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Tritium radiolabelling of PB28, a potent sigma-2 receptor ligand: pharmacokinetic and pharmacodynamic characterization

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Abstract—A potent sigma-2 receptor ligand, known as PB28, was tritium radiolabelled and biologically evaluated. The results showed that [^{3}H]PB28 and the corresponding unlabelled PB28 had superimposed pharmacodynamic properties. This radioligand appears as a potential candidate for receptor binding and in living cells assays.

Sigma receptors are endoplasmic binding sites clearly distinct from other known receptors both in pharmacology and structure. Two subtypes of sigma receptors have been identified, sigma-1 and sigma-2, different for distribution in tissues and for pharmacology and physiology.[1] Interest in sigma receptor research is growing as much evidence shows how sigma proteins, and in particular sigma-2 subtype, are markedly overexpressed in many tumour cell lines.[2,3] Thus, sigma receptor ligands are now being developed for the imaging of the two subtypes with noninvasive techniques (i.e., PET and SPECT) providing information about the stage of the tumour.[4,5] Besides this diagnostic importance, sigma-2 receptor agonists display a noteworthy therapeutic potential in the oncology field.

Despite these claimed potentials, sigma-2 receptors have not been isolated yet. In the last few years our group developed 1-cyclohexyl-4-[3-(5-methoxy-1,2,3,4-tetrahydro-naphthalen-1-yl)propyl]piperazine known as PB28 (Fig. 1).[6,7] This compound displayed high sigma-2 receptor affinity (K_i = 0.34 nM)[3,7] and at present, it is one of the best sigma-2 ligands known in the literature so that PB28 is commercially available as a pharmacological tool for sigma-2 receptor research. To contribute to the isolation and characterization of sigma-2 receptors, our group modified PB28 and linked it to a stationary phase.[8] The elution of the protein content from human SK-N-SH neuroblastoma cell line overexpressing sigma-2 receptors led to isolate several histone proteins. Therefore, we concluded that overexpressed sigma-2 receptors are likely histones or that sigma-2 ligands bind histones.

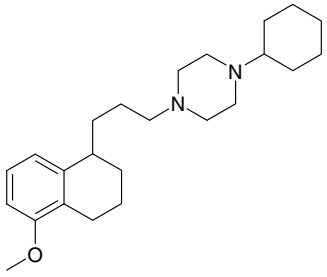

Figure 1. PB28 structure.

Several pharmacological investigations have been carried out both in living cells[3] and in guinea-pig ileum and bladder.[9] In all biological assays PB28 displayed sigma-2 receptor agonist properties. In fact, it showed cytotoxicity by activating apoptotic pathways through the Ca^{++} release by inositol 1,4,5-Tris–phosphate receptor modulation.[10] At present, PB28 is the first sigma-2 receptor ligand displaying high affinity and high agonist activity towards sigma-2 receptors reported in the literature. On the other hand, SM-21 is reported as the best sigma-2 ligand displaying antagonist activity.[11,12]

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Moreover, PB28 has been ^{11}C-radiolabelled as a potential probe for *in vivo* PET studies. Unfortunately, ^{11}C-PB28 failed as a PET probe due to non-specific binding in the CNS.[13]

In MCF-7/Adr cell line overexpressing sigma-2 receptor, our compound highly modulated P-gp mRNA level and also inhibited P-gp activity.[14] The unsolved question was to establish if these effects were sigma-2 receptor mediated or PB28 per se interfered with P-gp activity.

In order to better elucidate the role of sigma-2 receptors and the involvement of PB28 in P-gp modulating activity, we planned to ^{3}H-label PB28. In the present paper we reported the synthesis and the pharmacodynamic characterization of [^{3}H]PB28.[15]

The synthesis of [^{3}H]PB28 is depicted in Scheme 1 and partially differs from the one previously reported for nonradiolabelled PB28.[6]

