

Conjugation of haloperidol to PEG allows peripheral localisation of haloperidol and eliminates CNS extrapyramidal effects

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To link to this article DOI: <http://dx.doi.org/10.1016/j.jconrel.2020.02.037>

Publisher: Elsevier

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Conjugation of haloperidol to PEG allows peripheral localisation of haloperidol and eliminates CNS extrapyramidal effects

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Abstract

We have previously reported the synthesis of a poly(ethylene glycol)-haloperidol (PEG-haloperidol) conjugate that retained affinity for its target D₂ receptor and was stable in simulated physiological conditions. We hypothesised that this polymer-drug conjugate would localise haloperidol's activity either centrally or peripherally, dependent on the location of administration, due to the polymer preventing penetration through the blood-brain barrier (BBB). Herein, we validate this hypothesis using *in vitro* and *in vivo* studies. We first demonstrate, *via* a [³⁵S]GTPγS-binding assay, that drug activity is retained after conjugation to the polymer, supportive of retention of effective therapeutic ability. Specifically, the PEG-haloperidol conjugate (at 10 and 100 nM) was able to significantly inhibit dopamine-induced G-protein activation *via* D₂ receptors, albeit with a loss of potency compared to the free haloperidol (~18-fold at 10 nM). This loss of potency was further probed and rationalised using molecular docking experiments, which indicated that conjugated haloperidol can still bind to the D₂ receptors, albeit with a flipped orientation in the binding pocket within the receptor, which may explain the reduced activity. Finally, rat catalepsy studies confirmed the restricted permeation of the conjugate through the BBB *in vivo*. Rats treated intravenously with free haloperidol became cataleptic, whereas normal behaviour was observed in rats that received the PEG-haloperidol conjugate, suggesting that conjugation can effectively prevent unwanted central effects. Taken together these results demonstrate that conjugating small

molecules to polymers is effective at prohibiting penetration of the drug through the BBB and is a valid targeting strategy for drugs to facilitate peripheral (or central) effects without inducing side effects in other compartments.

Keywords:

Nanotechnology, nanomedicine, polymer-drug conjugate, blood-brain barrier, haloperidol, PEG.

1. Introduction

Penetration through the blood-brain barrier (BBB) is recognised as a significant challenge when developing therapeutic agents for diseases of the central nervous system, for example, for psychiatric disorders and Alzheimer's disease [1]. What is less well articulated, however, is the requirement to prevent certain peripheral therapeutic drugs from crossing the BBB [2,3]. This need can be demonstrated by considering first-generation antihistamine agents where beneficial peripheral effects can be compromised by unwanted sedative effects when the drug crosses the BBB and acts centrally [4]. Since the size is a key parameter affecting permeability across the BBB, with 500 Da typically being considered as the maximum threshold for BBB permeability, one strategy to reduce penetration through the BBB is to form a polymer-drug conjugate (PDC) [5,6]. A very limited number of studies (three to the best of our knowledge) have previously demonstrated that PDCs can indeed restrict drug activity to peripheral organs and prevent undesired effects in the CNS. A PDC of an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer and TNP-470, an anti-tumour agent, using a biologically labile tetrapeptide linker, significantly reduced TNP-470-related neurotoxicity in comparison to the free TNP-470 [7]. Movantik[®], a PDC in which naloxol (an opioid antagonist) is covalently linked to poly(ethylene glycol) (PEG) *via* a biologically stable ether linkage, has been used clinically to prevent opioid-induced constipation [8]. Third, and of particular relevance to the work presented here, we have reported the synthesis and characterisation of a non-prodrug PDC in which the D₂ antagonist haloperidol was conjugated to PEG *via* a biologically stable carbamate linkage (Fig. 1) [9]. We demonstrated stability of the linker *in vitro* and presented evidence of potential activity by demonstrating that the PEG-haloperidol conjugate retains binding affinity (albeit reduced) to D₂ receptors; we also showed initial evidence that PEG conjugation could prevent haloperidol crossing the BBB through a simple single *in silico* equation, based on methodology reported in Fu et al [10].

