Biological evaluation of PEG-based conjugates offering localisation of conjugated drugs at the desired compartment

A thesis submitted to the University of Reading in partial fulfilment for the degree of Doctor of Philosophy

School of Pharmacy

By

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

AZ ALDDIEN NATFJI

"O Allah, benefit me by that which You have taught me, and teach me that which will benefit me, and increase me in knowledge"

This work is dedicated to

the piece of my heart in Syria my beloved parents

Rajaa & Radwan,

My soulmate and partner in this achievement

my wife Dalal

&

the souls of people we wished them to be among us

to celebrate this moment

my aunt Rajaa Natfji

my aunt Wafaa Alhafez

&

my father in law

Nawaf Shaban Kabakibo

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Finally, may **Allah (SWT)** accept my work and support me to help humanity.

Abstract

Polymer-drug conjugates (PDCs) are drug delivery systems in which drug molecules are covalently linked to hydrophilic polymers. These systems have initially been developed to improve the anticancer efficacy and safety of chemotherapeutic agents based on the enhanced permeability and retention (EPR) effect. In these applications, the PDCs require the release of the conjugated drugs within the tumour tissues. However, there are few cases in which the release of the drug is not the aim. For example, Movantik®, the only available PDC on the market, was developed to prevent the unfavourable penetration of naloxol across the blood-brain barrier (BBB) while retaining its effect in the intestines. This system was designed as a non-prodrug employing PEG (as a polymer) and an ether bond as a linker. The current project focuses on the biological evaluation of utilising the PEGylation strategy to develop non-prodrug PDCs of haloperidol providing compartmentalisation of haloperidol at the intended site of action, which could allow potential non-CNS applications of the conjugated haloperidol.

In **Chapter 1**, a general introduction to the biological barriers, PDCs and their applications are discussed. The rationale behind this project is also provided.

Chapter 2 identifies the various non-cancer applications of PDCs and key features influencing the design of such systems for specific diseases are recognised.

Chapter 3 represents a systematic analysis of clinical studies of nanomedicines (including PDCs) used to treat solid tumours of different origins based on the EPR effect. From all studied cancers, ovarian, brain, stomach, breast, colon and colorectal, and pancreatic cancers showed the highest levels (up to >8-fold) of accumulation of nanomedicines compared to other tumours. Moreover, tumour size was another factor that impacted the accumulation of nanomedicines, with high levels of accumulation observed in large tumours (~5-fold) compared to medium or very large tumours. Other parameters such as perfusion levels, the presence of angiogenesis and inflammation in tumour tissues were identified as factors that might influence the magnitude of the EPR effect and, as a consequence, the accumulation of nanomedicines within tumour tissues. The chapter proposes two strategies to select patients who could potentially benefit from the increased accumulation of nanomedicines based on the EPR effect, which might enhance the clinical outcomes of using nanomedicines as personalised anticancer agents.

In **Chapter 4**, the feasibility of utilising the PEGylation strategy to prevent haloperidol diffusing through the BBB is demonstrated using different *in silico, in vitro* and *in vivo* approaches. The synthesis of a PEG-haloperidol conjugate was carried out (using PEG 6000 Da) by applying a protocol slightly modified based on our previously reported protocol. The *in vitro* binding assay indicated that the PEG-haloperidol conjugate had a retained activity through D_2 receptors, however, this was lower than that of the free drug (~18-fold at 10 nM). Molecular docking (MD) studies indicated that the conjugates exhibited a retained binding affinity for the D_2 receptors, and the binding pattern of the conjugate in the binding pocket explained the loss of the biological activity of the conjugates compared to the free haloperidol. *In vivo* studies on rats revealed that rats treated with PEG-haloperidol were not cataleptic in contrast to the free haloperidol treated rats, which indicated the prevented crossing of PEG-haloperidol into the CNS.

Chapter 5 describes potential applications of PEG-haloperidol conjugates in the field of cancer (acting *via* σ receptors) assessed using *in vitro* and *in silico* approaches. PEGs of two MWs (2000 and 6000 Da) were synthesised. PEG (2000 Da) enhanced the haloperidol's loading in the conjugate (~25% w/w) by ~3-fold compared with the loading of haloperidol in PEG

(6000 Da) conjugate. The cytotoxicity of the conjugates was evaluated using breast cancer cell lines (MCF-7 and MDA-MB 231). The application of the conjugates as potential antiproliferative agents was limited as their IC_{50} values were > 100 μM (compared to ~50 μM for the free haloperidol) for both cell lines. The conjugates were also tested for potential anti-migratory activity *in vitro* on vascular endothelial cells (HUVECs). The conjugates significantly inhibited the VEGF-stimulated migration of HUVECs (> 65% inhibition) although at a lower level compared to the free haloperidol (91% of inhibition). MD studies were performed and explained the loss of the biological activity of the conjugates compared to free haloperidol.

Chapter 6 indicates a preliminary evaluation of potential cardiovascular applications of PEG-haloperidol by studying its effects on human platelets' aggregation induced by CRP-XL or ADP. The results indicated that free and conjugated haloperidol (at all tested concentrations) did not significantly abrogate the platelets aggregation stimulated by the CRP-XL (mediated by GPVI receptors). Moreover, haloperidol and PEG-haloperidol inhibited, however, not significantly, ADP-induced aggregation of human platelets at concentrations ≥12.5 μM haloperidol equivalent, probably through $P2Y_1$ receptors. However, further studies by employing other agonists and/or increasing the incubation time, and using different methodologies are required to identify the final conclusion.

Chapter 7 represents the feasibility of using PEG-based PDC (designed as a non-prodrug system) to decrease or avoid the transfer of conjugated drugs through the human placenta. PEG, as a polymeric carrier, did not significantly affect the apoptosis or proliferation rates within placental explants when incubated up to 48 h, as indicated *via* immunohistochemistry staining. No signs of necrosis were observed when the explants were challenged with PEG as the released levels of lactate dehydrogenase from the explants did not significantly change. Treatment with PEG did not alter the normal function of the placental tissues where the secreted levels of hCG hormone from the explants were not significantly influenced by the polymer. Moreover, the cellular uptake studies of the PEG-Cy5.5 (dye) and PEG-haloperidol conjugates, used as model drugs, using fluorescent microscopy and RP-HPLC, respectively, showed complete absence of PEG-Cy5.5 from the placental tissues and limited uptake of PEG-haloperidol by the tissues compared to the free Cy5.5 and haloperidol, respectively. This indicated the potential efficiency of PEGylation strategy to design non-prodrug systems to treat illnesses during pregnancy without inducing negative effects on the developing fetus.

In **Chapter 8,** the key findings of this PhD project are summarised, critical evaluation of work-related aspects and potential future work is suggested. Specifically, taken together, the data presented in this thesis demonstrated the feasibility of using PEGylated macromolecules (designed as non-prodrug systems) to reduce or prevent the transfer of conjugated drugs across biological barriers while retaining their activity. This strategy would form a platform to design drug delivery systems for applications where specific compartmentalisation of the effects of drugs is required. Future work will look to investigate this further by exploring PEGylated systems of therapeutic agents of different classes for their potential clinical applications.

List of Publications

The present thesis produced the following papers

A. Dodd[+], **A.A. Natfji**[+], A. Evangelinos[+] A. Grigolettod, G. Pasut, F. Beards, L. Renshall, H.M.I. Osborn, F. Greco, L.K. Harris, Conjugation to PEG limits drug uptake by the placenta: potential applications for drug administration in pregnancy, J. Control. Release. (2020). *Manuscript in preparation*.

[+] **Authors have an equal contribution to this work.**

A.A. Natfji, C. Pegoraro, K.A. Watson, H.M.I. Osborn, F. Greco, A polymer-drug strategy to repurpose haloperidol as an anticancer agent acting through sigma (σ) receptors, Pharmaceutics. (2020). *Under revision*.

A.A. Natfji, D.O. Nikitin, I.I. Semina, R.I. Moustafine, V.V. Khutoryanskiy, H. Lin, G.J. Stephens, K.A. Watson, H.M.I. Osborn, F. Greco, Conjugation of haloperidol to PEG allows peripheral localisation of haloperidol and eliminates CNS extrapyramidal effects, J. Control. Release. 322 (2020) 227–235.

A.A. Natfji, H.M.I. Osborn, F. Greco, Feasibility of polymer-drug conjugates for non-cancer applications, Curr. Opin. Colloid Interface Sci. 31 (2017) 51–66.

A.A. Natfji, D. Ravishankar, H.M.I. Osborn, F. Greco, Parameters Affecting the Enhanced Permeability and Retention Effect: The Need for Patient Selection, J. Pharm. Sci. 106 (2017) 3179–3187.

Papers not related to the work of this project

S. Salimi, Y. Wu, M.I.E. Barreiros, **A.A. Natfji**, S. Khaled, R. Wildman, L.R. Hart, F. Greco, E.A. Clark, C.J. Roberts, W. Hayes, A 3D printed drug delivery implant formed from a dynamic supramolecular polyurethane formulation, Polym. Chem. 11 (2020) 3453–3464.

List of Conferences Attended

A.A. Natfji, J. Visconti, D. Nikitin, I. Semina, R. Moustafine, V. Khutoryanskiy, H. Lin, G. J. Stephens, H.M.I. Osborn, F. Greco, Polymer-drug conjugates as non-prodrug systems, a strategy offering peripheral localisation of drugs and retained biological activity, **The 10th APS International PharmSci Conference, Sep 2019, University of Greenwich, UK. Talk**.

A.A. Natfji, J. Visconti, D. Nikitin, I. Semina, R. Moustafine, V. Khutoryanskiy, H. Lin, G. J. Stephens, H.M.I. Osborn, F. Greco, Conjugating Haloperidol To A Polymer, A Potential Strategy To Localise Haloperidol's Effects Peripherally, **Controlled Release Annual Meeting & Exposition, Jul 2019, Valencia, Spain. Poster**.

A.A. Natfji, H.M.I. Osborn, F. Greco, Polymer-drug conjugates as non-prodrug systems, a strategy offering peripheral localisation of drugs and retained biological activity, **Pharmacy PhD Showcase, Apr 2019, University of Reading, UK. Talk**.

A.A. Natfji, J. Visconti, V.V. Khutoryanskiy, H.M.I Osborn, F. Greco, Exploring the suitability of a PEG-haloperidol conjugate for anticancer applications, **The 12th International Symposium on Polymer Therapeutics: From Laboratory to Clinical Practice, May 2018, Valencia, Spain. Short Talk & Poster**.

A.A. Natfji, J. Visconti, V.V. Khutoryanskiy, H.M.I Osborn, F. Greco, Polymer-haloperidol conjugate, a potential anticancer agent with peripherally localised effects, **Pharmacy PhD Showcase, Apr 2018, University of Reading, UK. Talk**.

A.A. Natfji, D. Ravishankar, H.M.I Osborn, F. Greco, Towards better applications of EPR effect-dependent nanomedicines for treating solid tumours, **The 8th APS International PharmSci Conference, Sep 2017, University of Hertfordshire, UK**. **Poster**.

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Abbreviations

a_nO: number of oxygen atoms; A_{pol}: polar surface area; ΔG°_{W}: computed solvation free energy in water; μ_1^{PS}: a variable related to polar surface; *: the experimentally determined log P` was used in these equations; # rot. Bonds: the number of rotatable bonds; 11-β-hydroxysteroid dehydrogenase 2: 11-βHSD2; 4G: glycine-glycine-glycine-glycine; 5-HT: serotonin; 5-HT_{2A} receptors: serotonin receptors; *A* logP: Ghose and Crippen $logP_{o/w}$; a_nN: number of nitrogen atoms; A: overall hydrogen bond acidity; AA: arachidonic acid; ABC: accelerated blood clearance; ADA: adenosine deaminase; ADP: adenosine diphosphate; AHPP: 4-amino-6-hydroxypyrazolo[3,4-D]pyrimidine; Ala: alanine; AlogP: Ghose and Crippen logP; AMD: age-related molecular degeneration; ANOVA: analysis of variance; ANS: 8-anilionaphthalene-1-sulfonic acid; Apaf-1, apoptotic protease activating factor 1; AR6: number of aromatic 6-membered rings; AsnPhePhe: asparagine-phenylalanine-phenylalanine; Asp: aspartic acid; B: overall hydrogen bond basicity; BBB: blodd-brain barrier; BCRP: breast cancer resistance protein; BF: blood flow; BIndx: Balaban index; CAD: coronary artery disease; CLL: chronic lymphocytic leukemia; clog P: calculated log P; CLogP: calculated log partition coefficient; ClogP: calculated logP; CMC: critical micelle concentration; CNS: central nervous system; Cox1: cyclooxygenase1; CRP-XL: cross-linked collagen related peptide; Cy5.5: cyanine-5.5 ; D- (Asp)8: D-aspartic acid peptide; D: degradable; D_2: dopamine 2; d^2X^v: second order difference valence molecular connectivity; Da: Dalton; dd: double distilled; DDS: drug delivery system; DPSA: dynamic polar surface area; E: enzymatic degradation; E: excess molar refraction; EC_{50}: half maximal effective concentration; EDTA: ethylenediaminetetraacetic acid; ES_sum_sBr: sum of individual atom level E-state values of all bromine atoms; E_{sb}: stretch-bend energy of a molecule; Fc/Mc: the ratio of the concertation of a drug in fetal blood to the concentration of the drug in the maternal blood; FDA: food and drug administration; FISA: Hydrophilic component of solvent accessible surface area; G: generation; GFAL: glycine-phenylalanine-alanine leucine; GFGG: glycine-phenylalanine-glycine-glycine; GFLG: glycinephenylalanine-leucine-glycine; GG: glycine-glycine; GGPNle: glycine-glycine-proline-norleucine; GL: glycine-leucine; Glu: glutamic acid; GP IIb/IIIa: glycoprotein IIb/IIIa receptors; G_{solv}: solvation free energy; GTP: guanosine-5'-triphosphate; H: hydrolytic degradation; HALO: sum of halogen atoms (Br, F, Cl); NO2: number of NO_2 groups; SU: number of sulfur atoms; HBA: number of hydrogen bond acceptor; HBAC: number of solute hydrogen bond acceptor; HBDN: number of solute hydrogen bond donor; hCG: human chorionic gonadotropin; HEMA: 2-hydroxyethyl methacrylate; HER-2: human epidermal growth factor receptor 2; His: histidine; HPMA: *N*-(2-hydroxypropyl)methacrylamide; HS^T(arom): the hydrogen E-State index for aromatic CHs; HS^T(HBd): hydrogen E-State index for hydrogen bond donors; HUVEC: human umbilical vein endothelial cell; IC_{50}: half maximal inhibitory concentration; I/R: ischemia reperfusion; I_1: an indicator variable, I_1= 1 for a compound containing a carboxylic acid fragment, I_1= 0 otherwise; I_3: indicator variable, I_3= -1 for compounds with COOH, 1 for compounds with amino nitrogen, and 0 for other compounds; Ile: isoleucine; IP: ionization potential; Jurs-WNSA-3: surface weighted charged partial surface area; LA: contribution to $LogP_{oct}$ from atoms other than oxygen and nitrogen; Lact2G: lactic acid-glycine-glycine; Lact4G: lactic acid-glycine-glycine-glycine-glycine; LDH: lactate dehydrogenase; LMHC: low molecular weight hydroxyethyl chitosan; log k_{BMC}: logarithm of the retention factor in biopartitioning micellar chromatography; log P: calculated octanol-water partition coefficient; log $P_{o/w}$: octanol/water partition coefficient; log $D_{7.4}$: logarithm of distribution coefficient in octanol/water at pH 7.4; logS: solubility; M–ESP: mean – ESP (molecular electrostatic potential); Mass: MW; *MC* log P_{ow}: log $P_{o/w}$ calculated using Monte Carlo simulations; Met: methionine; MIC: minimum inhibitory concentration; MlogP: Moriguchi log P; MMPs: matrix metalloproteinases; mPEG: methoxy PEG; MRP: multidrug resistance proteins; MVOL: molecular volume; MW: Molecular weight; MWEI: molecular

