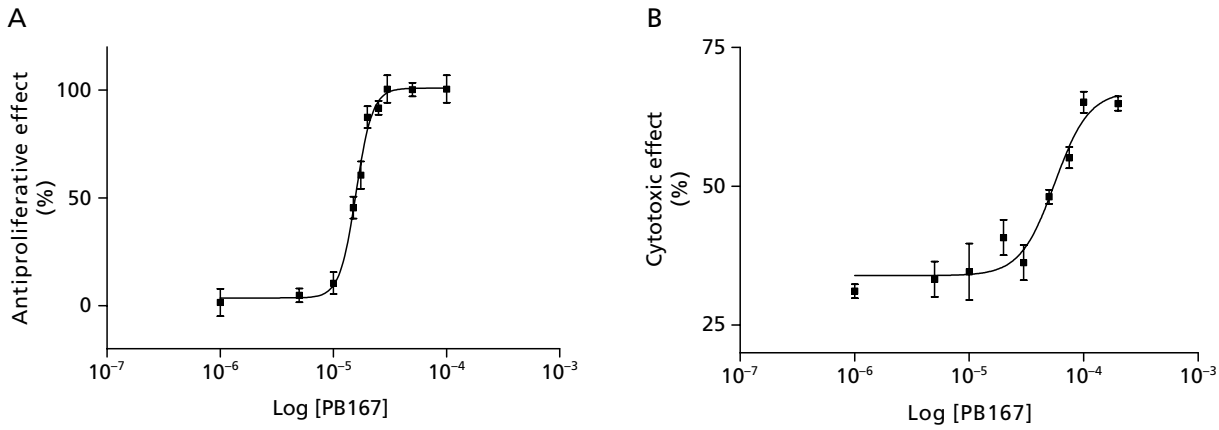



**Figure 4** Representative curves from three experiments in triplicate of antiproliferative (A) and cytotoxic (B) effects for PB167 in EMT-6 cell line.

results encouraged us to verify if this compound could be developed as a radiotracer for in-vivo PET in order to visualize $\sigma_2$ receptors expressed in EMT-6 cells when implanted in mice. Moreover, this perspective was favourably considered because PB167 bears a methoxy substituent on the tetraline nucleus, an easy point for $^{11}C$ labelling.

PB167 displayed good $\sigma_2$ receptor affinity and moderate selectivity towards the $\sigma_1$ receptor ($K_i(\sigma_1/\sigma_2) = 55$-fold) in EMT-6 cell membranes. These results were substantially in accordance with the corresponding $\sigma$ receptor affinity values previously reported. Furthermore, PB167 caused marked antiproliferative and cytotoxic effects that were consistent with the $\sigma_2$ agonist activity previously determined in the isolated guinea-pig bladder by a functional assay to determine only $\sigma_2$ receptor activity.

The antiproliferative and cytotoxic effects exerted by PB167 in the EMT-6 cells disclosed a new perspective for in-vivo diagnostic and therapeutic performances of $\sigma$ ligands towards solid tumours overexpressing $\sigma$ receptors.

This preliminary study suggests that the EMT-6 cells implanted in mice can be a good tool for evaluating the proliferation of solid tumours. Moreover, the ligand PB167 can be positively considered for a radiosynthesis preparation in order to suitably visualize $\sigma_2$ receptors by the in-vivo PET technique and correlate their presence to tumour proliferation.

Compound PB167 could radiolabel several areas in the central nervous system where $\sigma_2$ receptors have been recognized by autoradiographic visualization in rats using [$^3$H]-DTG (Bouchard & Quirion 1997). In this study a high level of $\sigma_2$ receptors was found in the motor cortex, nucleus accumbens, substantia nigra, central grey matter, oculomotor nucleus and cerebellum. On the other hand, it is true that high ClogP values could determine high specific binding as for PB167 (ClogP = 6.24). The [$^{11}$C]-PB167 radiosynthesis and its in-vivo PET evaluation in EMT-6 cells will be presented in a later study.

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