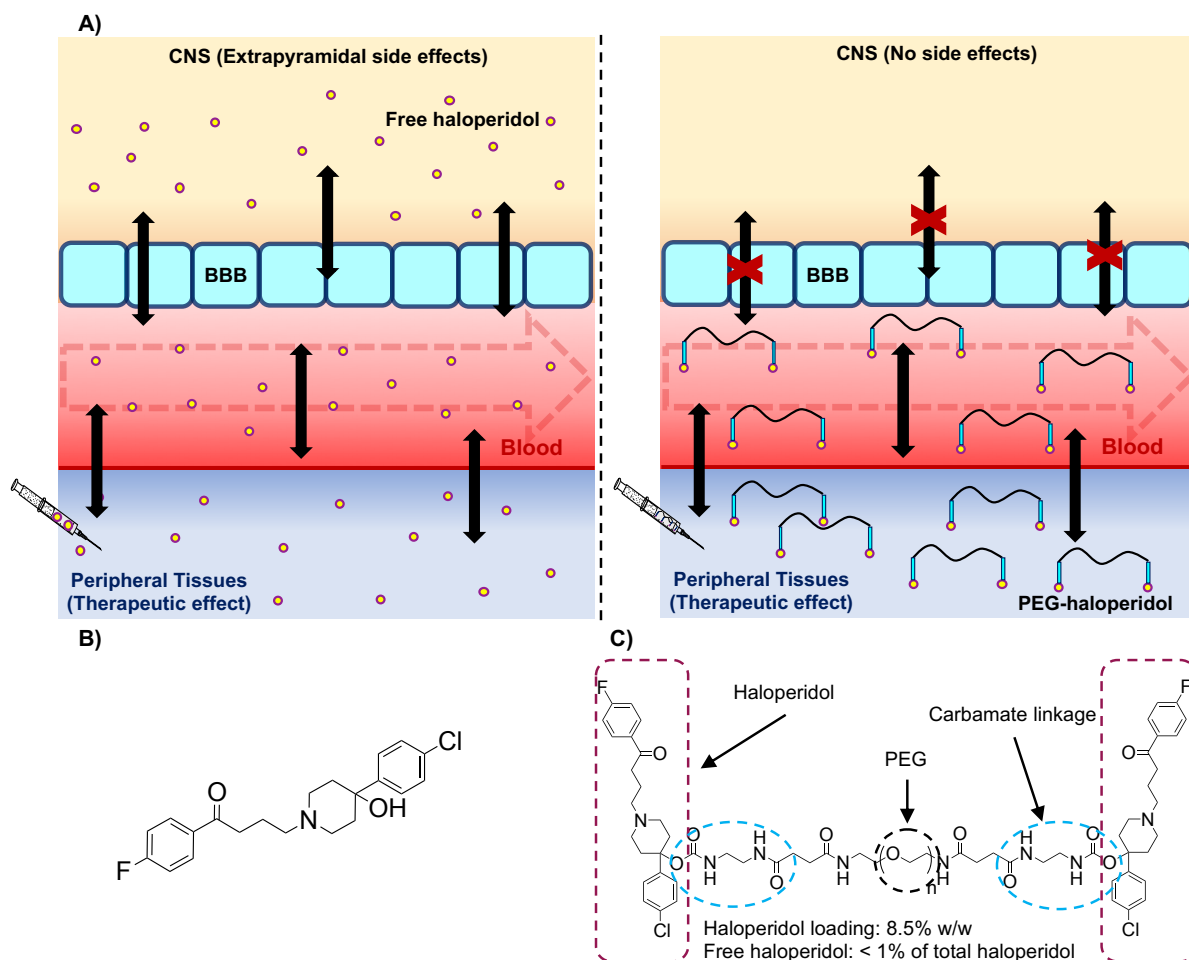


Fig. 1. Panel A, Distribution of free haloperidol in the central nervous system (CNS) and peripheral tissues after its peripheral administration and the ability of the conjugate to localise the effect of haloperidol peripherally; Panel B, Chemical structure of haloperidol; Panel C, Chemical structure and characterisation of PEG-haloperidol.

In the present study, and for the first time, we robustly demonstrate the feasibility of using PEG to prevent penetration of the haloperidol through the BBB using *in vitro* and *in vivo* approaches. Specifically, the pharmacological activity of the PEG-haloperidol conjugate was assessed *in vitro* by measuring the inhibition of dopamine-induced [35 S]GTP γ S-binding via D₂ receptors. Next, we interpret the retained biological potency of conjugated haloperidol by looking at the effect of PEGylation on haloperidol binding to D₂ receptors using *in silico* molecular docking studies. Finally, and of particular significance, we extended our study and evaluated the penetration of peripherally administered PEG-haloperidol conjugate through the BBB *in vivo*. This was carried out by recording catalepsy in rats, as an indication of haloperidol-induced CNS extrapyramidal side effects.

We demonstrate that the PEG conjugation strategy used was capable of preventing the penetration of conjugated haloperidol through the BBB and propose that such strategies can prevent unwanted central side effects of peripherally administered drugs (and/or vice versa),

and would form a strong base to re-direct the use of haloperidol, and similar drugs, to treat peripheral non-CNS diseases such as cardiovascular diseases and cancers.

2. Materials and methods

2.1. Materials

Alpha,omega-di-succinimidyl ester poly(ethylene glycol) (MW 6,429 Da) was obtained from Iris Biotech GmbH, Germany. Haloperidol was purchased from Sigma-Aldrich (UK). The radioligand [³⁵S]GTPγS was purchased from PerkinElmer (UK). All other chemicals and solvents were purchased from Fisher Scientific (UK) and Sigma-Aldrich (UK), respectively and utilised without any further purification unless otherwise stated.

2.2. The synthesis and characterisation of PEG-haloperidol

PEGylation of haloperidol was carried out as described previously with minor modifications [9]: anhydrous DMF was replaced with anhydrous THF, and NHS-PEG-NHS was purchased rather than activating PEG-COOH in house. Further details are provided in the Supporting Information.