weight; n R11: the number of the fusion of 6- and 7-membered rings; n: number of compounds: r: correlation coefficient; N/A: not applicable/not stated; N+O: total number of nitrogen and oxygen atoms; $n_{acc,solv}$: no of hydrogen bonds acceptors in aqueous medium; nCl: the number of Cl atoms; No AromaticRings: number of aromatic rings; n_{pol}: number of polar atoms (nitrogen, oxygen, and attached hydrogens); OCT: optimal cutting temperature; OR: oxidation responsive; $P2Y_1$: $P2Y_{12}$: ADP receptors; PAA: poly(acrylic acid); PAHA: poly[α,β-(*N*-2-hydroxyethyl DLaspartamide)]-poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)]; PAMAM: poly(amidoamine); PAR1: PAR4: protease-activated receptors; PB: placenta barrier; PCGA: 3rd PC of molecular geometry; PCGC: 1st PC of molecular geometry; PCL: polycaprolactone; PDC: polymer-drug conjugate; pDMAEMA: poly(dimethylamino)ethyl methacrylate; PEG: poly(ethylene glycol); PGA: polyglutamic acid; PGH2: prostaglandin H2; Phe: phenylalanine; PHEA: α,β-poly[(*N*-2-hydroxyethyl)-DL-aspartamide]; PHPA: poly[α,β-(*N*-3-hydroxypropyl-DL-aspartamide)]; PHPMA: poly*N*-(2 hydroxypropyl)methacrylamide; PMAA: poly(methacrylic acid); PNSA-2: partial negative solvent accessible surface area multiplied by negative charge; Rotlbonds: number of rotatable bonds; PRP: platelet-rich plasma:PSA: polar surface area, nm^2; PSA: polar surface area; PSA: polar surface area; PVP: poly(vinyl pyrrolidinone); q_C^{max}: the maximum Mulliken charge-derived descriptor on the carbon atom; q_F^{max}: Mulliken charge-derived descriptor on the on the fluorine; (q_H^{max} -q_H^{min}); qp_EA(eV): PM3 calculated electron affinity; qp_num-amine: number of non-conjugate amine groups; qp_PSA: van der Waals surface area of polar nitrogen and oxygen; R_2: excess molar refraction; RadOfGryation: radius of gyration; ROA: route of administration; R_{og}: Radius of gyration; ROTB: number of rotatable σ-bonds; Rotlbonds: Number of rotatable bonds; RP-HPLC: reversed-phase high performance liquid chromatography; S: polarizability/ dipolarity; S_dsN: N connected by a double and single bond; S_sssCH: CH connected by three single bonds; S_sssN: N connected by three single bonds; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SASA: solvent accessible surface area; SAXS: small-angle x-ray scattering; SCID: severe combined immunodeficiency disease; SEC: size exclusion chromatography; Ser: serine; SMA: styrene-maleic acid; SP: van der Waals surface; sPEG: star PEG; SPW: water accessible hydrophilic surface; TBS: Tris-buffered saline; TEM: transmission electron microscopy; TFPI: tissue factor pathway inhibitor; Thr: threonine; TP: thromboxane receptor; TPSA (NH): the topological polar surface area, where each N-containing fragment in the molecule contributes to the total value; TPSA: topological surface area; (a_acid+a_base): sum of number of acidic and basic atoms; Λ_{alk}: *LogP value (by experiment,* log P_{cych}); Trp: tryptophan; TXA2: thromboxane A2; Tyr: tyrosine; V: molar volume, nm^3; Val: valine; VEGF: vascular endothelial growth factor; V_M or V_m: Molar volume; V_m: molar volume; Volume: Molecular volume; VXBAL: VTOT (total variance of ESP) x BALESP (balance parameter to the ESP); CHBBA: covalent hydrogen bond acidity; V_χ: McGown volume ($cm^3\ mol^{-1}/100$); M_0, M_2, M_3: moment descriptors, obtained by calculations; V_χ: McGown volume; $\alpha_2\beta_1$, GPVI receptors: collagen receptors; α^H_2: overall hydrogen bond acidity; β^H_2: overall hydrogen bond basicity; ΔE_{total}, $\Delta E_{trosion}$: intermolecular descriptors related to interactions between a solute and the dimyristoylphosphatidylcholine (DMPC) water model; Δlog P= $logP_{oct}$ – $logP_{cych}$; μ_1^{AM}: a variable related to atomic mass; μ_1^H: a variable related to hydrophobicity; μ^2: the dipole moment; π^H_2: polarizability/ dipolarity.

Chapter 1
General introduction

1.1. Introduction

Since the 1950s, when the first drug delivery system (DDS) was reported to provide a sustained release of dextroamphetamine for 12 h, DDSs have been designed and developed to improve the clinical outcomes of therapeutic agents [1]. Enhancing penetration through biological barriers in order for drugs to reach their active sites has been often considered a major challenge when designing a new therapeutic agent [2]. However, in other scenarios, unwanted accumulation in specific organs or tissues, due to the unfavourable diffusion of drug molecules through biological barriers, led to serious side effects and catastrophic consequences [3,4]. This was the case for thalidomide, that was initially introduced to treat morning sickness during pregnancy. The diffusion of this small molecule through the placenta to the fetus caused serious teratogenic effects. As a consequence, thalidomide was excluded for many years from being explored for its other possible clinical applications [5]. Therefore, in few specific cases, there is a need to design DDSs that would allow the effects of some drugs to be localised to a specific compartment of the body to minimise or even prevent the associated side effects of the parent drug due to undesirable distribution throughout the body. This, in turn, would allow repurposing of many groups of old drugs, which have unfavourable accumulation in various tissues and cause side effects, to treat variable diseases (rare and common) [6,7].

The research presented in this thesis aims to evaluate the feasibility of DDS designed based on the concept of **polymer-drug conjugates (PDCs)** to prevent the conjugated drug (**haloperidol**) from crossing **biological barriers** (**the blood-brain barrier and the placenta**). In addition to that, it aims to assess the **retained activity** of the conjugated drug and the possibility of **repositioning** its therapeutic effects in another compartment of the body.

Therefore, a general background to biological barriers, the concept of PDCs and their applications, and an overview of repurposing of drugs are presented here to clarify the rationales behind this work (**Figure 1.1**). Thereafter, the structure of the thesis is presented.

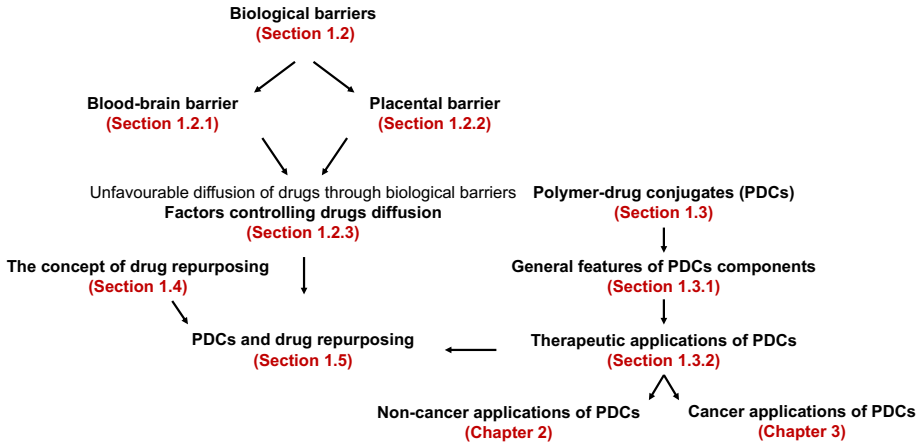

Figure 1.1. Schematic representation of the organisation of the general background of this project.

1.2. Biological barriers

The human body has defence barriers that efficiently protect organs and tissues from any harm that could affect their normal biological functions [8,9]. These barriers vary in their mechanisms of protection, as some act physically (such as the skin) while others have more complicated features (e.g. the blood-brain barrier (BBB) and the placental barrier (PB) [10]) with the presence of efflux mechanism (P-glycoprotein) [11], secretory and enzymatic activities (expression of alkaline phosphatase to degrade chemicals) [12,13] to protect their respective tissues. In addition to their protective function, they have unique structures to ensure an adequate supply of oxygen, nutrient and the other vital molecules to these organs [14–16]. Amongst the various biological barriers, of particular relevance to this thesis, are the BBB and the PB and as such, they are discussed in more depth in subsequent sections.

1.2.1. The BBB

The BBB is a term used to describe the unique structure of tightly constructed endothelial cells forming a non-fenestrated monolayer surrounding the central nervous system (CNS). This feature of the cells makes the BBB restrictive in nature [17]. However, the BBB is not 100% an impermeable barrier, as it exhibits paracellular and transcellular permeability, with almost 12 m^2 surface area of endothelial cells available for cellular exchange and transportation [18–20]. The physiology of the BBB is maintained by neurovascular units which consist of a wide variety of cells including vascular cells (such as endothelial, smooth muscle cells and pericytes) and glial cells (microglia and astrocytes), combined with tight junctions and a basal membrane with surrounding neurons. These collections of cells play a vital role in regulating the features of the BBB such as its integrity, cell-matrix interactions, angiogenesis and neurogenesis [21–24]. In addition to that, these cells control the permeability and transportation across the BBB *via* influencing the expression of ion channels, transporters, efflux pumps and other regulatory molecules [17,20,25–29]. In addition to the restricted transportation to the CNS, the BBB has another way of protection characterised by its metabolic activity. The presence of different enzymes, such as alkaline phosphatase and γ-glutamyl transpeptidase, plays an essential role in decomposing the chemical molecules penetrating the BBB [30,31].

Transportation across the BBB

The transportation through the BBB is mediated by different mechanisms. Paracellular and transcellular passive diffusion, active transportation in both its forms (influx and efflux and

transcytosis) have been reported as mechanisms controlling the passage of the molecules into the CNS (**Figure 1.2, Table 1.1**), discussed further in references [32–34]).

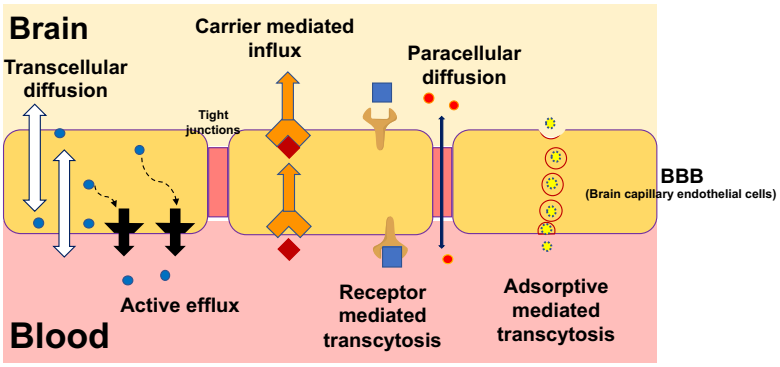

Figure 1.2. Cartoon representation of mechanisms of transportation across the BBB (adapted from [35–37]).

Table 1.1. Potential mechanisms of transportation through the BBB.

Mechanism	Substrates	Ref
Paracellular diffusion	Water soluble molecules, ions, leukocytes	[38]
Transcellular passive diffusion	Lipophilic molecules (MW <400-600 Da), might be prevented by conjugating drugs to polymers	[39]
Active efflux transportation (P-glycoprotein, MRP*, BCRP+)	Already penetrated molecules: CNS acting drugs (antiepileptic and anticancer agents)	[40]
Carrier mediate influx (active or passive)	Amines, amino acids, choline, monocarboxylates, nucleosides	[41]
Receptor mediated transcytosis	Peptides, proteins (insulin, cytokines, etc.), some nanoparticles	[42,43]
Adsorptive-mediated transcytosis	Albumin	[44]

*: multidrug resistance proteins; +: breast cancer resistance protein.

Although drugs can penetrate through the BBB *via* different mechanisms, passive diffusion is the primary mechanism for drugs to penetrate the BBB [33]. The amount of drugs or compounds that permeate through the BBB can be measured using two mathematical approaches; the log BB and log PS (log permeability surface-area coefficient) [10,45].

The log BB is calculated using the following equation (Eq1):

$$Log\ BB = \log \frac{(\text{Steady state concentration of a drug in the brain})}{(\text{Steady state concentration of the drug in the blood})}$$

However, the log PS can be calculated using (Eq2):

$$Log\ PS = \log \frac{(\text{Observed permeability across the BBB})}{(\text{Surface area of capillary endothelium of the brain})}$$

The log BB value has been considered as a simple method to predict the penetration of compounds through the BBB. Several attempts have been carried out using *in silico* models to foresee the penetration of drugs through the BBB, and consequently predict their compartmental localisation [46]. However, the log PS value is more complicated and usually requires *in vivo* microsurgical operations to directly measure the apparent permeability of free molecules across the BBB, which reflects its limited applicability [47].

Due to its strictly protective function to regulate the transportation of xenobiotics into the CNS, extensive research has been carried out to improve the penetration of pharmacologically active agents through the BBB to treat CNS related diseases (Alzheimer, psychosis, tumours, etc.) [48–50]. However, in some specific cases, drugs that act in the peripheral system can unfavourably accumulate in the CNS and exert CNS side effects. This was the case of the 1st generation of antihistamine (which causes sedation) and antimuscarinic agents for treating overactive bladder (which induce cognitive impairment in elderly), where their CNS associated side effects have always negatively impacted their clinical applications [51,52]. Therefore, developing new DDSs offering selective compartmental localisation of the therapeutic effects of the drugs remains an unmet challenge.

1.2.2. The PB

The placenta is the only organ that contains two genetically different tissues, and it starts to develop and grow at the beginning of each pregnancy [53,54]. It has a complicated histological structure with several layers of different types of cells that form its unique disc shape [10]. The chorionic villi form the basic units of the placental structure. These vascular projections of the fetal tissues are surrounded by chorion, which consists of two layers of cells: the inner cytotrophoblast and the outer layer of syncytiotrophoblast localising within the intervillous space and directly contacting the maternal blood [55]. As the gestation progresses, a single layer of the syncytiotrophoblast acts as a separating layer between the endothelium of the fetus blood vessels and the maternal blood, with the diminished presence of cytotrophoblast layer [56].

The placental terminal villi provide a large surface area of 12-14 m^2 for exchange and transportation of gases and nutrition with fetal tissues [55,56]. It is obvious that the physiological function of the placenta is to maintain the healthy development of the fetus and this is achieved in different ways. The placenta provides adequate blood supply of oxygen and nutrients to the fetus, and helps the fetus to develop its immune system by transferring maternal antibodies from the mother to the fetus [57,58]. Moreover, the placental tissues protect the fetus from the effects of xenobiotics, where it controls the delivery of compounds to the fetal tissues. Furthermore, the placenta exhibits liver-like function by producing specific enzymes protecting the fetus, such as 11-β-hydroxysteroid dehydrogenase 2 (11-βHSD2) which inactivates the maternal cortisol [59]. In addition to that, the placenta secretes various hormones such as progesterone, oestrogen, placental lactogen and human chorionic gonadotrophin (hCG) which regulate the development of the embryo [60,61].

Transportation across the PB

Most of the maternally administered medications can penetrate the PB and reach the fetus. This is desirable when the fetus has health conditions which require treatments such as fetal arrhythmia or immature fetal lungs [62,63]. However, the transported drugs could also negatively affect the fetus with high risks of impairing the fetal development and growth or even inducting teratogenic effects [64].

Scientists have recognised three types of transplacental drug delivery depending on the concentrations of a drug administered to the mother in both fetal and maternal circulations. These are summarised in (**Table 1.2**) [65,66].

Table 1.2. Types of placental transfer of drugs [65,66].

Type of transfer	F_c/M_c	Example
Complete transfer	=1	Ampicillin, morphine, paracetamol, lorazepam, methyldopa
Incomplete transfer	<1	Cefotaxime, lidocaine, propranolol, phenobarbital PAMAM-drug conjugate (*ex vivo* study)
Exceeding transfer	>1	Ketamine, valproic acid, diazepam

*F_c/M_c: the ratio of the concertation of a drug in fetal blood to the concentration of the drug in the maternal blood.

With similarity with the BBB, the same transportation mechanisms are also present in the placenta (**Table 1.3, Figure 1.3**), with passive diffusion being the predominant feature for controlling the passage of chemical molecules to the fetal tissues (reviewed further in references [67,68]).

Therefore, designing DDSs to compartmentalise the therapeutic effects of drugs in the dame's bloodstream would protect the fetus, which provides a new angle to treat health complications during pregnancy.

Table 1.3. Potential mechanisms of transportation through the placenta [65,66,69,70].