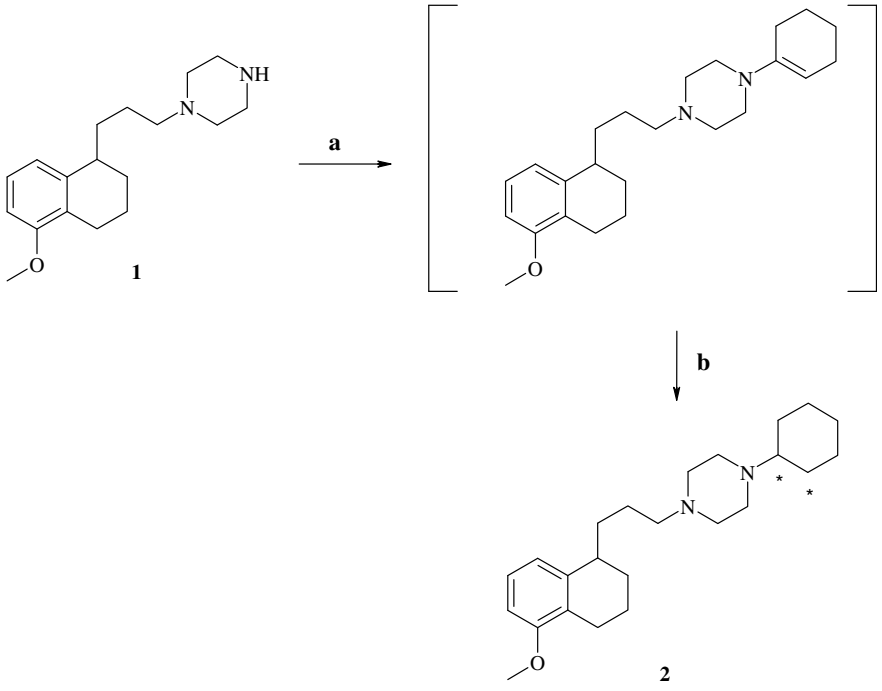
The synthetic strategy was aimed to label the compound in the final step. The already known derivative **1**[6] was condensed with cyclohexanone providing the corresponding enamine, which was reduced with $NaBT_4$ (specific activity 15 Ci/mmol) to the final [^{3}H]PB28. The enamine reduction by $NaBT_4$ was easy and quantitative. [^{3}H]PB28 was obtained in high yield after being purified by column chromatography (silica gel, eluent $CHCl_3$/MeOH 19:1) and the radiochemical purity (>96%) was determined by TLC. [^{3}H]PB28 UV–vis spectrascopy was carried out in order to compare the spectroscopic properties of unlabelled PB28 with those of labelled PB28 (Fig. 2A and B), respectively). [^{3}H]PB28 was dissolved in absolute EtOH and its specific activity was determined (8 mCi/mmol).

[^{3}H]PB28 was tested in biological assays using standard protocol[16] and its K_d value (0.12 nM) was determined in rat liver, masking sigma-1 receptors with (+)-pentazocine, as K_{off}/K_{on} ratio (Figs. 3 and 4, respectively). These kinetic constants were obtained by association experiment ($K_{on} = 0.1836 \pm 0.057$ min^{-1} nM^{-1} and $t_{1/2} = 3.77$ min) and dissociation experiment ($K_{off} = 0.0220 \pm 0.0096$ min^{-1} and $t_{1/2} = 45$ min). In each kinetic experiment 120 nM [^{3}H]PB28 was employed with the maximal receptor occupancy obtained at this concentration. Moreover, as depicted in Figure 5, in the rat liver membranes [^{3}H]PB28 displayed $K_d = 0.13 \pm 0.03$ nM by saturation analysis. Furthermore, high sigma-2 receptor density was found (497 ± 34 fmol/mg of protein) in this tissue. K_d found by saturation analysis matched with the corresponding K_d found in kinetic experiments (0.13 nM and 0.12 nM, respectively).

Starting from these results, competition binding assays were performed using 12 nM [^{3}H]PB28 and evaluating the receptor affinity of sigma-2 receptor reference compounds such as DTG, haloperidol and PB28, (Fig. 6).

K_d value of radiolabelled PB28 from kinetic experiments (0.12 nM) fitted the K_i value of the corresponding unlabelled PB28 (0.34 nM) already reported.[7] Moreover, in binding competition assays, the reference compounds, DTG, haloperidol and PB28 displayed K_i values (42.0 nM, 5.85 nM, 0.57 nM, respectively) superimposable on those determined in standard protocol with [^{3}H]DTG as radioligand (28.2 nM,[17] 7.38 nM,[3] 0.34 nM,[17] respectively).

Since binding results of labelled ($K_d = 0.12$ nM) and unlabelled PB28 ($K_i = 0.34$ nM) were comparable, it is



Scheme 1. Radiosynthesis of [^{3}H]PB28. Reagents: (a) cyclohexanone, TFA; (b) $NaBT_4$.