2.3 *In vitro* assessment of the biological activity

Cell culture

CHO expressing dopamine D₂ receptors were cultured in Dulbecco's modified Eagle's medium (high glucose, supplemented with 1% L-glutamine; Sigma-Aldrich, UK), 5% fetal bovine serum (Gibco™, UK), 1% non-essential amino acids (Gibco™, UK). To maintain selection pressure, 500 µg/mL of geneticin (Gibco™, UK) was added to the growth medium. The cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C.

Cell membrane preparation

Cell membranes from CHO cells stably expressing the dopamine D₂ receptors were prepared as previously described in [11]. Briefly, confluent cells in 175 cm² flasks were washed twice with 5 mL HEPES buffer (20 mM HEPES, 1 mM EDTA, and 1 mM EGTA; pH 7.4). Cells were then detached from the flask surface using glass beads and ~ 8 mL of HEPES buffer. The collected cells were homogenised using an Ultra-Turax homogeniser (4 x 10 second run). The homogenate was centrifuged at 1000 g for 10 min at 4 °C. The supernatant was then

centrifuged at 39000 g for 1 h at 4 °C. Next, the pellet was resuspended in HEPES buffer and stored as aliquots at -80 °C. The amount of protein (D₂ receptor) was determined using Lowry *et al* method [12]. Further details are provided in the Supporting Information.

[³⁵S]GTPγS-binding assay

Cell membrane (20 μg) was incubated with dopamine (a D₂ receptor agonist) at a range of concentrations 1 nM to 10 mM either alone or with: a) the D₂ receptor antagonist haloperidol; b) PEG-haloperidol; or c) PEG-COOH (6,000 Da) (negative control) at two concentrations (Table 1) in 900 μL HEPES buffer (20 mM HEPES, 60 mM NaCl, 3 mM MgCl₂ and 1 mM EGTA, 0.5 mg /mL fatty acid - free BSA; pH 7.4) supplemented with (100 μM) dithiothreitol and (10 μM) GDP for 30 min at 30 °C.

Table 1. Drugs and their concentrations used in the [³⁵S]GTPγS-binding.

Drugs	Tested concentrations
Dopamine	1 nM-10 mM
Dopamine + Haloperidol	1 nM-10 mM of dopamine + 10 nM or 100 nM of haloperidol
Dopamine + PEG-haloperidol	1 nM-10 mM of dopamine + 10 nM and 100 nM of PEG-haloperidol (haloperidol equivalent)
Dopamine + PEG-COOH	1 nM-10 mM of dopamine + 10 nM and 100 nM of PEG-COOH (haloperidol equivalent)

To initiate the reaction, [³⁵S]GTPγS (100 μL) was added to give a final concentration of 100 pM [³⁵S]GTPγS. The reaction mixture was further incubated for 30 min at 30 °C. The reaction was then terminated by rapid filtration through Whatman filters (GF/C), using a Brandel cell harvester, followed by 4 cycles of wash with ice-cold PBS (~ 4 mL, 5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 3 mM KCl, 0.14 M NaCl; pH 7.4) to remove unbound [³⁵S]GTPγS. The filters were soaked in 2 mL of scintillation fluid overnight and the bound radioactivity ([³⁵S]GTPγS) was determined *via* liquid scintillation counting. All data were performed in triplicate. pEC₅₀ values were determined, using GraphPad Prism (v5.04) software.

2.4. Molecular Docking

Molecular docking studies were performed using the programme Surflex-Dock (SFXC) [13], as provided by Sybyl-X 2.1. The X-ray crystallographic structure of human D₂ receptor complexed with risperidone (an antagonist) was retrieved from the Protein Data Bank (PDBid 6CM4, 2.87 Å resolution) [14]. The protein structure was prepared for docking, using the Biopolymer Structure Preparation Tool, with the implemented default settings provided in

the SYBYL programme suite. The 3D structures of haloperidol and PEG-haloperidol (1 to 5 ethylene oxide monomers) were drawn using ChemDraw Professional 16, MarvinSketch 18.8.0 and Maestro program [15]. Preparation of the ligands was carried out using the Ligand Preparation tool in Sybyl-2.1. Further details of the docking procedure can be found in the Supporting Information. The docking results were visualised using the programme PyMOL and MAESTRO [15–17] and the molecular interactions of the docked ligands were analysed using the programme MAESTRO. Potential hydrogen bonds were assigned if the distance between two electronegative atoms was less than 3.3 Å, whereas any separation greater than 3.3 Å, but less than 4.5 Å, was considered a van der Waal interaction. Further details are provided in the Supporting Information.

2.5. *In vivo* catalepsy recording test

Animals

10 Female and male Wistar rats weighing (mean weight 315 g) were used in this study. All animals were kept in standard vivarium conditions with a natural light-dark cycle, and they were allowed to access a full-balanced diet. All usage of the animals and the experimental procedures were in compliance with the International Recommendations of the European Convention for the Protection of Vertebrates, and the rules of laboratory practice for carrying out preclinical research in the Russian Federation (GOST G 51000.3-96 and 51000.4-96). All studies were approved by the ethical review committee (approval no. 10 from 19th December 2017).