Mechanism	Substrates	Ref
Passive diffusion (trans or paracellular)	Paracetamol, lipophilic molecules (MW <400-600 Da)	[71]
Carrier mediated influx (facilitated diffusion)	Amino acids, monoamines nucleosides	[70]
Active efflux transportation (P-glycoprotein, MRP 1-3*, $BCRP^+$)	Already penetrated molecules: digoxin, methotrexate	[68]
Pinocytosis	Not enough data are available	[56]

*: multidrug resistance proteins; $^+$: breast cancer resistance proteins.

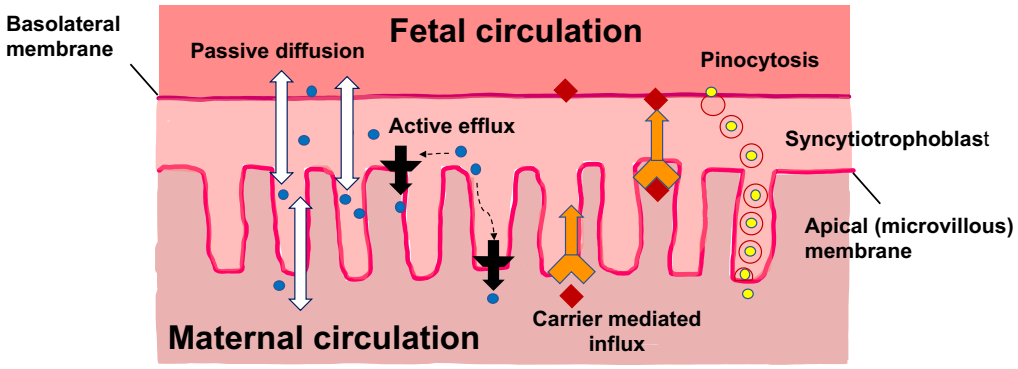

Figure 1.3. Cartoon representation of mechanisms of transportation across the human placenta (adapted from [56,70]).

1.2.3. Diffusion across the BBB and PB

As stated previously (**sections 1.2.1** and **1.2.2**), passive diffusion forms the predominant mechanism to cross both the BBB and the PB [39,71]. The physicochemical properties of passively diffusing drugs have been identified as major factors influencing their transfer *via* this mechanism. Small lipophilic drugs with MW <400-600 Da have been shown to freely diffuse through both barriers [10,72,73]. Therefore, it has been suggested that altering the solubility and the size of such drugs would dramatically affect their diffusion through these barriers and localise their effects at the desired compartment [74]. One strategy that could be employed to modify the physicochemical properties of drugs to reduce or even avoid their penetration across the BBB and PB is to covalently conjugate them to hydrophilic polymers, as further discussed in **section 1.3**.

1.3. PDCs

The first attempt to conjugate drugs to polymers was reported in the 1960s, where penicillin-related antibiotics were conjugated to polyvinylpyrrolidone [75–77]. However, the concept of the strategy was introduced, for the first time, by Helmut Ringsdorf in 1975. He initially called this system "pharmacologically active polymers or polymeric drugs" [78,79]. These nanosized DDSs were based on designing and developing therapeutic macromolecules, in which one or more drug molecules are conjugated to a hydrophilic polymer through covalent bonds. A targeting moiety could be additionally linked to the polymeric backbone to enhance the selectivity of the system (**Figure 1.4**). For reviews of PDCs, see, for examples, references [80–82].

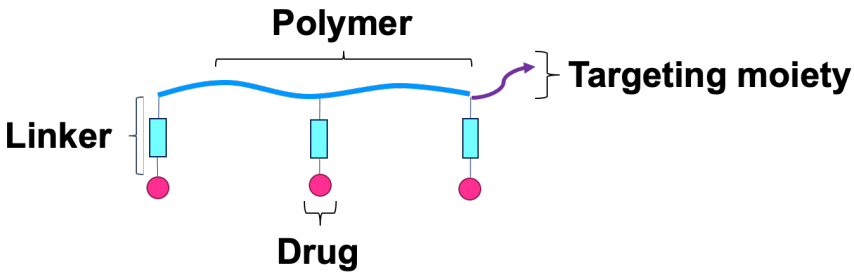

Figure 1.4. General structure and components of PDCs.

1.3.1. General features of PDCs components

Since that date of proposing the concept of PDCs, extensive research has been performed to identify the criteria to select the components of the PDC system (i.e. the polymer, the drug, the linker and the targeting moiety) that allow the design of therapeutically active agents [83].

Polymers

Polymers used to design PDCs must have specific properties. They should be water-soluble and contain at least one functional group that allows direct conjugation with a drug or the addition of a specific spacer [84]. Therefore, the maximum loading capacity of polymers (the number of functional groups available for conjugation) is another factor to be considered when selecting them to design PDCs [85]. The maximum loading capacity varies among polymers, from 1 in some poly(ethylene glycol) PEG polymers to more than 200 in poly(L-glutamic acid) PGA or even more in other polymers such as dextran depending on their molecular weight [86–88]. Polymers with higher loading capacity are usually preferred as they allow fewer polymers to be administered to achieve the required dose of the conjugated drug. In addition to that, they should be biocompatible and not generate any toxic metabolites or induce any immune reactions [79]. Moreover, polymers should not accumulate within the body and they should undergo biodegradation or complete excretion for non-biodegradable polymers. Therefore, the size of the polymers should be taken into consideration when designing PDCs [89]. For biodegradable polymers, their MW will affect the circulation time of the system in the bloodstream. However, and because they will be metabolised by the body, the risk of their unfavourable accumulation within the body is low [90]. On the other hand, the size of non-biodegradable polymers influences their final fate in the body. Therefore, their molecular weights should be below the renal excretion threshold of molecules (MW <40 kDa) to ensure their excretion *via* kidney filtration and reduce the risk of accumulation [91]. Several natural or synthetic polymers, with branched or linear structures, have been employed in designing PDCs, (**Figure 1.5, Table 1.4)**.

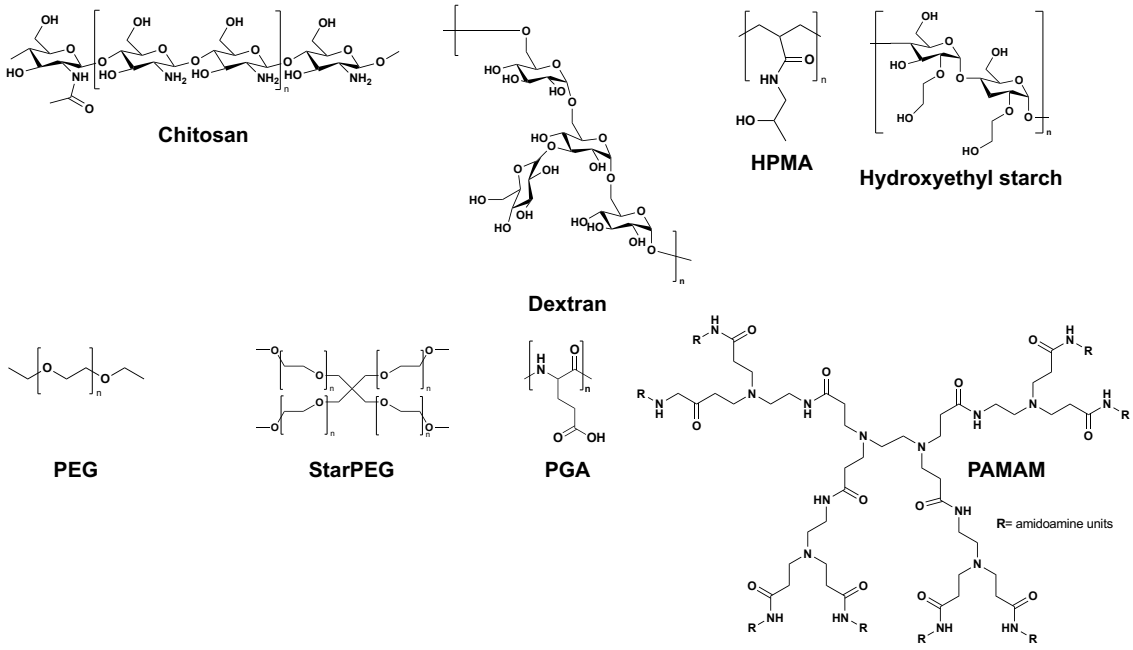

Figure 1.5. Chemical structures of commonly used polymers in designing PDCs.

Table 1.4. Examples of polymers used in designing PDCs.

Polymers	Nature /Structure	PDCs	Examples of applications	Polymer's degradability	Ref
Chitosan	Natural/Linear	Chitosan-stavudine	HIV	Biodegradable	[92]
Dextran	Natural/Linear	Dextran-methylprednisolone	Immunosuppression	Biodegradable	[93]
HPMA*	Synthetic/ Linear/branched	HPMA copolymer - doxorubicin	Cancer	Non-biodegradable/ backbone-degradable	[94]
Hydroxyethyl starch	Natural/Linear	Hydroxyethyl starch-hydroxychloroquine	Cancer	Biodegradable	[95]
PEG	Synthetic/Linear	PEG-naloxol	Opioid-induced constipation	Non-biodegradable	[96]
StarPEG	Synthetic/Branched	sPEG-nystatin	Fungal infection	Non-biodegradable	[97]
PAMAM**	Synthetic/Branched	PAMAM G5-methotrexate	Rheumatoid arthritis	Non-biodegradable	[98]
PGA	Synthetic/Linear	PGA-paclitaxel	Cancer	Biodegradable	[99]

*: *N*-(2-hydroxypropyl)methacrylamide; **: Poly(amidoamine)

Drugs

The chemical structure of a drug to be conjugated should have at least one functional group to allow the conjugation with a polymer. Several functional groups have been utilised to establish the linkage between the drug and the polymer such as a hydroxyl group, an amine group or a sulfhydryl group [100–102]. However, for a limited number of studies (including the work presented in this thesis) where PDCs were designed as non-prodrug systems (i.e. there is no liberation of the conjugated drug), the functional groups of the drug employed in the conjugation reaction should not be essential for the biological activity of the drug or it will be abrogated due to conjugation [74]. The drug should also be detectable using an appropriate analytical technique. Furthermore, the drug should also be potent, where less potent drugs

would require to be administered at high doses. This would prevent the administration of large quantities of the conjugated drug (depending on the % of loading) [83].

Chemical linkers

For the most therapeutic applications of PDCs, the release of the conjugated drug at the targeted site is essential to exert the biological activity, therefore, the linker should show stability until reaching the site of action [103]. However, in other cases, the liberation of the linked pharmacophore must be avoided while retaining its therapeutic activity [74]. Therefore, the linkers could be categorised into two groups: cleavable linkers (which have been utilised in the vast majority of PDCs designed as prodrug macromolecules) and non-cleavable linkers (which have been used to design non-prodrug PDCs). The selection of cleavable linkers is mostly based on the unique features of the diseases to be treated and the cellular compartments they will occupy (such as overexpression of specific enzymes, oxidation level and specific pH within the tissue and cellular compartments) [82,104]. These disease-specific stimuli would initiate the selective release of the conjugated drugs only at the selected sites of action and not in the normal tissues, **Table 1.5**.

Table 1.5. Examples of stimuli used to release drugs from PDCs, and type of linkers used.

Stimuli	Linker example	PDCs	Application	Ref
pH	Ester	Dextran-naproxen	Colitis	[105]
	Amide	Chitosan-kartogenin	Osteoarthritis	[106]
	Hydrazine	PGA-doxorubicin-aminoglutethimide	Cancer	[107]
Enzymes	Ester	PEG-acyclovir	Viral infection	[108]
	Amide	PGA-dopamine	Cancer	[86]
	Dipeptide linker	PEG-2G-PTD7	Septicaemia	[109]
Redox activity	Disulphide	PAMAM (G3.5)-*N*-acetyl cysteine	Neuroinflammation	[110]

On the other hand, for cases where preventing the release of conjugated drugs is the aim, ether and carbamate groups could be employed to design biologically stable PDCs that act as non-prodrug macromolecules [74,111].

Targeting moiety

The fourth, however, an optional, component of the general structure of PDC design is the targeting moiety [83]. Although the majority of PDCs have been designed with the aim of passive accumulation within the tissues (utilising the physicochemical properties of their polymers and the pathophysiological features of the treated tissues), active targeting has also been employed to improve the selectivity of PDCs [82]. Several cancers or non-cancer tissues (with specific pathological conditions, see **Chapter 2**) have been identified to express "tissue-

specific" receptors, enzymes or other markers [112,113]. These specific markers are essential to select the appropriate targeting group to graft the PDCs with [114].

As previously mentioned, PDCs could be designed as prodrug or non-prodrug macromolecules with respect to the disease and/or the drug to be conjugated. The key differences between the component of both systems and their design are summarised in (**Figure 1.6**).

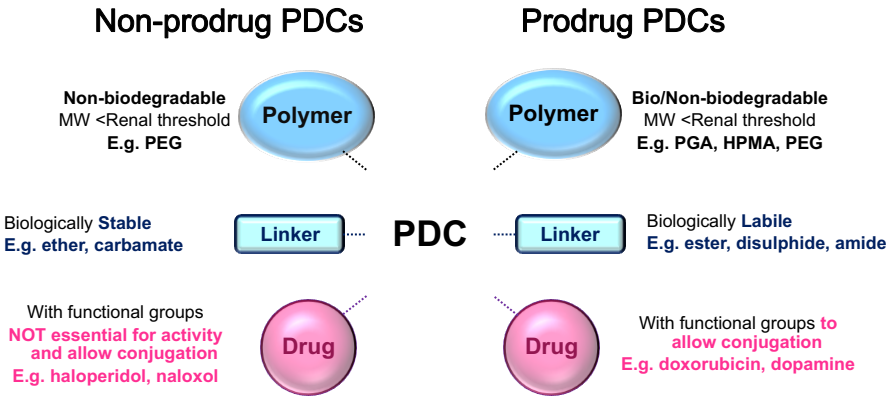

Figure 1.6. Schematic represntation of differences of the design of PDCs.

1.3.2. Therapeutic applications of PDCs

Since their first introduction in 1975, PDCs were extensively applied in the field of cancer [113]. However, they were also developed to treat diseases other than cancers [115]. For these diseases, the rationales and the key features underpinning the design of PDCs vary according to the disease (discussed in detail in **Chapter 2** [72]). However, in the case of cancer applications of PDCs, the rationales in most cases were improving the pharmacokinetics of the chemotherapeutics, preventing the side effects associated with conventional forms and providing site targeted release of the anticancer agents [116]. The leaky blood vessels and the impaired lymphatic drainage of solid tumours allow preferential passive accumulation of PDCs within these tissues. This phenomenon was defined for the first time in the 1980s by Maeda *et al* as "the enhanced permeability and retention effect (EPR effect)" and formed the traditional purpose in designing PDCs and other nanomedicines (e.g. liposomes, nanoparticles, polymeric micelles, polymer-protein conjugates and antibody-drug conjugates [117]) to treat several solid tumours [82,118,119]. Since that date, several PDCs were developed and tested on different levels (preclinical and clinical studies) as nanosized chemotherapeutic agents, however, none has reached the market. The gap between the optimistic preclinical data and outcomes of the clinical studies is highly related to the heterogenicity and complexity of the EPR effects [120]. This has, indeed, required extra efforts to further understand this phenomenon for better utilisation in treating solid tumours, further discussed in **Chapter 3** [121].

1.4. The concept of drug repurposing

Drug repurposing (drug repositioning, drug re-tasking or drug reprofiling) is a concept used to identify new clinical applications for already approved or under-researched drugs [122,123]. Repurposing of drugs is more advantageous than developing entirely novel compounds for the same indications for several reasons [122]. New mechanisms of action or pathways could be further explored and studied when drugs are repurposed, which gives the opportunity to understand the pathology of diseases. It offers, also, the use of already studied drugs deemed to be at least clinically safe. Therefore, the risk of failure at subsequent trails is relatively low compared to a new medication. In addition to that, low investments, in terms of time and cost, are required. This is because many stages of clinical studies required for the approval for novel drugs are no longer needed as some data are available from previous applications [124]. This, in turn, could at least reduce the cost of bringing a new chemical compound to the market from ~$3 billion to $300 million in the case of a repurposed drug [125].

Drugs repositioning is not without challenges, for example, due to the risk of failure of translating the preclinical studies to clinical ones. In addition to that, toxicity profiles and side effects associated with the administration of these medications are also persistent challenges [124,125]. Examples of repositioned drugs are presented in **Table 1.6**.

Table 1.6. Examples of repurposed drugs.