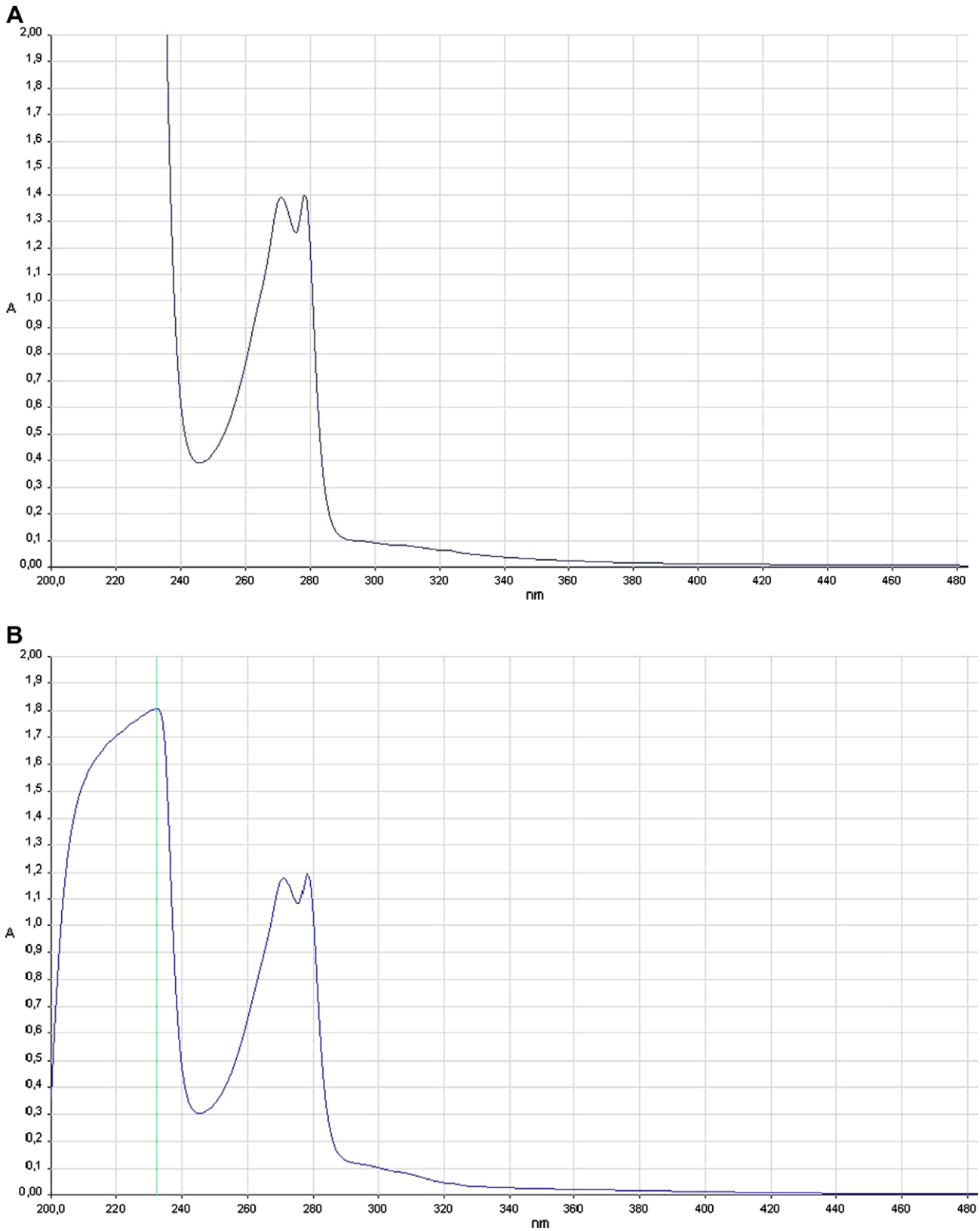


Figure 2. (A) Unlabelled 10^{-3} M PB28 UV spectra (EtOH, λ_{max} 277 nm, $\varepsilon = 1396$). (B) 10^{-3} M [^{3}H]PB28 UV spectra (EtOH, λ_{max} 277 nm, $\varepsilon = 1396$).

possible to assess that PB28, both in labelled and in unlabelled form, selectively binds sigma-2 receptors.

Recently, Xu et al.[18] reported the radiosynthesis and the biological evaluation of a selective sigma-2 ligand known as [^{3}H]RMH-1. This compound, when tested as unlabelled compound, displayed moderate sigma-2 receptor affinity ($K_i = 10.3$ nM) and sigma-1/sigma-2 selectivity ratio >400. When this compound was tested as [^{3}H]RMH-1, it displayed sigma-2 receptor affinity

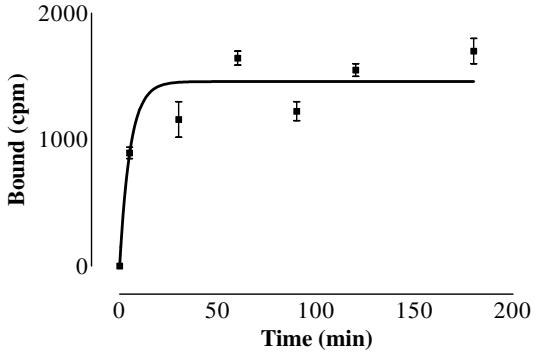


Figure 3. Sigma-2 association experiment.