Catalepsy test

In this test, the front paws of the animal were gently placed on a horizontal plastic bar located at a height of 10 cm in a wooden box. The time the animal spent in maintaining this abnormal posture was recorded. A commercial formulation of haloperidol was used as received (5 mg/mL haloperidol sterile solutions containing lactic acid, Ozone Pharmaceutica Ltd, Russian Federation); PEG-haloperidol was dissolved in sterile water. All drugs were administered intravenously at a dose of 1 mg/kg (haloperidol equivalent) *via* the tail vein (mixed male and female rats, 5 rats/group). The animals were placed on the bar at different time intervals for 3 hours after the administration. A scoring system (from 1 to 6) was used to rate the time the rat spent on the bar (0–10 s = 1, 11–20 s = 2, 21–30 s = 3, 31–40 s = 4, 41–50 s = 5 or 51–60 s or more = 6). A score of at least 3 was used as a cut-off score of catalepsy [18].

2.6. Statistical analysis

Data are presented as mean \pm s.e.m (standard error of mean, n=3 unless otherwise stated).

Statistical significance was predicted using the unpaired Student's t-test or one-way ANOVA (Analysis of Variance) test prior to Bonferroni's post hoc test. Statistical significance (*: $p < 0.05$, ***: $p < 0.001$, ns: not significant) was set in the parameters.

3. Results and Discussion

This work studied the impact of conjugating haloperidol to PEG through a biologically stable linker on the biological activity of haloperidol and its ability to penetrate the BBB.

Bifunctional conjugation of PEG was chosen to balance the need for appropriate drug loading of haloperidol (w/w) with using an appropriate PEG chain length that would prevent penetration of the conjugated haloperidol through the BBB whilst also producing a clinically relevant half-life. The average haloperidol content in the conjugate, as determined *via* ^1H -NMR spectroscopic analysis, is 8.5% w/w and that is equivalent to an average (1.6) haloperidol molecules per PEG chain. Biological activity was assessed *in vitro* by assaying inhibition of dopamine-mediated G protein turnover *via* D_2 receptors by PEG-haloperidol as a non-prodrug system. This was followed by *in silico* molecular docking studies to understand the consequence of conjugation on the biological activity of haloperidol. The restricted penetration of PEGylated haloperidol through the BBB was confirmed by recording catalepsy in rats.

3.1. PEG-haloperidol inhibition of dopamine-induced activity in D_2 receptors

In order for the PDC to be effective as a non-prodrug therapeutic agent, it is essential that it retains biological activity at the target of the free drug. Since our PDC contained haloperidol, we sought to determine the ability of PEG-haloperidol to inhibit dopamine-induced G protein activation as an indication of its retained biological activity on D_2 receptors. We have previously demonstrated that PEG-haloperidol can bind to the D_2 receptors in the system used [9]. Here, we investigated the effects of PEGylation of haloperidol on its ability to modulate agonist-induced G protein turnover *via* D_2 dopamine receptors using the well-established [^{35}S]GTP γS -binding assay. Dopamine was used as a control that exhibits full agonist stimulation, whereas free haloperidol and PEG were used as a positive and negative control, respectively. The ligands (haloperidol and PEG-haloperidol) were tested at 10 and 100 nM, based on our previous findings [9]. Indeed, dopamine alone was able to induce G protein

activation (EC_{50} = 0.26 μ M, Table 2, Fig. 2), in agreement with the literature [19]. As expected, the addition of free haloperidol was able to significantly inhibit dopamine-induced stimulation of [35 S]GTP γ S-binding (dopamine EC_{50} increased to 11.5 and 91.8 μ M, in the presence of haloperidol at 10 and 100 nM, respectively, Table 2, Fig. 2).

Table 2. Inhibition of dopamine-stimulated [35 S]GTP γ S-binding by haloperidol, PEG-haloperidol, and PEG. Values are expressed as Mean \pm SEM (n=3).

Drug	p EC_{50}	EC_{50} (μ M)
Dopamine	6.59 \pm 0.07	0.26
Dopamine + haloperidol 10 nM	4.94 \pm 0.04 ***	11.5
Dopamine + haloperidol 100 nM	4.05 \pm 0.09 ***	91.8
Dopamine + PEG-haloperidol 10 nM (haloperidol equivalent)	6.19 \pm 0.02 *	0.65
Dopamine + PEG-haloperidol 100 nM (haloperidol equivalent)	5.81 \pm 0.11 ***	1.64
Dopamine + PEG (control for the conjugate with 10 nM, haloperidol equivalent) ‡	6.52 \pm 0.06 ^{ns}	0.3
Dopamine + PEG (control for the conjugate with 100 nM, haloperidol equivalent) ‡	6.39 \pm 0.09 ^{ns}	0.43

‡ no haloperidol present in the PEG controls, and the concentration of PEG in the PEG controls was identical to the concentration of PEG in the conjugate which the PEG acted as control for.

Interestingly, PEG-haloperidol retained biological activity with significant inhibition of the dopamine-induced stimulation of [35 S]GTP γ S-binding at 10 and 100 nM (haloperidol equivalent). However, dopamine EC_{50} (0.26 μ M) was left-shifted relative to that of free haloperidol in the presence of 10 and 100 nM conjugated haloperidol (haloperidol equivalent) to 0.65 and 1.64 μ M, respectively (Table 2, Fig. 2).