Drug	Original/initial indication	New/proposed indication	Ref
Celecoxib	Osteoarthritis, rheumatoid arthritis	Familial adenomatous polyposis, colorectal cancer	[126]
Haloperidol	Antipsychotic	Bacterial and fungal infection, Covid-19, cancers	[127–130]
Minoxidil	Hypertension	Alopecia	[131]
Rituximab	Various cancers	Rheumatoid arthritis	[132]
Sildenafil	Angina	Male erectile dysfunction	[133]
Thalidomide	Morning sickness	Multiple myeloma	[133]

1.5. PDCs and drug repurposing

One strategy that could be utilised to improve the safety profiles of drugs and reduce their associated side effects (especially with unfavourable penetration of drugs across biological barriers) is the PDC concept [116]. However, it is important to stress that the developed PDCs are new chemical materials which requires their safety to be assessed in order to ensure they pass all Regulatory Approval Standards before reaching the market, reviewed further in reference [134].

1.6. Project Aims

Unfavourable accumulation within specific tissues (such as the brain and the placenta) has been considered a major hurdle affecting repurposing of several drugs by impacting their safety profiles [135,136]. Haloperidol, a D_2 receptor antagonist used to treat psychosis, has many potential non-CNS applications (through several receptors) [128,129,137]. However, these applications are limited by its penetration across biological barriers such as the BBB and the PB [138,139]. The concept of polymer-drug conjugate has been established as a strategy to prevent diffusion across biological barriers while providing localised effects at the intended site of action [80,116]. PEGylated naloxol (Movantik®) was the first PDC to reach the market and was designed to localise the effects of naloxol, an opioid antagonist, in the intestine (without diffusing through the BBB) after oral administration [96].

Therefore, the overall aim of this PhD project was to evaluate the PEGylated haloperidol conjugate for its impaired penetration across the BBB and the PB and to explore its potential therapeutic applications as a compartmentalised therapeutic agent.

The organisation of the thesis is summarised in (**Figure 1.7**). The general background of this thesis has also included, in addition to the general introduction chapter, **Chapters 2** and **3**. The first objective of the current project was to critically appraise PDCs developed to treat non-cancer diseases and consider the key rationales underpinning their design. This was reviewed in **Chapter 2**. Then, in **Chapter 3,** clinical studies related to the application of nanomedicines to treat solid tumours (accumulating *via* the EPR effect) were analysed to identify the key parameters influencing the EPR effect and to determine factors affecting patient selection for these treatments. Thereafter, the feasibility of the PEGylation strategy to prevent penetration of conjugated drugs penetration across biological barriers was explored and the potential compartmentalised therapeutic effects were studied (**Figure 1.7**). The ability of the conjugation strategy to retain the biological activity of haloperidol through D_2 receptors while preventing its penetration across the BBB was evaluated in **Chapter 4** using different approaches (*in vitro*, *in silico* and *in vivo*). Potential repurposed therapeutic applications of PEG-haloperidol (acting peripherally) were assessed by exploring its potential activity *via* different receptors for different medical conditions, namely σ receptors in cancer cell lines and GPVI and $P2Y_1$ receptors of the human platelets. Potential antiangiogenic activity of PEG-haloperidol conjugates (acting *via* σ receptors) was explored *in vitro* and *in silico* and the effect of PEG size was assessed as well in **Chapter 5**. Proposed cardiovascular applications of the PEG-haloperidol conjugate were studied by exploring, *in vitro*, the effects of the conjugate on the aggregation of human platelets (through GPVI and $P2Y_1$ receptors), presented in **Chapter 6**.

The feasibility of PEGylation to develop non-prodrug macromolecules as DDSs reducing the transfer of drugs across the placenta was studied *ex vivo* in **Chapter 7**. The overview of the project is summarised in **Figure 1.7**.

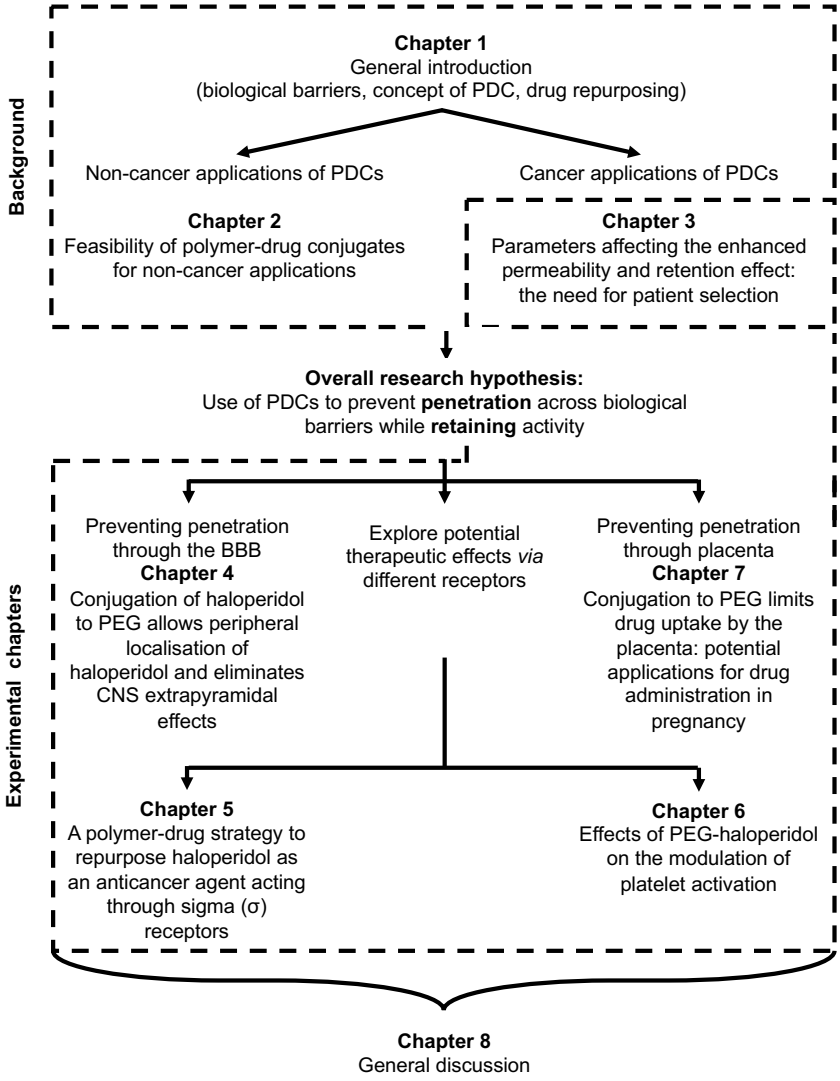

Figure 1.7. Schematic representation of the organization of the content of this PhD thesis.

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Chapter 2
Feasibility of polymer-drug conjugates for non-cancer applications

Chapter summary:
In this chapter, the rationale and key features for designing PDCs for treating diseases other than cancer are discussed.

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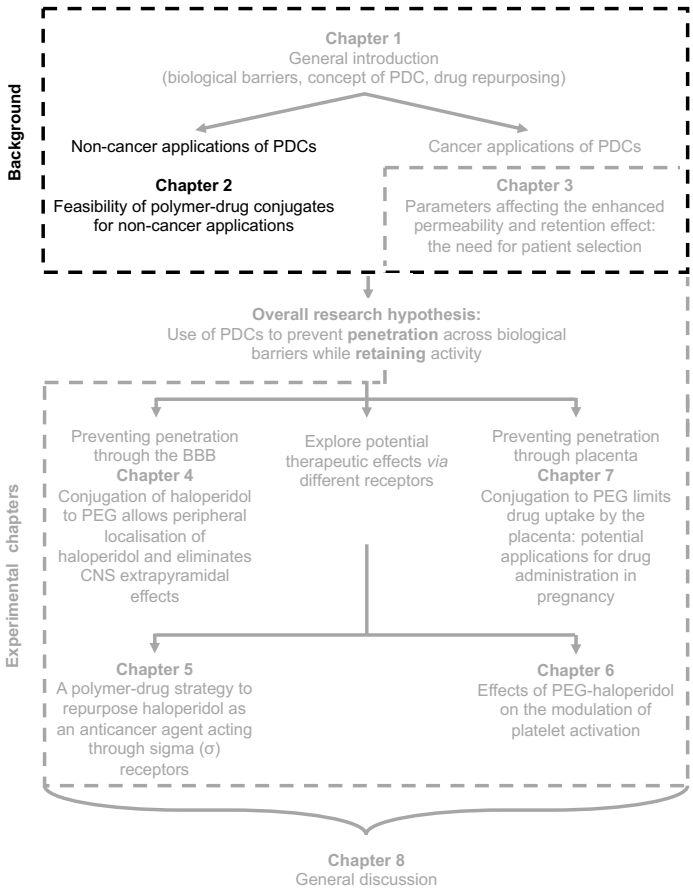

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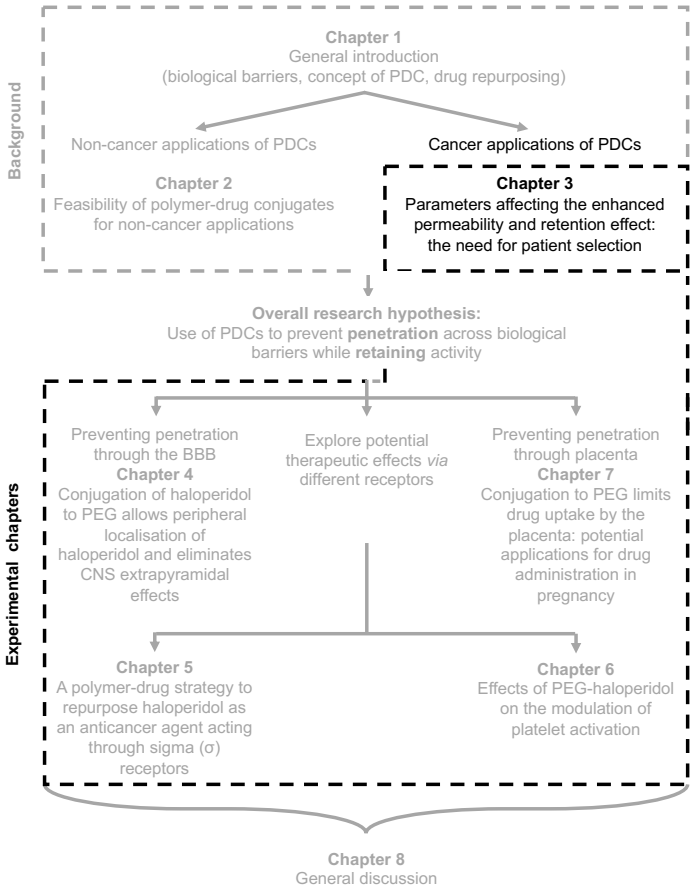
Chapter 3
Parameters affecting the enhanced permeability and retention effect: the need for patient selection

Chapter summary:
In this chapter, clinical studies that used nanomedicines to treat a range of solid tumours are analysed to decipher the key parameters that influence the magnitude of the EPR effect. In addition, two strategies to select patients who might be most suited for treatment with nanomedicines are suggested.

Bibliographic details: *A.A. Natfji, D. Ravishankar, H.M.I. Osborn, F. Greco, Parameters Affecting the Enhanced Permeability and Retention Effect: The Need for Patient Selection, J. Pharm. Sci. 106 (2017) 3179–3187.*

Author Contributions: *F.G and H.M.I.O. designed, reviewed and supervised the study. A.A.N performed the search, data analysis and prepared the original draft of the manuscript. A.A.N and D.R constructed figures and edited the manuscript. All authors edited the manuscript.*



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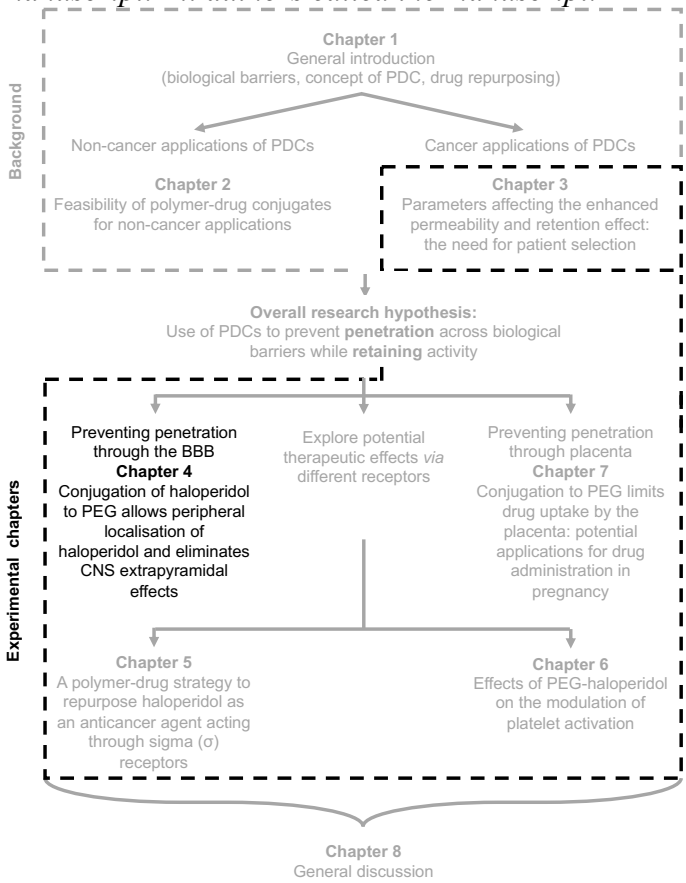
Chapter 4
Conjugation of haloperidol to PEG allows peripheral localisation of haloperidol and eliminates CNS extrapyramidal effects

Chapter summary:
In this chapter, the impact of conjugating haloperidol to PEG on its pharmacological activity and its ability to penetrate the BBB are evaluated via in silico, in vitro and in vivo studies. The result indicated that PEG-haloperidol preserved its activity through D_2 receptors, and, this was lower than that of free haloperidol. In vivo studies on rats revealed the impaired ability of PEG-haloperidol to cross the BBB which was indicated by the absence of catalepsy.

Bibliographic details: *A.A. Natfji, D.O. Nikitin, I.I. Semina, R.I. Moustafine, V. V. Khutoryanskiy, H. Lin, G.J. Stephens, K.A. Watson, H.M.I. Osborn, F. Greco, Conjugation of haloperidol to PEG allows peripheral localisation of haloperidol and eliminates CNS extrapyramidal effects, J. Control. Release. 322 (2020) 227–235.*

Author Contributions: *H.M.I.O and F.G designed, reviewed and supervised the study. A.A.N carried out the synthesis and characterisation of the PEG-haloperidol conjugate. A.A.N performed in silico calculations of log BBB. A.A.N designed and D.O.N carried out the in vivo study under the supervision of I.I.S, R.I.M, and V.V.K. L.H and A.A.N carried out the binding study under the supervision of G.J.S. A.A.N performed in silico MD studies under the supervision of K.A.W. A.A.N performed all data analysis, constructed figures and prepared the original draft of the manuscript. All authors edited the manuscript.*



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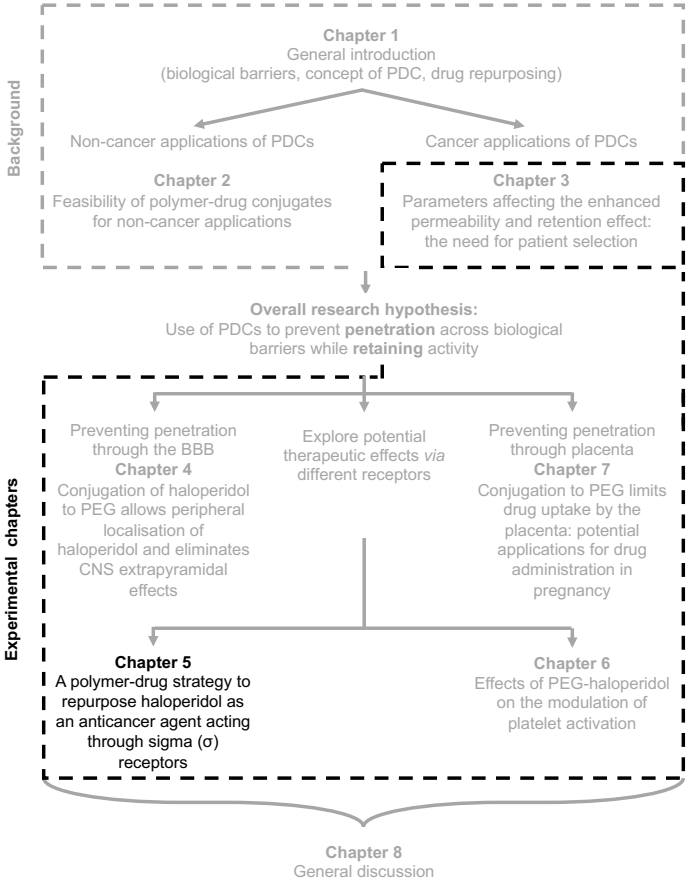
Chapter 5
A polymer-drug strategy to repurpose haloperidol as an anticancer agent acting through sigma (σ) receptors

Chapter summary:
In this chapter, the potential anticancer effects (through σ receptors) of PEG-haloperidol conjugates prepared using 2 different MW of PEG are explored via in silico and in vitro studies. Using of PEG (2000 Da) to prepare the conjugate increased the loading of haloperidol in the conjugate by 3-fold when compared to PEG (6000 Da). In vitro cytotoxicity studies on 2 breast cancer cell lines indicated the limited application of the conjugates as antiproliferative agents. The conjugates exhibited anti-migratory effects on the VEGF-induced migration of HUVECs, however, at lower levels when compared with free haloperidol.