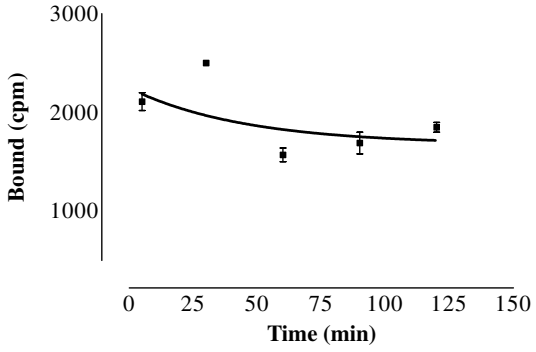


Figure 4. Sigma-2 dissociation experiment.

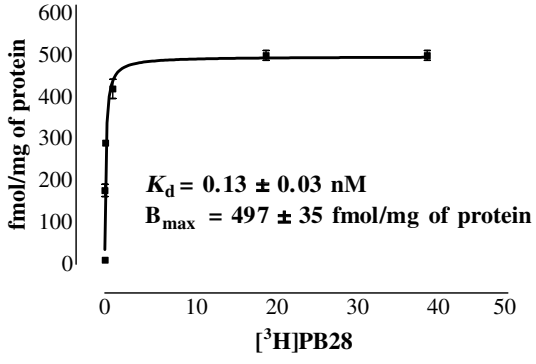


Figure 5. Saturation binding analysis with [^{3}H]PB28 towards sigma-2 receptors in rat liver membranes.

higher than the unlabelled counterpart (K_d = 0.66 nM). Moreover, several reference compounds have been tested for determining their sigma-2 receptor affinity employing [^{3}H]RMH-1 as radioligand in competition binding assays. These compounds displayed sigma-2 affinity values comparable to the corresponding results obtained using [^{3}H]DTG. These results led the authors to suggest that [^{3}H]DTG and [^{3}H]RHM-1 bound the same sites. However, the discrepancy between cold and labelled RHM-1 remains to be clarified yet. By contrast, [^{3}H]PB28 and the unlabelled counterpart, displayed similar value (K_d = 0.12 nM and K_i = 0.57 nM, respectively). However, [^{3}H]PB28 displayed lower selectivity (about 40-fold) towards sigma-1 receptor (K_i = 13.6 nM) than [^{3}H]RMH-1.

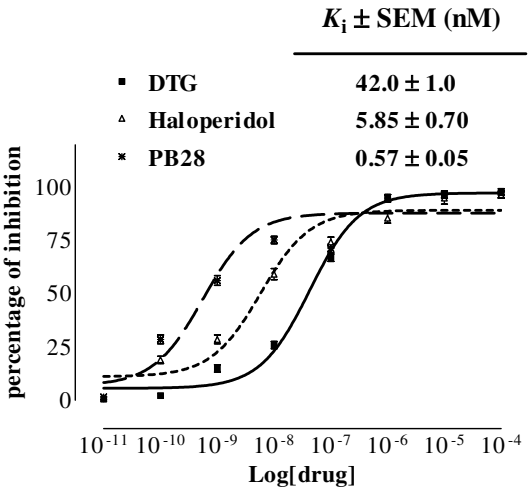


Figure 6. Inhibition curves in sigma-2 competition binding assay.

These results evidenced that [^{3}H]PB28 showed a worse selectivity towards sigma-1 receptor than [^{3}H]RMH-1, but the receptor binding profile towards sigma-2 receptor of our radiolabelled compound is more reliable.

These preliminary results prompted us to plan future experiments on living cells in order to identify the localization of sigma-2 subtypes in the different cell compartments.

In conclusion we have developed an efficient method to radiolabel PB28 starting from the readily obtainable intermediate **1** through an easy reductive amination of the cyclohexanone. The biological results given by [^{3}H]PB28 and its unlabelled counterpart were perfectly superimposed.

References and notes

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The dissociation kinetic experiment was performed by equilibrating for 120 min at 25 °C the samples containing 120 nM [^{3}H]PB28 and 500 μg rat liver cell membranes in 50 mM Tris–HCl, pH 8.0, in a total volume of 500 μL. Moreover, 1 μM (+)-pentazocine was added to mask σ_1 receptors. At equilibrium, an excess of 10 μM DTG was added for σ_2 dissociation experiment and the samples were filtered and washed.
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