However, the conjugated haloperidol increased dopamine EC_{50} (0.26 μ M) more modestly than the free drug with EC_{50} of 0.65 vs 11.5 μ M at a concentration of 10 nM (haloperidol equivalent) and 1.64 vs 91.8 μ M at 100 nM (haloperidol equivalent) (Table 2, Fig. 2).

Thus, both PEGylated and free haloperidol could inhibit the dopamine-stimulated G protein activation, as the log concentration-response curves for dopamine were shifted to the right in a progressive manner by increasing concentrations of haloperidol and PEG-haloperidol, which were both significantly different from dopamine (P < 0.05). However, the potency of the conjugated haloperidol was weaker than that of free haloperidol (~18 fold at 10 nM and ~56 fold at 100 nM), these values are in line with our previous binding study [9].

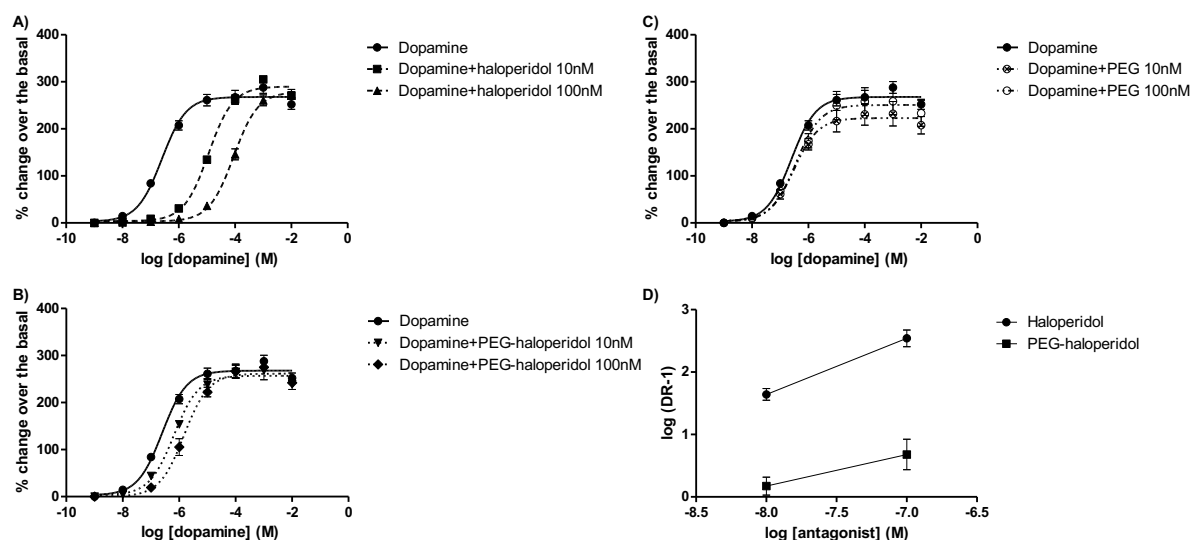


Fig. 2. Effects of haloperidol, PEG-haloperidol and PEG on dopamine stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. Dopamine stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to D_2 receptors was assessed in the presence of two concentrations of tested compounds (10 and 100 nM, haloperidol equivalent). Panels **A**, **B** and **C**, Dopamine log concentration-response curves in the presence of haloperidol, PEG-haloperidol and PEG, respectively. Panel **D**, Estimates of haloperidol and PEG-haloperidol effects using dose-ratios. Data are presented as Mean \pm SEM, $n=3$.

Inhibitory effects of free and conjugated haloperidol on $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ -binding assay can also be visualised using dose-ratio data derived from the same log concentration-response curves (Fig. 2. D). Based on the two concentrations, it was confirmed that PEGylated haloperidol was less effective at inducing a response than free haloperidol, and at 100 nM, PEGylated haloperidol had more pronounced effects than at 10 nM. Of potential interest was that slopes generated by this analysis can be estimated to 0.90 for free haloperidol vs 0.50 for PEGylated haloperidol. These data are consistent with the hypothesis that the conjugated haloperidol may behave differently from free haloperidol (whose action is consistent with competitive antagonism), in that PEGylated haloperidol may act non-competitively.

These data are likely to be related to the lower affinity of PEG-haloperidol for the D_2 receptors, due to conjugation (as further explored in section 3.2) and due to the reduced dynamic equilibrium during the experiment [20]. This condition might be related to the low diffusion rate and reduced uptake of conjugated haloperidol compared to the free haloperidol by D_2 receptors of the isolated membrane due to conjugation to a large molecule.

Importantly, free PEG had no significant effect on dopamine-induced G protein turnover ($P > 0.05$), which indicated that the inhibition induced by PEG-haloperidol was related to the effects of haloperidol moiety on D_2 receptors.