Bibliographic details: *A.A. Natfji, C. Pegoraro, K.A. Watson, H.M.I. Osborn, F. Greco, A polymer-drug strategy to repurpose haloperidol as an anticancer agent acting through sigma (σ) receptors, Pharmaceutics. (2020). Under revision.*

Author Contributions: *H.M.I.O and F.G designed and supervised the study. MD studies were supervised by K.A.W. A.A.N preformed the synthesis and characterisation of PEG-haloperidol conjugates, in vitro and MD studies, data analysis and prepared the original draft of the manuscript. A.A.N supervised C.P's work on the preliminary synthesis of PEG-haloperidol conjugate (2000 Da) and initial MD studies. All authors edited the manuscript.*



Article

A polymer-drug strategy to repurpose haloperidol as an anticancer agent acting through sigma (σ) receptors

Az Alddien Natfji [1], Camilla Pegoraro [1,2], Kimberly A. Watson [3], Helen M. I. Osborn [1] and Francesca Greco [1,*]

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Received: date; Accepted: date; Published: date

Abstract: We have recently reported that conjugation of haloperidol to poly(ethylene glycol) (PEG) prevents its passive diffusion through the BBB and eliminates CNS side effects, with potential for peripheral therapeutic applications of haloperidol. Here we investigate a polymer-drug conjugate strategy to repurpose haloperidol as an anticancer agent acting through sigma (σ) receptors. PEG (MW 2000 and 6000 Da)-haloperidol conjugates were synthesised. The lower molecular weight PEG (2000 Da) allowed a dramatic increase in haloperidol's loading in the conjugate (from ~ 8 to ~25%w/w) and maintained a high purity (free haloperidol < 0.2%). Direct anticancer effects were assessed against MCF-7 and MDA-MB 231 breast cancer cell lines. The IC_{50} of both conjugates were > 100 μM (compared to ~50 μM for haloperidol), limiting their antiproliferative applications. The conjugates were then tested for anti-angiogenic activity *in vitro*. Both conjugates significantly inhibited the migration of vascular endothelial cells (> 65% inhibition) although to a lesser extent than free haloperidol (91% inhibition). Molecular docking studies demonstrated that indeed the conjugates retained affinity for σ receptors, and explained the partial loss of activity. Taken together these data indicate polymer conjugation as a feasible strategy for repositioning haloperidol as a promising antiangiogenic agent with limited CNS side effects.

Keywords: Polymer-drug conjugates, tumours, haloperidol, PEG, angiogenesis, sigma receptors.

1. Introduction:

Upon their identification in 1976, sigma (σ) receptors were initially classified as one of the subclasses of opioid receptors [1]. However, subsequent studies reported that these receptors are not related to the opioid receptors and form a unique family of receptors with two identified receptor subtypes (σ_1 and σ_2) [2]. Recently, different biological molecules have been identified as proposed endogenous ligands for these receptors, for example: steroid hormones, choline and dimethyltryptamine [3,4]. Several reports showed that the molecular and the biological actions of σ receptors remain uncertain [5,6]. However, they have been found to exert their effects *via* regulating and modulating several membrane proteins including ion channels (such as Ca^{+2} channels) and other G protein-coupled receptors (such as dopamine receptors) [7–9]. Therefore, they have been suggested to mediate the actions of various therapeutic agents of several pharmacological classes such as neuroprotective, immunosuppressing, and antipsychotic agents [6,10]. Both σ receptor subtypes are present in various tissues including the central nervous system (CNS) and other peripheral organs such as liver, heart, kidney, etc [11]. Several studies have identified different locations of these receptors such as the plasma membrane, endoplasmic reticulum and its mitochondrial-associated membrane and the nuclear envelop [8]. Interestingly, σ receptors have also been reported to be overexpressed in different

cancer cell lines and cancer tissues biopsies, including breast, brain, colon, ovarian, and pancreatic tumours [12–14]. They have been shown to orchestrate the growth of tumour cells by influencing their proliferation and potentiating angiogenesis leading to more aggressive tumour cells. Therefore, these receptors have been attracting attention as potential targets for therapeutic and diagnostic purposes for several tumours *in vitro*, *in vivo* and clinically [15–18].

Haloperidol, a dopamine (D_2) receptor antagonist used as an antipsychotic drug, has shown an affinity towards both subclasses of σ receptors (as antagonist and agonist for σ_1 and σ_2 receptors, respectively) [19]. Several studies documented promising haloperidol's therapeutic effects against several solid tumours *in vitro* and *in vivo* which are well summarised in this review [20]. Therefore, it has been suggested the repurposing of haloperidol as an anticancer agent *via* two mechanisms. Direct effects of haloperidol on tumour growth were confirmed by inhibiting proliferation (*via* inducing apoptosis) and motility with mitotic arrest effects in tumour cells [21,22]. In addition, haloperidol was suggested to indirectly suppress tumour growth by affecting the angiogenesis process within tumour tissues [23,24]. However, treatments with the conventional forms of haloperidol are usually accompanied with CNS side effects (extrapyramidal effects). These unwanted effects are due to the antagonistic activity of haloperidol on the CNS dopaminergic system as a consequence of the passive diffusion of haloperidol's small molecules through the blood-brain barrier (BBB) [25]. Therefore, these central side effects are a major hurdle limiting the repurposing of haloperidol as an anticancer agent clinically. To overcome the problem of the unwanted biodistribution of haloperidol to the CNS, we previously developed a biologically stable polymer-drug conjugate system for haloperidol by covalently linking it to a non-biodegradable hydrophilic polymer, poly(ethylene glycol) (PEG), through a biologically stable carbamate linkage to restrict its penetration through the BBB [26]. This demonstrated that the conjugation strategy prevents haloperidol from crossing through the BBB. Moreover, the conjugate retained its antagonistic effects on D_2 receptors (but with less potency compared to the free haloperidol) [27]. These findings suggested the feasibility of using PEGylated haloperidol for peripheral applications.

In the current study, we investigate one such application by proposing the use of PEG-haloperidol as an anticancer agent. Specifically, PEG-haloperidol conjugates, synthesised using two different molecular weight of PEG (6000 and 2000 Da), were assessed *in vitro and in silico*. The direct anticancer effects of the PEG-haloperidol conjugates were assessed using two breast cancer cell lines (MCF-7 and MDA-MB 231). Then, we assessed the abilities of the PEG-haloperidol conjugates to inhibit angiogenesis (indirectly affecting tumour growth) *via* monitoring their effects on the migration of human umbilical vein endothelial cells (HUVEC). Finally, we employed *in silico* molecular docking studies to understand the impact of conjugation on the activity of haloperidol *via* the σ receptors.

2. Materials and Methods

2.1. Materials

Alpha,omega-di-succinimidyl ester poly(ethylene glycol) (MW 6429 and 1983 Da) were purchased from Iris Biotech GmbH, Germany. Haloperidol was purchased from Sigma-Aldrich, UK. All other solvents and chemicals were purchased from Sigma-Aldrich, UK and Fisher Scientific, UK and were used without further purification unless otherwise stated. Deuterated solvents for NMR spectroscopic analysis were obtained from Sigma-Aldrich, UK and Cambridge Isotope Laboratories, UK.

2.2. Synthesis of PEG-haloperidol conjugates:

Two molecular weights of PEG (6000 Da and 2000 Da) were used to synthesise PEG-haloperidol conjugates. The conjugation of haloperidol to PEG was carried out following a protocol previously developed by our group to synthesise PEG-haloperidol (6000 Da) [26,27]. To the first time, we present the synthesis of PEG-haloperidol conjugate (2000 Da). The protocol of PEG-haloperidol conjugates

synthesis includes two steps: first haloperidol was modified with an amine handle to allow conjugation; second modified haloperidol was conjugated to *N*-Hydroxysuccinimide (NHS) activated PEG of two MW (i.e. 6000 and 2000 Da).

2.2.1. Modification of haloperidol with an amine handle at the hydroxyl group:

This step was carried out according to our previously published protocol [26,27]. The final product haloperidol amino-ethyl carbamate (haloperidol-AEC) was characterised using ^{1}H and ^{13}C NMR spectroscopic analysis. The product was then conjugated to NHS activated PEGs.

2.2.2. Conjugation of modified haloperidol (haloperidol-AEC) to NHS activated PEG of two MW:

The conjugation of modified haloperidol to NHS-PEG-NHS (6000 Da) was carried out according to our previously published protocol [26,27]. The same protocol was applied here to conjugate haloperidol-amine handle to NHS-PEG-NHS (2000 Da). Modified haloperidol (haloperidol-AEC) (4 equiv., 277 mg, 600 µmol) was dissolved in anhydrous $CHCl_3$ (10 mL) and *N*,*N*-diisopropyl-ethylamine (DIPEA) (4 equiv., 105 µL, 600 µmol) was added to the solution under an inert atmosphere. NHS-PEG-NHS (MW 1983, 297 mg, 150 µmol) was dissolved in $CHCl_3$ (5 mL) and added dropwise. The reaction was stirred for 3 days during which time it was protected from light. The reaction mixture was evaporated under vacuum to ~7 mL. The purification of the final product was carried out using column chromatography (silica gel, chloroform–methanol mixture containing 90:10 to 80:20). The final product was dissolved in ~7 mL of water and freeze dried for 3 days. PEG-haloperidol (2000 Da) was characterised using ^{1}H and ^{13}C NMR spectroscopic analysis and RP-HPLC. **Yield**: (71%). **^{1}H NMR** ($CDCl_3$, 700 MHz): 10.97 (s, CO-CH_2-CH_2-COO*H*, PEG), 8.01 (dd, *J*=7 Hz, 7 Hz, F-Ar-*H*, HA-AEC), 7.3 (dd, *J*=7 Hz, 7 Hz, Cl-Ar-*H*, HA-AEC), 7.13 (t, *J*=7 Hz, F-Ar-H, HA-AEC), 6.92 (s, NH, HA-AEC), 6.37 (s, NH, HA-AEC), 3.66-3.61 (m, O-C*H*$_2$-C*H*$_2$, PEG), 3.58-3.55 (m, NH-C*H*$_2$-CH_2-O, PEG), 3.52-3.45 (m, NH-C*H*$_2$-CH_2-NH, HA-AEC), 3.44-3.38 (m, NH-CH_2-C*H*$_2$-NH, N-(C*H*$_2$-CH_2), HA-AEC), 3.35-3.30 (m, N-(C*H*$_2$-CH_2) HA-AEC), 3.29-3.23 (CO-*CH*$_2$-CH_2-CH_2, HA-AEC),), 3.21-3.15 (m, N-(CH_2-C*H*$_2$) HA-AEC), 3.13-3.08 (m, N-(CH_2-C*H*$_2$) HA-AEC) 2.78-2.70 (m, (CO-*CH*$_2$-CH_2-CO, PEG), 2.66-2.63 (m, (CO-CH_2-*CH*$_2$-CO, PEG), (CO-CH_2-CH_2-C*H*$_2$, HA-AEC)), 2.62-2.58 (m, (N-(CH_2-C*H*$_2$) HA-AEC, (CO-CH_2-C*H*$_2$-COOH, PEG)), 2.49-2.44 (m, (N-(CH_2-C*H*$_2$) HA-AEC, (CO-CH_2-C*H*$_2$-COOH, PEG)), 2.37-2.3 (m, CO-CH_2-C*H*$_2$-CH_2, HA-AEC).**^{13}C NMR**, ($CDCl_3$, 125 MHz): 197.05 (Ar-*C*O-CH_2-, HA-AEC), 173.4 (NH-*C*O-CH_2, PEG), 172.76 (CH_2-CH_2-*C*OOH, PEG), 165.96 (Ar-F, HA-AEC), 154.73 (*C*O-NH-CH_2- CH_2, HA-AEC), 141.27 (Ar-Cl, HA-AEC), 133.63 (Ar-Cl, HA-AEC), 132.77 (Ar-F, HA-AEC), 130.81 (Ar-F, HA-AEC), 128.78 (Ar-Cl, HA-AEC), 126.02 (Ar-Cl, HA-AEC), 115.9 (Ar-F, HA-AEC), 70.54 ((O-*C*H$_2$-*C*H$_2$), PEG), 70.10 ((*C*(O-CO)-Ar, HA-AEC), 69.5 (NH-*C*H$_2$-*C*H$_2$-(O- *C*H$_2$-*C*H$_2$), PEG), 69.54 (NH-*C*H$_2$-*C*H$_2$-(O-*C*H$_2$-*C*H$_2$), PEG), 48.36 (NH-*C*H$_2$-*C*H$_2$-NH, HA-AEC), 42.00 (*C*H$_2$-CH$_2$-N-*C*H$_2$-*C*H$_2$, HA-AEC), 41.71 (*C*H$_2$-CH$_2$-N-*C*H$_2$-*C*H$_2$, HA-AEC), 39.42 (*C*H$_2$-*C*H$_2$-N-*C*H$_2$-*C*H$_2$, HA-AEC), 39.34 (*C*H$_2$-*C*H$_2$-N-*C*H$_2$-*C*H$_2$, HA-AEC), 35.65 (CO-*C*H$_2$-*C*H$_2$-*C*H$_2$, HA-AEC), 33.26 (CO-*C*H$_2$-*C*H$_2$-CO, PEG), 31.96 (*C*H$_2$-*C*H$_2$-CO-OH, PEG), 31.60 (*C*H$_2$-*C*H$_2$-CO-OH, PEG), 25.44 (CO-*C*H$_2$-*C*H$_2$-*C*H$_2$, HA-AEC) 18.37 (CO-*C*H$_2$-*C*H$_2$-*C*H$_2$, HA-AEC).

2.3. Cell culture:

Human breast adenocarcinoma cells (MCF-7), the triple negative breast cancer cell line (MDA-MB 231) and HUVEC cell line were purchased from the European Collection of Cell Cultures (ECACC). MCF-7 cells were cultured in RPMI-1640 (with L-glutamine, Gibco™, UK) medium supplemented with 5% FPS (Gibco™, UK). For MDA-MB 231, the culture medium was Dulbecco's modified eagle's medium (1g/L glucose, with L-glutamine, Gibco™, UK) supplemented with 10% FBS (Gibco™, UK). HUVECs were cultured in EGM-2 (EBM with SingleQuotes™ kit: foetal bovine serum (FPS), fibroblast growth factor B, epidermal growth factor, vascular endothelial growth factor (VEGF), insulin-like growth

factor-1, heparin, hydrocortisone) (Lonza, Belgium). The cells were incubated at 37 °C and 5% CO_2. For HUVECs, the cells were at passage 3 when used in the experiment and were not further sub-cultured.

For *in vitro* cells experiment, a stock solution of free haloperidol was prepared in DMSO and dilutions were carried out using the appropriate growth medium. The concentration of DMSO was kept below ≤0.5% in all test concentrations. As PEG-haloperidol (6000 and 2000 Da) and PEG-COOH (6000 and 2000 Da) are water-soluble, stock solutions were prepared by dissolving the materials in 1% or 0.2 % DMSO solution in the appropriate growth medium. All prepared treatments were sterile filtered through 0.22 μm filters.