Given the loss in potency of the conjugate compared to free haloperidol and the administration as a conjugate rather than a free drug, one might conclude that the conjugate would need to be administered at a significantly higher (and, arguably, not practical) dose

than the free drug. In fact, other factors are expected to counterbalance the need for an increased dose, namely: (a) increased bioavailability from administering the conjugate *i.v.* (100%) compared to oral administration of haloperidol (60%) [21]; (b) expected decrease in plasma protein binding compared to free haloperidol, which is heavily bound to plasma proteins (only ~8% of total haloperidol in serum is available as the free form in the blood [22,23]); (c) if required, loading can be further increased by decreasing PEG's molecular weight. Taking (a) (b) and (c) together, we are estimating that the dose required, will be practically feasible.

3.2. Molecular Docking

In light of the *in vitro* [³⁵S]GTPγS -binding assay and our previous study of D₂ receptor affinity binding of haloperidol and PEG-haloperidol [9], we conducted a molecular docking study in order to understand the impact, at a molecular level, of conjugating haloperidol to a polymer on its binding to D₂ receptors.

First, we investigated the interactions of free haloperidol with D₂ receptors. Haloperidol (Fig. 3) was docked sequentially into the binding sites, retrieved from [24], the X-ray crystallographic structure of human D₂ receptor complexed with risperidone (PDB code 6cm4, 2.87 Å resolution) [14], using a previously validated docking procedure.

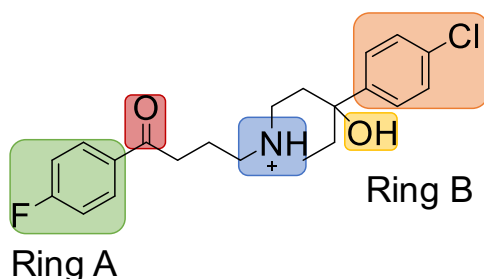


Fig. 3. The chemical structure of haloperidol at pH 7.4 representing different ring structures and functional groups.

The results showed that the space occupied by haloperidol was in agreement with data previously reported for haloperidol interacting with a homology model developed for D₂ receptors [24–27] (Fig. 4A, I). A 3.0 Å salt bridge interaction was formed between the protonated nitrogen of the piperidine in haloperidol and Asp 114 (TM3 of the D₂ receptor). In addition, a 3.4 Å hydrogen bond was established with Ser 197 on TM5. While distant to what has been previously reported [24], ring A of haloperidol formed complementary heteroatom interactions with Trp 386 and Phe 390 at 4.9 Å and 5.3 Å, respectively. Moreover, our results showed that the carbonyl group of the butyrophenone fragment was involved in a hydrogen bond with Cys 118 (TM3) at 3.2 Å (Fig. 4A, II). Both ring B and the

hydroxyl group of haloperidol (which is axial) were solvent accessible (Fig. 4A, III). Ring A was buried in the pocket and parallel to Trp 386 (TM6), while its fluorine atom was directed to Ile 128 (TM3) (Fig. 4A, IV). It is important to highlight that we found the molecular orientation of haloperidol within the D₂ receptor to be different from the previously reported docking studies of haloperidol with a 180° rotation of predicted orientations [24–26]. However, our results are in line with recently published data predicting the binding pathways of haloperidol in D₂ receptors [27].

haloperidol 3D structure with 1 ethylene oxide monomer, which provides an indication of the possible poses of longer conjugated haloperidol molecules within the D₂ receptor. The remaining 4 conjugate haloperidol structures provided similar results to the monomeric conjugate (data not shown). The molecular docking studies of PEG-haloperidol conjugates revealed that the salt bridge between the protonated nitrogen of the piperidine ring in conjugated haloperidol and Asp 114 (TM3), which is essential for the biological activity, was preserved at 2.6 Å, however, the poses of conjugated haloperidol were flipped in the binding pocket compared to free haloperidol. Ring A and the carbonyl group both became solvent accessible and these were not involved in any significant interactions (Fig. 4B, I-III). However, as observed for the docked free haloperidol, ring B of conjugated haloperidol formed heteroatom interactions with Trp 386 and Phe 390 at 4.8 Å and 5.1 Å, respectively (Fig. 4B, II). Ring B was buried in the pocket facing Ile 128 (TM3), instead of ring A observed for free haloperidol (Fig. 4B, IV). These findings may provide possible explanations for the retained but reduced biological potency of PEGylated haloperidol *in vitro*. The retained biological activity for conjugated haloperidol might be directly related to the salt bridge interaction with a key Asp 114 residue, which both free and conjugated haloperidol are able to exploit. This aspartate residue plays an important role in specific binding of ligands to G protein-coupled receptors, and mutations, typically to alanine, result in a loss of ligand binding to the receptors [28,29]. On the other hand, the change in space occupied by haloperidol after its conjugation could account for the reduction in activity (Fig. 5)

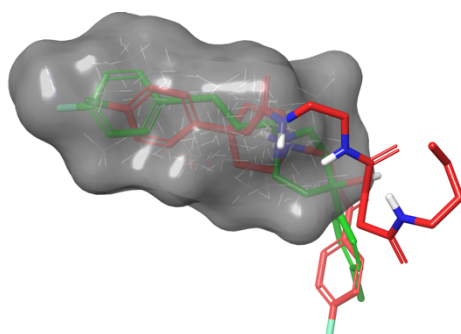


Fig. 5. Overlaid docked poses for haloperidol (in green) and PEG-haloperidol (in red) in the binding pocket.

The conjugation of haloperidol to PEG apparently results in a flipped orientation in the binding mode, making ring A and the carbonyl group (a butyrophenone moiety) solvent-accessible rather than binding deep into the pocket of the D₂ receptor. These results are in agreement with other experimental studies, which support the observation that the

butyrophenone moiety must bind deep into the binding pocket in order to retain binding affinity towards D₂ receptors [30,31]. Furthermore, the involvement of the hydroxyl group of haloperidol in the conjugation to PEG might be another reason for the reduced affinity and activity of PEG-haloperidol compared to free haloperidol. Different studies have reported that the presence of a hydroxyl group in the haloperidol structure at a position bound to the piperidine ring is not essential for haloperidol to bind to D₂ receptors, however, its presence significantly increases affinity towards these receptors [32–34].

Although the PEG-haloperidol conjugate contains two haloperidol moieties, it is anticipated that, similarly to the unconjugated form, only one haloperidol will bind to the receptor due to the architectural restrictions in the binding site. It is possible that the other haloperidol could independently and simultaneously bind to a different D₂ receptor nearby, but this is likely to be disfavoured from entropy considerations.

3.3. Prevention of permeability, in vivo catalepsy recording test

As a preliminary screen to determine the enthalpic impact of conjugation on permeability across the BBB, theoretical calculations were carried out using literature-retrieved equations to predict log BB values for both haloperidol and the conjugate. These demonstrated that, as per our design, in all cases, PEG-haloperidol was dramatically less permeable than the free haloperidol (Fig. S1, Fig. S2, Table S3, Table S4) (Supporting Information). However, it should be noted that these models do not account for entropic factors (which would also disfavour permeability across the BBB) and for pathways alternative to passive diffusion (which could potentially determine some permeability across the BBB). Therefore, to robustly test our hypothesis that polymer conjugation can be used to prevent permeability across the BBB, the conjugate was evaluated *in vivo*. Therefore, the cataleptogenic effects of PEG-haloperidol were monitored in rats. Neuroleptic drugs (typical antipsychotics such as haloperidol) are well-known to induce catalepsy *via* inhibitory effects on the nigrostriatal dopamine system [35]. In addition, cataleptic symptoms in animals are used to predict the unwanted extrapyramidal side effects induced by antipsychotics due to their permeation through the BBB [36].

With the aim of assessing the penetration of free or conjugated haloperidol through the BBB, catalepsy was recorded in rats, at different time intervals, after the peripheral administration of the drug. Cataleptic responses were readily apparent in animals treated with haloperidol. The cataleptic effects of haloperidol commenced after 5 min of administration and lasted for at least 180 min (the duration of the test) (Fig. A).