2.4. In vitro antiproliferative study:

The direct antiproliferative effects of free and PEGylated haloperidol and PEG-COOH of two different molecular weights were evaluated against MCF-7 and MDA-MB 231 cells, using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, UK) assay [28,29]. Briefly, the cells were seeded into a 96-well plate at a density of 4 x 10^4 cells/mL and 2 x 10^4 cells for MCF-7 and MDA-MB 23, respectively. After 24 h of incubation, to allow cell attachment, the cells were treated with freshly prepared media of free or conjugated haloperidol or PEG-COOH with different concentrations (haloperidol equivalent) and incubated for 67 h. After incubation with the treatment, the MTT assay was carried out by adding 20 μL of MTT solution (5 mg/ml, prepared in PBS, pH 7.4) into each well and further incubating for 5 h. The purple crystals of formazan formed were dissolved in DMSO (100 μL) after removing the medium, drug and MTT solutions [30]. After incubation for 30 min, the plate was read at 570 nm (using SPECTRA max UV spectrometer). All experiments were performed in at least three independent repeats and the data were presented as a mean ± the standard error of mean (SEM). The data were analysed using MS Excel 2016 and the pIC50 value was calculated using GraphPad Prism v8.0 software [30].

2.5. *In vitro cell migration (scratch assay)*

The biocompatibility of free and PEGylated haloperidol and PEG-COOH of different molecular weights were first assessed against HUVECs, using trypan blue exclusion assay [31]. HUVECs were seeded into a 96-well plate at a density of 6 x 10^4 cells/mL. After 24 h of incubation, the cells were treated with freshly prepared media of free or conjugated haloperidol or PEG-COOH with different concentrations (haloperidol equivalent) and incubated for 24 h (in the case of HUVECs). After incubation with treatments, the solutions were removed, and the cells were washed once with PBS (100 μL). Then, trypsin-EDTA (50 μL) was added and the cells were further incubated for 5 min. EGM-2 medium (50 μL) was then to the wells. Aliquots of the cell suspensions (20 μL) were diluted 1:1 with trypan blue (Sigma Aldrich, UK) (0.2% in PBS) and viable HUVECs were counted using a haemocytometer. The experiment was repeated in triplicate and cell viability was expressed as % of control (cells treated with compound free medium) [31].

For wound healing (scratch assay), HUVECs were seeded in 12-well plates at 3 x 10^4 cells/well (1 mL/well) and cultured for 48 h. Then, the media were removed, and the cells were treated with serum starvation medium (0.1% serum in EGM-2) for 24 h to inactivate cell proliferation. The cells were treated for 1 h with 1 mL of medium containing VEGF (Peprotech, UK) (10 ng/mL) and either haloperidol (20 μM), PEG-haloperidol or PEG-COOH of different molecular weights at (20 μM, haloperidol equivalent). After incubation with treatments, the cell monolayer was scratched using a sterile 100 μL pipette tip, and then the cells were washed twice with PBS to remove all cell debris. PBS was removed and 1 mL of media containing previous treatments were added. Images of the scratch were taken at t = 0 h. The cells, after treatment, were incubated in standard tissue culture conditions before taking images of the scratch at 12 h post treatment. The assay was performed in triplicate and each treatment of a compound was carried out in duplicate, in each experiment. The area of the wound not covered by the migrated cells was quantified using ImageJ software (1.52K). Data were normalised to the absence or presence of VEGF

(VEGF-free and VEGF-enriched medium were set at 0% and 100%, respectively), using the following equation:

$$\frac{100 \times (\%\ of\ Drug\ induced\ wound\ closure\ -\ \%\ of\ VEGF\ free\ medium\ induced\ wound\ closure)}{(\%\ of\ VEGF\ induced\ wound\ closure\ -\ \%\ of\ VEGF\ free\ medium\ induced\ wound\ closure)}$$

2.6. Molecular docking studies for designing PEG-haloperidol conjugate

Molecular docking studies were carried out using the programme Surflex-Dock (SFXC) [32], as provided by Sybyl-X 2.1, according to a previously published procedure [27,30]. The X-ray crystallographic structure of human σ_1 receptor complexed with PD144418 (an antagonist) was obtained from the Protein Data Bank (PDBid 5hk1, 2.51 Å resolution) [33]. The preparation of chain B for docking, extracted from the trimeric architecture of the receptor protein, was performed using the Biopolymer Structure Preparation Tool (the implemented default settings in the SYBYL programme suite were employed) [34]. The validation of the accuracy of the docking procedure, used in this study, was conducted *via* docking the PD144418 ligand (extracted from the coordinate file, obtained from the Protein Data Bank PDB; PDBid 5hk1) into the prepared chain B of the receptor [34]. The root mean square deviation (RMSD) value obtained between the native co-crystallised ligand bound conformation and the docked conformation (as generated by the docking algorithm) was found to be 0.11 Å (confirmed using PyMol and PDBeFold) [35,36], which was well within the 2 Å grid spacing used in the docking procedure. This finding indicated that the docking method to be used was valid and reliable. The 3D structures of haloperidol and PEG-haloperidol (1 to 6 ethylene oxide monomers) were drawn using MarvinSketch 18.8.0 and Maestro program [37]. Molecular docking studies were performed using the programme Surflex-Dock (SFXC) [32], as provided by Sybyl-X 2.1. Ligand preparation and molecular docking were carried out according to the previously published procedure [27].

PyMOL and MAESTRO programmes were used to visualise the docking results and the programme MAESTRO was used to analyse the molecular interactions of the docked ligands [35,37,38]. Potential hydrogen bonds were assigned if the distance between two electronegative atoms was < 3.6 Å, whereas a van der Waal interaction was assigned if the separation is >3.6 Å, but < 4.5 Å.

2.7. *Statistical analysis*

Data were presented as mean ± SEM (standard error of mean, n=3 unless otherwise stated).

Statistical significance was predicted using one-way Analysis of Variance (ANOVA) test followed by Bonferroni's *post hoc* test. Statistical significance (ns: non-significant, *: $P < 0.05$, **: $P < 0.005$) was set in the parameters.

3. Results and Discussion

3.1. *Synthesis of PEG-haloperidol conjugates*

By applying the same protocol for the synthesis of the PEG-haloperidol conjugate (6000 Da) [27], we conjugated haloperidol to PEG (2000 Da) through its tertiary hydroxyl group *via* a carbamate linkage (Figure 1).

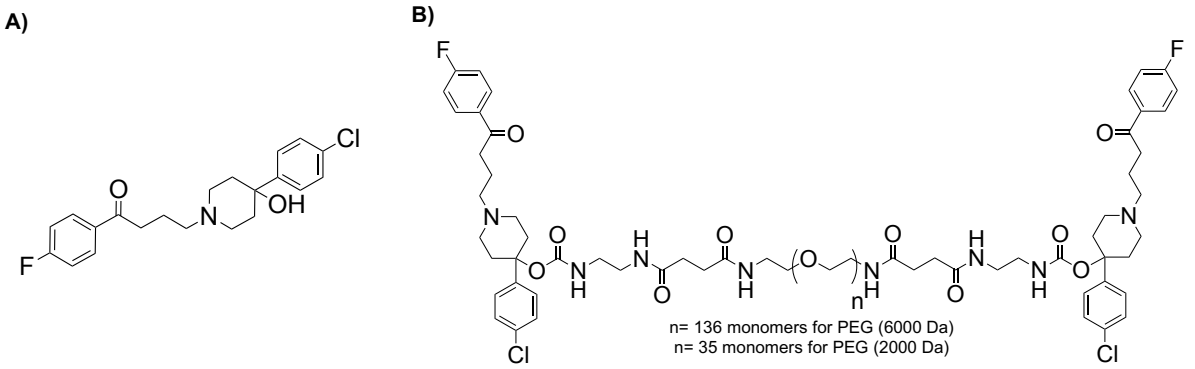

Figure 1. Chemical structures of A) haloperidol; B) PEG-haloperidol conjugates.

It has been previously reported that conjugating haloperidol through its tertiary hydroxyl groups to different cationic lipids did not significantly impair haloperidol's activity through σ receptors [39]. Therefore, we hypothesised that conjugation of haloperidol to PEG through this group would preserve, to an extent, its biological activity *via* these receptors.

Two molecular weights were selected, in order to vary the loading of haloperidol, with the conjugate derived from PEG (2000 Da) being anticipated to have a higher loading of haloperidol compared to the previously reported PEG-haloperidol conjugate (6000 Da) [27].

The novel PEG-haloperidol conjugate (2000 Da) was successfully synthesised with excellent purity and a significant increase in the total content of haloperidol (Table 1).

Table 1. Characterization of PEG-haloperidol conjugates.

Characterisation	PEG-haloperidol (6000 Da)*	PEG-haloperidol (2000 Da)
Loading via ^{1}H-NMR spectroscopic analysis	8.5% (w/w)	25.5% (w/w)
Loading of haloperidol molecules per PEG chain	1.6	1.8
Free haloperidol content via RP-HPLC	< 0.2%	< 0.2%

*Data for PEG-haloperidol were previously reported in [27].

Conjugating haloperidol to PEG (2000 Da) afforded a 3-fold increase in the total content of haloperidol compared to PEG-haloperidol (6000 Da). This increase in drug loading therefore provides an opportunity to increase the administered dose of PEG-haloperidol to counterbalance any possible loss of the biological activity of haloperidol after the formation of a polymer-drug conjugate.

3.2. In vitro antiproliferative study:

As a first step to investigate the potential application of PEG-haloperidol as an anticancer agent, direct antiproliferative effects of free haloperidol and PEG-haloperidol of the different polymer sizes (6000 and 2000 Da) were evaluated on MCF-7 and MDA-MB 231 breast cancer cell lines (both known to overexpress σ receptors [40]). Concentration-dependent MCF-7 and MDA-MP 231 responses were assessed *via* the MTT assay. The IC_{50} profiles and values of haloperidol, PEG-haloperidol (6000 Da) and PEG-haloperidol (2000 Da) are presented in (Table 2 and Figure 2).

Table 2. pIC_{50} values of free and conjugated haloperidol (2 different MW) on MCF-7 and MDA-MB cells. Values are expressed as mean ± SEM (n≥3).

Drug	MCF-7 cells		MDA-MB 231	
	pIC_{50}	**IC_{50} (μM)**	**pIC_{50}**	**IC_{50} (μM)**
Haloperidol	4.28 ± 0.02	52.00	4.35 ± 0.02	44.31
PEG-haloperidol (6000 Da)	3.87 ± 0.05	134.02	3.9 ± 0.02	124.8

PEG-haloperidol (2000 Da)	3.6 ± 0.03	252.9	3.64 ± 0.01	230.4

The IC_{50} values of free haloperidol on MCF-7 and MDA-MB 231 cells were 53 μM and 44.31 μM, respectively. These findings were in good agreement with different studies in which the cytotoxic effects of haloperidol on MCF-7 and MDA-MB 231 cell lines were evaluated [39,41]. PEG-haloperidol (6000 Da) showed antiproliferative effects on MCF-7 and MDA-MB 231 cells, albeit, at higher concentrations compared to the free haloperidol with IC_{50} values of 134.02 μM and 124.8 μM, respectively. Interestingly, PEG-haloperidol (2000 Da) showed significantly lower cytotoxic effects on the cells compared to the free haloperidol and PEG-haloperidol (6000 Da) with IC_{50} values of 252.9 μM and 230.4 μM on MCF-7 and MDA-MB 231 cells, respectively. As expected, the carriers (native PEGs) did not show cytotoxic effects on either cell line indicating the cytotoxic effects of the conjugates are due to the presence of haloperidol (Figure 2).

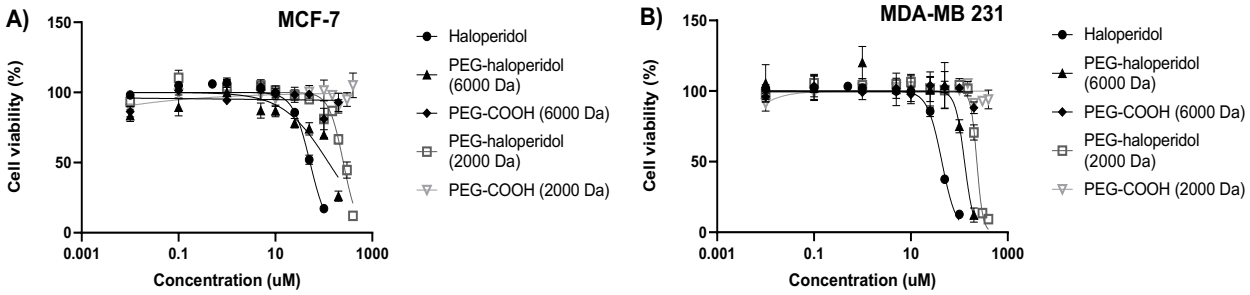

Figure 2. Determined IC_{50} of free haloperidol and PEG-haloperidol (6000 and 2000 Da) against MCF-7 and MDA-MB 231 cancer cell lines. Data are presented as mean ± SEM (n≥3).

The loss of activity of PEG-haloperidol conjugates might be a consequence of the conjugation to a polymer (further studied in Section 3.4).

The significant reduction in potency of PEG-haloperidol (2000 Da) compared to PEG-haloperidol (6000 Da) might be related to the decreased availability of the conjugated haloperidol to bind to the receptors. An effect related to the possibility of forming micelles, as the conjugate consists of hydrophobic molecules of haloperidol and a hydrophilic polymeric chain. This would be in agreement with another study documenting the anticancer activity of doxorubicin conjugated to a stearic acid-*g*-chitosan oligosaccharide, where the cytotoxic effects of doxorubicin were higher than its micellar forms [42]. In addition, others have reported that the formation of micelles of PEGylated phospholipids was affected by the length of the PEG chain. The critical micelle concentration was directly dependent on the length of the hydrophilic chain of amphiphilic PEGs [43]. These results highlight the need to consider the structural features of the polymeric chain to which haloperidol is conjugated, for example to consider the molecular weight and the hydrophilicity of the polymer [44].

3.3. Antiangiogenic activity

Having looked at direct antiproliferative effects of the conjugates on tumour cells, we evaluated, here, our conjugates for the indirect potential anticancer effects *via* inhibiting angiogenesis. Endothelial cell migration is considered very critical to induce angiogenesis and wound healing. Different cytokines and receptors are involved in this physiological , and at time pathological, process [45,46]. The chemokine VEGF has been identified to modulate the neovascularisation of tumours by regulating the proliferation, differentiation and migration of endothelial cells [47,48]. Many angiogenic modulators have been developed to treat angiogenesis-related diseases. However, their associated side effects, due to lack of selectivity, have negatively impacted their clinical applications [49]. Recently, σ receptors have been shown to play an important role in controlling angiogenesis in tumour tissues, therefore, they have been suggested as potential therapeutic targets [2]. Several studies have reported the evaluation of

antitumour agents on HUVECs by studying their effects on the high rate of migration of HUVECs induced by VEGF [50,51]. Interestingly, these cells also express σ receptors [52]. Here, we studied and evaluated the ability of conjugated haloperidol to inhibit the VEGF-induced migration of HUVECs as an indication of inhibiting angiogenesis using the well-established *in vitro* cell migration/scratch assay [53]. It is important to highlight that haloperidol has been shown to inhibit angiogenesis induced in tumours *via* acting through σ receptors [3].

First, we determined the viability of the HUVECs after treatment with 5, 20, and 100 µM of free haloperidol, PEG-haloperidol conjugates (6000 and 2000 Da) and PEG-COOH (6000 and 2000 Da) (haloperidol equivalent). This was carried out to ensure that any effects on HUVECs migration are not related to the cytotoxic effects of the tested compounds. The treatment with 5 and 20 µM (a concentration that was chosen to study cell migration based on literature data [23,54]) of free haloperidol did not significantly affect cell viability while some toxicity seen at 100 µM, in line with data reported by others [55,56]. Interestingly, no cytotoxicity was detected when HUVECs were treated with the conjugated forms of haloperidol and native PEGs at the same equivalent dose of free haloperidol (Figure 3A).