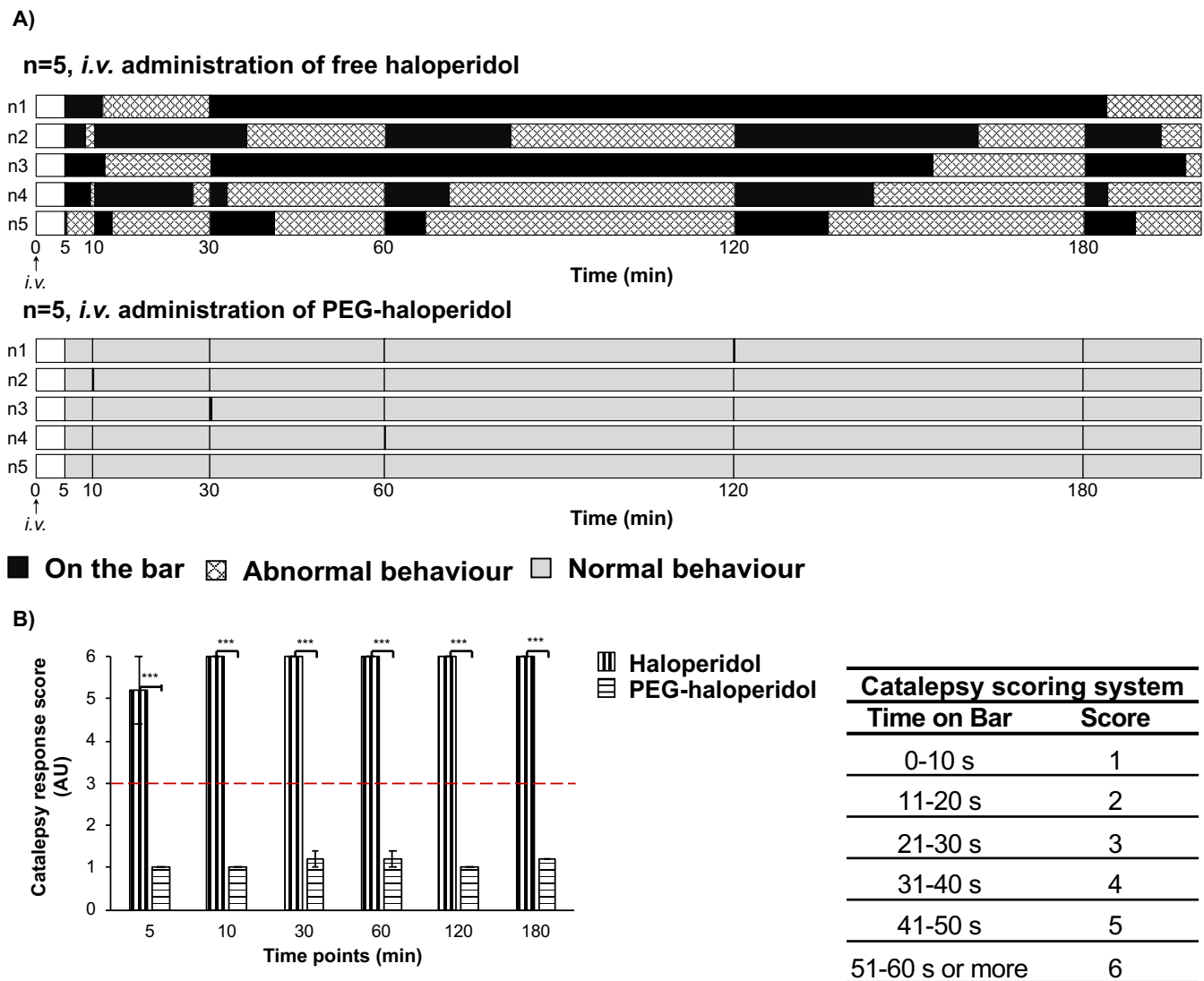


Fig. 6. *In vivo* catalepsy test in rats. Panel **A**, Time (min) recorded chart representing episodes of catalepsy induced after *i.v.* administration of free haloperidol and PEG-haloperidol; Panel **B**, Catalepsy response scores for free haloperidol and PEG-haloperidol and the scoring system used. The red dashed line represents the catalepsy cut off (Mean \pm SEM, $n=5$). n represents a single animal in the experiment.

Even when the rats fell from the bar, they exhibited abnormal behaviour characterised by reduced activity. All haloperidol treated animals achieved scores higher than the catalepsy cut-off score (i.e. >3) (Fig. B). Rats showed normal behaviour with no sign of catalepsy was recorded, with significant differences to haloperidol treated animals ($P<0.001$) (Fig. A, 6B). The *i.v.* treatment of rats with free haloperidol led to episodes of catalepsy, consistent with antagonism of dopamine effects in the brain, in agreement with previous works [37–40]. However, when PEG-haloperidol was administered, the CNS-associated side effects of haloperidol on rats were absent. The normal behaviour seen in PEG-haloperidol treated animals is most likely related to the absence of haloperidol in the CNS. The lack of catalepsy observed following administration of the conjugate indirectly shows that haloperidol is not

released from PEG in plasma. This is desirable and in line with our previously reported stability data [9]. Furthermore, PEG is well known to be biologically stable. Therefore, with respect to overall metabolism, it is likely that the conjugate will be excreted unmodified in the urine, following glomerular filtration, in line with other studies on PEG 6000 that report elimination (96%), renally [41].

It is important to highlight that haloperidol and PEG-haloperidol were *i.v.* injected at a dose ~13 fold higher than the dose required to induce catalepsy in rats after *s.c.* injection [42]. As such, the lack of catalepsy observed after injection of the conjugate is most likely the result of the conjugate preventing haloperidol's permeability across the BBB, rather than the result of a loss of potency.

Hence, we demonstrate that the conjugation strategy used was capable of preventing the penetration of conjugated haloperidol through the BBB.

4. Conclusions

This study uses a PEG-haloperidol conjugate as a non-prodrug system to validate the hypothesis that conjugation to a polymer can localise a drug peripherally and avoid central effects. The approach relies on (a) polymer conjugation being effective at preventing BBB permeation and (b) the conjugated drug therapeutic activity being retained. *In vivo* studies in rats demonstrated strong evidence for (a), in that no unwanted cataleptogenic effects were observed when the rats were treated intravenously with PEG-haloperidol, in marked contrast to rats treated with haloperidol. With respect to (b), *in vitro* studies showed that PEG-haloperidol was biologically active as an antagonist of D₂ receptors by inhibiting the dopamine-induced stimulation of [³⁵S]GTPγS-binding (although some loss of activity was observed). Molecular docking studies also confirmed that after conjugation, haloperidol preserved its ability to bind to D₂ receptors, whilst also providing a possible explanation for the reduced activity of conjugated haloperidol compared to the free haloperidol. Taken together these findings suggest that PEGylation *via* a biologically stable linkage is a highly viable strategy to minimise penetration of a therapeutic drug through the BBB hence allowing drugs to be localised either within the CNS, or within the peripheral system, dependent on the point of administration. Future work will now look to interrogate this further, for example to realise the clinical applications of this work by probing the therapeutic activity of the conjugate for peripheral applications.

Acknowledgements

The authors would like to thank the Council for At-Risk Academics (Cara) and the University of Reading for academic placement and financial support. The authors would like to thank Jessica Visconti for her preliminary contributions to the *in silico* permeability study. The authors would also like to thank the University of Reading for the provision of the Chemical Analysis Facility.

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