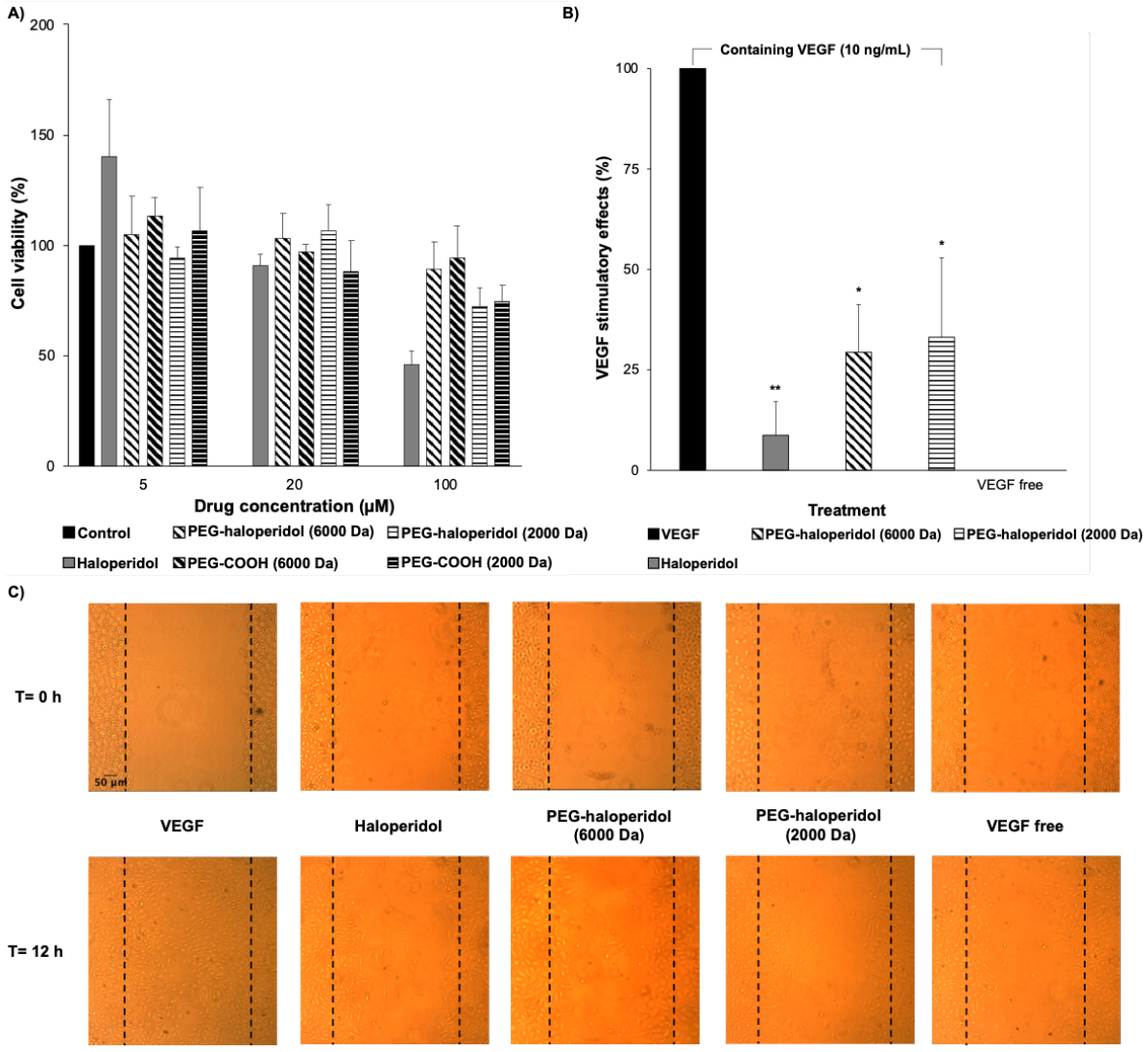

Figure 3. Biocompatibility and cell migration assays on HUVEC. A) Cell viability of HUVECs in the presence of different concentrations of tested compounds, Data are presented as mean ± SEM, n=3; B) The quantification of the inhibitory effects of haloperidol and PEG-haloperidol conjugates (6000 and 2000 Da) on VEGF induced migration of HUVECs, expressed as percentage of the scratched area covered

by the cells after 12 h migration. Data were normalised to the absence or presence of VEGF (VEGF-free and VEGF-enriched medium (VEGF) were set at 0% and 100%, respectively). The data are expressed as the mean ± SEM, n = 3. Statistical significance was estimated with respect to the positive control by one-way ANOVA, followed by Bonferroni's post hoc test ($^{*}P < 0.05$, $^{**}P < 0.005$). C) Images representing the scratch and cell migration of HUVECs at 0 and 12 h post treatments.

Therefore, the cell migration studies on HUVECs were carried out using 20 μM (haloperidol equivalent) for all tested compounds. VEGF (10 ng/mL) was used as a control that exhibits full stimulation of the migration of HUVECs, whereas free haloperidol and native PEGs were used as a positive and negative control, respectively. Haloperidol, at this sub-cytotoxic concentration, significantly inhibited the VEGF-induced migration of HUVECs by 91% ($P < 0.005$) (Figure 3B, 3C) after 12 h of incubation. These findings are in good agreement with other studies that showed the antiangiogenic effects of haloperidol [23,54].

Interestingly, PEG-haloperidol (6000 and 2000 Da) conjugates showed promising antiangiogenic effects and significantly reversed the VEGF-stimulated migration of HUVECs for 12 h, however, at a slightly lower level compared to the free haloperidol with inhibition of HUVECs' migration by 71 and 67%, respectively ($P < 0.05$) (Figure 3B, 3C) (further studied in Section 3.4). These effects of the conjugates on HUVECs are similar to the antiangiogenic effects observed when the cells were treated with bevacizumab (an FDA approved antiangiogenic agent for treating metastatic tumours) [57]. The antiangiogenic effects demonstrated by free haloperidol and its PEG conjugates are suggested to be directly related to their action *via* the σ_1 receptors. It has been recently reported that prodrugs of haloperidol exhibited antiangiogenic activity through their antagonistic effects mainly on σ_1 receptors [24]. As expected, both native PEGs did not influence the stimulatory effects of VEGF on the migration of HUVECs ($P > 0.05$) (data not shown). These findings demonstrate that the PEG-haloperidol conjugates induced inhibition of VEGF's stimulatory effects due to the haloperidol moieties. It is important to highlight that there was no significant impact of the PEG's size on the antiangiogenic effects of the conjugated haloperidol. However, using the lower molecular weight PEG would make the therapeutic applications of PEG-haloperidol as an antiangiogenic agent further feasible as it allows higher loading of haloperidol which could counterbalance the observed loss of the activity compared to the free haloperidol. The conjugation of haloperidol to PEG would prevent the normal biodistribution of haloperidol to the CNS and its associated extrapyramidal side effects [27]. Consequently, more haloperidol would be available in the circulation with an expected half-life of the conjugates of more than 36 h as a result of PEGylation [58,59]. This extended circulation would offer further availability of the conjugated haloperidol to exert its antiangiogenic activity at the required site of action.

3.4. Molecular docking studies:

The molecular docking studies were conducted to further understand the reduced antiangiogenic activity of conjugated haloperidol that was observed *in vitro* with a focus on binding to σ_1 receptors [15,60]. Therefore, we investigated the impact of the conjugation strategy upon binding. at the molecular level, and, as a consequence, to provide a plausible explanation for the observed antagonistic effects of PEG-haloperidol conjugates on σ_1 receptors *in vitro*.

Firstly, we studied the interactions of haloperidol with a σ_1 receptor. Our preliminary docking studies showed that several amino acid residues located in the B chain of the homotrimer structure of human σ_1 receptor were the main binding sites of the ligand (data not shown), therefore, this chain was extracted and used throughout the molecular docking studies for free and PEG-haloperidol [34]. Haloperidol (Figure 4) was sequentially docked into the binding sites of the B chain of the σ_1 receptor, retrieved from the literature [33,34], using a previously validated docking procedure [34].

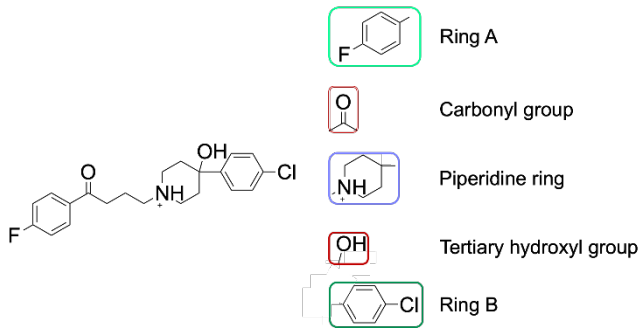

Figure 4. The chemical of haloperidol at biological pH and its rings and functional groups.

The molecular docking results revealed that free haloperidol occupied the same space reported elsewhere [33], where the molecule occupied the β-barrel region of the receptor close to the 4α and 5α helices of the protein structure (Figure 5A). In addition to that, the protonated nitrogen of the piperidine ring of haloperidol formed a 3.1 Å salt bridge with residue Glu 172. The formation of salt bridge with this amino acid residue has been shown to be obligatory for haloperidol binding and to exert its activity through σ_1 receptors [61]. The carbonyl group of haloperidol also established a hydrogen bond with Thr 181 at 3.6 Å. Hydrogen bonds also were detected between the hydroxyl group of haloperidol and Ser 117 and Tyr 120 at 3.0 Å and 3.3 Å, respectively (Figure 5B). Moreover, ring A of haloperidol formed a heteroatom interaction with Tyr 103 at 3.8 Å. In addition to that, ring B of haloperidol was close to Asp 126 and formed hydrogen bonds at 3.0 Å (Figure 5C). Importantly, these findings are in good agreement with recently published data of the interaction of haloperidol with human σ_1 receptors [33,34].

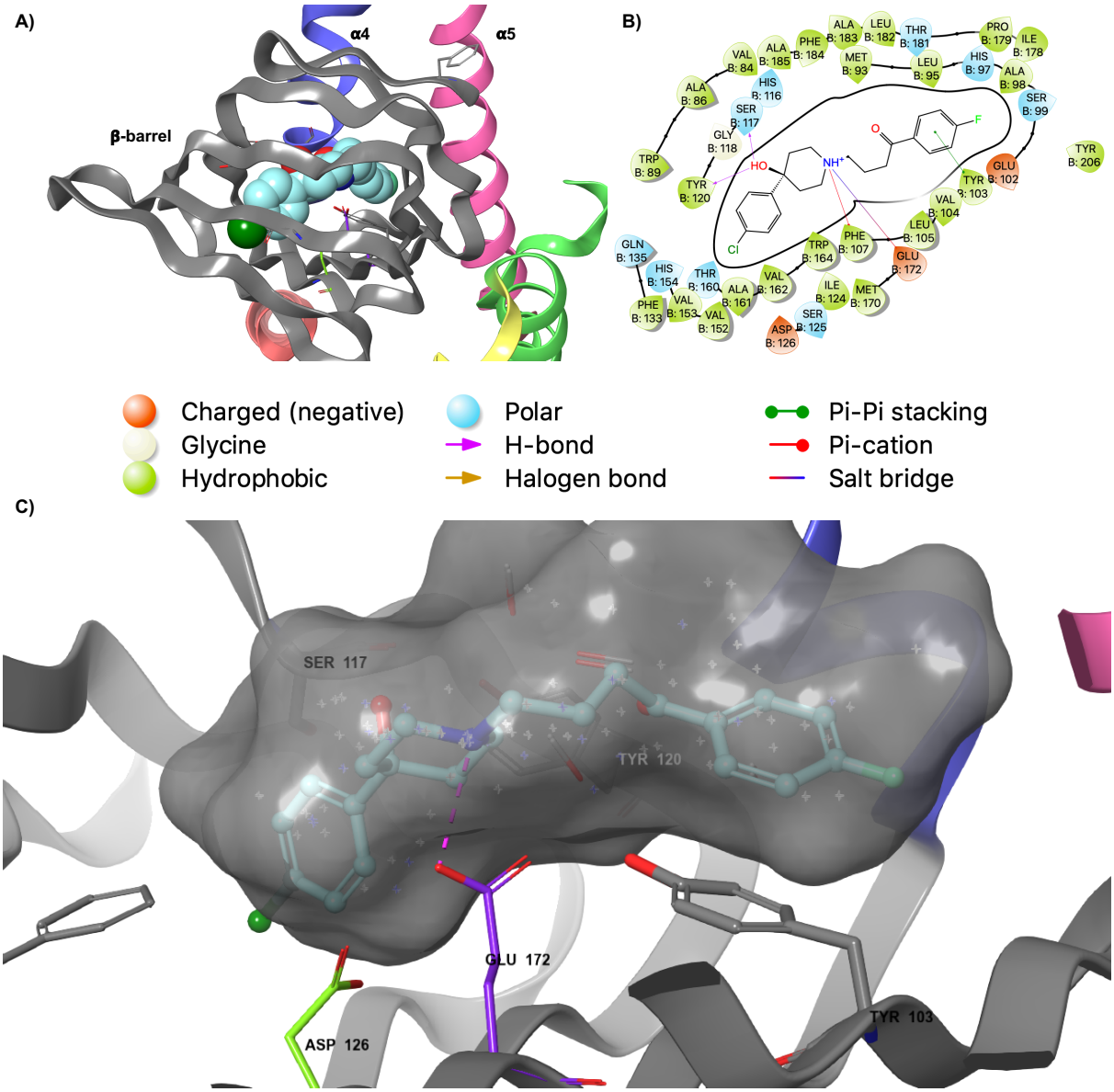

Figure 5. A cartoon representation of free haloperidol docking in the chain B of σ1 receptor. (A) data of free haloperidol in the binding pocket of the chain B of the σ1 receptor; (B) details of free haloperidol-σ1 receptor (chain B) interactions; (C) the salt bridge interaction of free haloperidol and the chain B of the σ1 receptor, and the alignment of ring B of free haloperidol in the binding pocket.

In the case of PEG-haloperidol conjugates, six 3D structures of PEG-haloperidol conjugates were prepared using several units of ethylene oxide monomers (ranging from 1 to 6 monomers) in the PEG polymeric chain to mimic the binding of PEG-haloperidol conjugate to chain B of the homotrimer of the σ1 receptor. The data displayed in this study reveal the 3D structure of PEG-haloperidol with 1 ethylene oxide unit representing possible orientations of conjugated haloperidol (with longer units) within the σ1 receptor. It is important to highlight that the remaining 5 conjugate haloperidol structures (2 to 6 units per polymeric chain) showed conformations within the σ1 receptor in binding modes similar to those revealed by the PEG-haloperidol with 1 ethylene oxide monomer (data not shown).

Our preliminary molecular docking studies of PEG-haloperidol conjugates (using several units) indicated that only a few conformations exhibited similar binding patterns to that of free haloperidol. Therefore, and to maximise the overall accuracy of the estimation of the PEG-haloperidol (1 monomer)

binding patterns, the number of the predicted conformations was increased from 20 to 100 conformations in the docking procedure.

Interestingly, 10 conformations showed binding modes of PEG-haloperidol in agreement with those observed for free haloperidol (Figure 6A). Specifically, the salt bridge of 3.1 Å was retained between the protonated nitrogen of the piperidine ring in the PEG-haloperidol conjugate with Glu 172, and ring B of the conjugate was close to Asp 126 (Figure 6B,C). Furthermore, hydrogen bonds of 2.7 Å and 2.8 Å were formed between the carbonyl groups in the PEG chain and Tyr 120 and Thr 181, respectively. In addition, the end of the ethylene oxide chain extends outside the hydrophobic binding pocket (Figure 6C) [33]. These observations predict the possible behaviour of longer-chain PEG-haloperidol conjugates upon binding to the receptor.

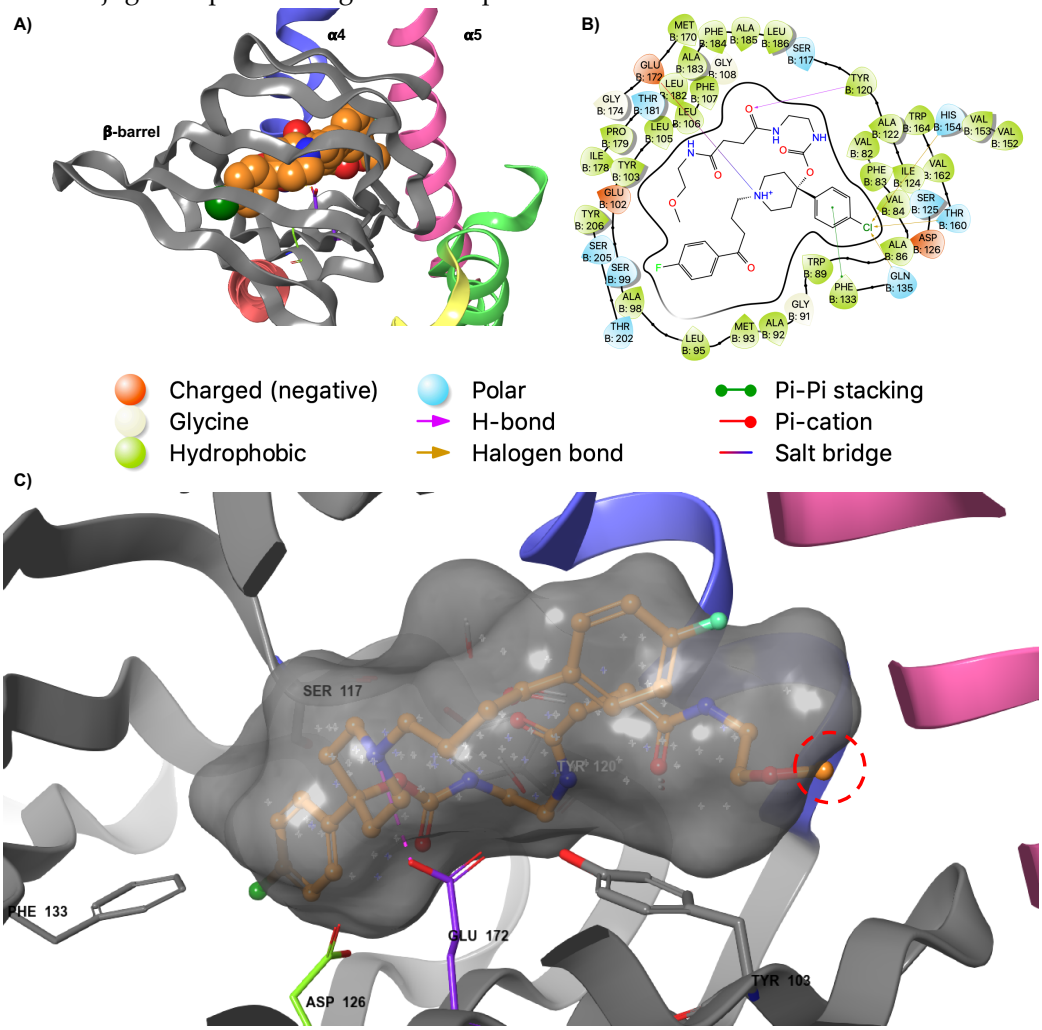

Figure 6. A cartoon representation of PEG-haloperidol docking in the chain B of σ1 receptor. (A) data of PEG-haloperidol in the binding pocket of the chain B of the σ1 receptor; (B) details of PEG-haloperidol-σ1 receptor (chain B) interactions; (C) the salt bridge interaction of PEG-haloperidol and the chain B of the σ1 receptor, and the alignment of ring B of PEG-haloperidol in the binding pocket. The dotted circle indicates the accessibility of the monomer of PEG chain out of the binding pocket.

However, 20 conformations showed a different binding pattern (Figure 7), where PEG-haloperidol was flipped in the binding pocket and ring A became close to the Asp 126 while a 3.8 Å salt bridge between the protonated nitrogen of the piperidine ring with Glu 172 was formed (Figure 7A). In addition, the protonated nitrogen of the piperidine ring was involved with an N-O bridge with Asp 126 at 4.8 Å. The carbonyl groups of the polymer side chains were also involved in hydrogen bonds with

Tyr 103, Tyr 120 and Thr 181 at 3.9 Å, 3.0 Å and 2.6 Å, respectively (Figure 7B,C). Moreover, as seen for the above mentioned 10 conformations, the monomer side chain of PEG-haloperidol extends out of the binding pocket, suggesting possibly orientations of longer chains of PEG-haloperidol conjugates (Figure 7C).

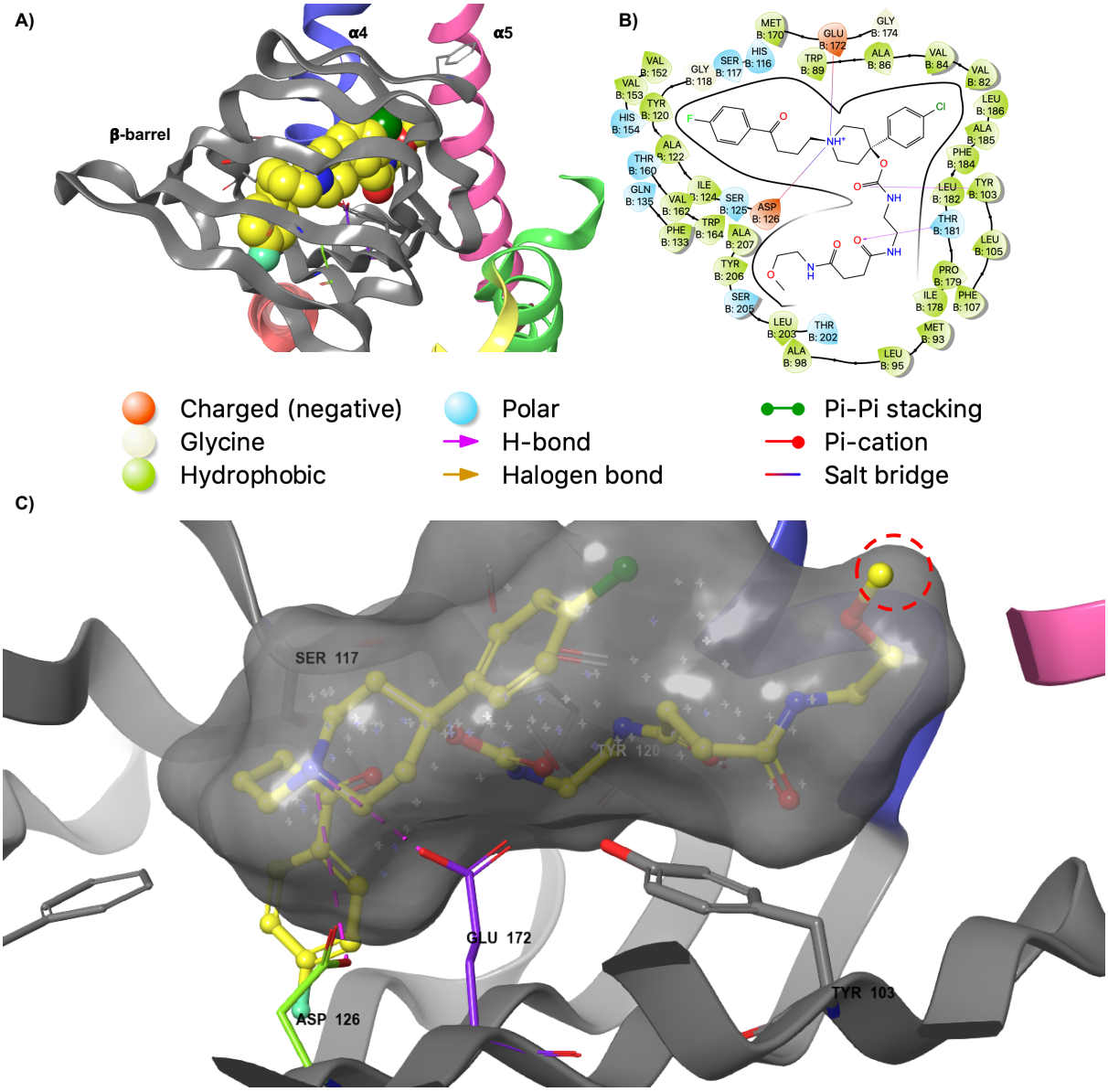

Figure 7. A cartoon representation of PEG-haloperidol docking in a σ_1 receptor showing different poses with different interactions. (A) PEG-haloperidol in a flipped pose in the binding pocket of the chain B of the σ_1 receptor; (B) details of PEG-haloperidol-σ_1 receptor (chain B) interactions; (C) the salt bridge interaction of PEG-haloperidol and the chain B of the σ_1 receptor, and the alignment of ring A of PEG-haloperidol. The dotted circle indicates the accessibility of the monomer of PEG chain out of the binding pocket.

However, the remaining 70 conformations exhibited completely different poses that showed neither the salt bridge between the protonated nitrogen of the piperidine ring with Glu 172 nor the favourable positioning of ring B close to Asp 126 was observed. In addition to that, either rings or ethylene oxide monomer of conformations became more solvent accessible. The molecular docking

studies results explain the consequence of PEGylation on the in vitro effects of haloperidol as a σ_1 receptor antagonist, where the conjugated forms of haloperidol showed retained activity, however, at lower levels compared to the free haloperidol. This reduction in the biological activity of PEG-haloperidol might be due to two reasons. First, the salt bridge interaction between the protonated nitrogen of the piperidine ring in the PEG-haloperidol conjugate with Glu 172 was observed only in 30% of the total conformations. It has been reported that a salt bridge with Glu 172 of the σ_1 receptor is directly related to the biological activity, and a mutation, typically to glycine, resulted in a reduction of ligand binding and loss of activity by at least 90% [33,62]. Second, changes in the orientation of ring B within the binding pocket in the σ_1 receptor were exhibited by 90% of the total predicted conformations. Ring B of haloperidol (the shortest hydrophobic region of σ_1 receptor's antagonists) has been indicated previously to occupy the space close to Asp 126 [63]. Although our results revealed that Asp 126 was only involved with a N-O bridge with the protonated nitrogen of conjugated haloperidol, in some flipped conformations, the position of ring B close to this amino acid residue appears to play a crucial role. It may minimise the rearrangement of the binding pocket, which could make the formation of a salt bridge with the crucial Glu 172 more readily accessible and, consequently, induce biological activity by the ligand [61].

4. Conclusion:

The present study employs the concept of polymer-drug conjugates to repurpose the antipsychotic drug haloperidol for possible anticancer applications *via* σ receptors.

The conjugation strategy is based on covalently linking haloperidol to PEG, through a carbamate linkage, to avoid its penetration through the BBB and prevent associated CNS side effects. The anticancer effects (either direct as antiproliferative or indirect as antiangiogenic) of the PEG-haloperidol conjugates, of two molecular weights (6000 and 2000 Da), were assessed *in vitro* and *in silico*. The conjugates did not exhibit potent cytotoxic effects against MCF-7 and MDA-MB 231 cells, which prevented their potential use as antiproliferative agents for these breast cancer cell lines. Interestingly, however, PEG-haloperidol conjugates significantly inhibited VEFG-mediated cell migration of HUVECs by at least 67%, which is comparable to bevacizumab' antiangiogenic effects on the same cells. However, some loss of activity was observed compared to the free haloperidol. The molecular docking studies provided insight into the consequence of conjugation haloperidol, upon binding to a σ_1 receptor. Specifically, conjugated haloperidol retained activity *via* σ_1 receptor, however, at a lower extent compared to the free haloperidol. In conclusion, the results of this study suggest the feasibility of repositioning haloperidol as a conjugate from a typical antipsychotic drug to a promising adjuvant antiangiogenic agent with limited CNS side effects.

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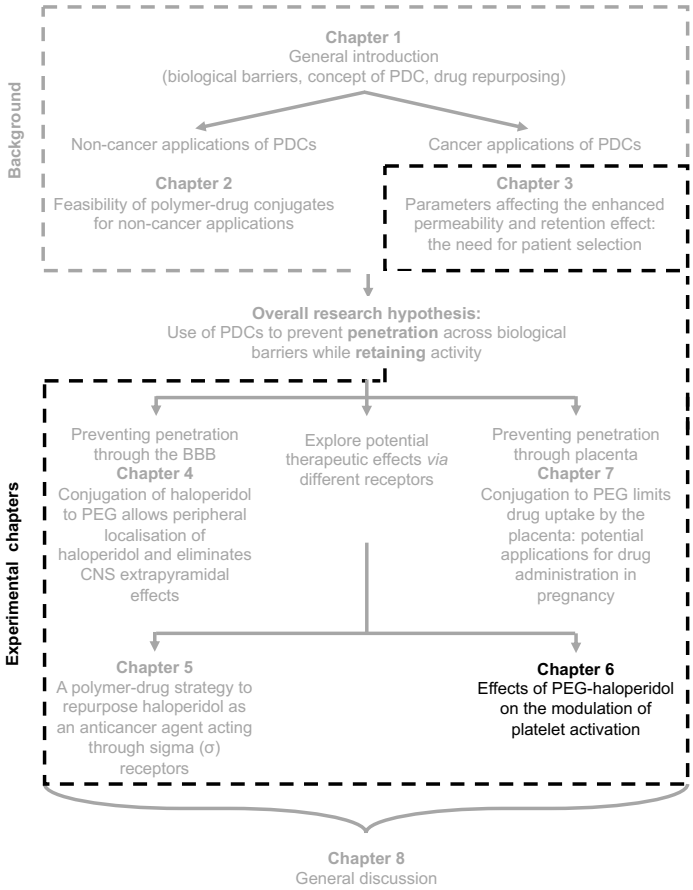
Chapter 6
Effects of PEG-haloperidol on the modulation of platelet activation

Chapter summary:
In this chapter, a preliminary study was carried out to evaluate any potential effect of PEG-haloperidol on CRP-XL- or ADP-stimulated aggregation of human platelets. The results indicated that PEG-haloperidol conjugate has no significant effect on agonists-induced platelet aggregation. Further research is required to determine the impact of this PEG-haloperidol in parallel comparison with haloperidol and other similar compounds.

*This chapter was written as a **manuscript** in consistency with thesis structure.*

Author Contributions: *S.V, H.M.I.O. and F.G designed, reviewed and supervised the study. A.A.N performed the experiments, data analysis and prepared the original draft of the manuscript. All authors edited the manuscript.*



Effects of PEG-haloperidol on the modulation of platelet activation

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Abstract

Haloperidol has been shown an ability to modulate platelet activation stimulated with collagen and adenosine diphosphate (ADP). However, repositioning haloperidol for potential non-CNS applications is limited due to the distribution of its small molecular structure into the central nervous system (CNS), its primary site of action, resulting in extrapyramidal side effects. We have recently reported the feasibility of designing a PEG-haloperidol conjugate to localise haloperidol outside the CNS by preventing its permeation across the blood-brain barrier, which may allow its non-CNS applications. The purpose of this study was to assess the effects of PEG conjugated haloperidol as a potential inhibitor of human platelet aggregation *in vitro* using two agonists to stimulate the aggregation of platelets. Our results revealed that free and PEG-haloperidol (at 3.125-100 μM, haloperidol equivalent) did not significantly inhibit the aggregation induced by the cross-linked collagen related peptide (CRP-XL) which binds to GPVI receptors. This might suggest haloperidol could work through another collagen receptor (α2β1). In addition to that, haloperidol and PEG-haloperidol showed inhibition, however, not significant, on ADP-stimulated aggregability of human platelets at ≥12.5 μM haloperidol equivalent, most probably mediate *via* $P2Y_1$ receptors. However, these preliminary findings require further investigation to fully understand the mechanism of action of PEG-haloperidol for its possible antiplatelet effects.

Keywords: Polymer-drug conjugate, PEG, haloperidol, platelets, aggregation.

6.1. Introduction

Platelets are anucleated, small circulating blood cells (with a diameter of 2–4 μm). They circulate in the blood for ~7 to 10 days. Platelets form the second most abundant cell type of the whole blood with a population of almost 10^{12} cells at any time.[1] They are produced from their parental cells, megakaryocytes in the bone marrow, *via* a process controlled by thrombopoietin (the liver-derived growth factor), while their elimination occurs largely in the spleen. They are multifunctional but primarily responsible for maintaining haemostasis upon vascular injury.[2] In addition to that, these short living and multi-responding cells play vital roles in thrombosis (i.e. formation of blood clots within the vasculature), wound healing, inflammation, immunity, atherosclerosis and tumour metastasis.[3] Several cardiovascular disorders such as stroke and ischemic heart diseases have been shown to be pathologically related to thrombosis. Therefore, antiplatelet agents are among the most commonly prescribed therapeutic agents in the world in order to prevent any potential thrombotic risks.[4] Given the serious side effects associated with the currently prescribed anti-platelet drugs such as aspirin and clopidogrel, there is an increased need to develop safer and more pharmacologically effective classes of antiplatelet agents.[5] Repurposing of drugs for their off-target therapeutic applications has recently become a promising strategy to identify new applications of existing drugs.[6] For example, haloperidol, a D_2 receptor antagonist used to treat psychosis, has shown potential non-CNS therapeutic applications including its ability to modulate platelets function by inhibiting platelets aggregation *via* different pathways (**Figure 6.1).** [7–11]

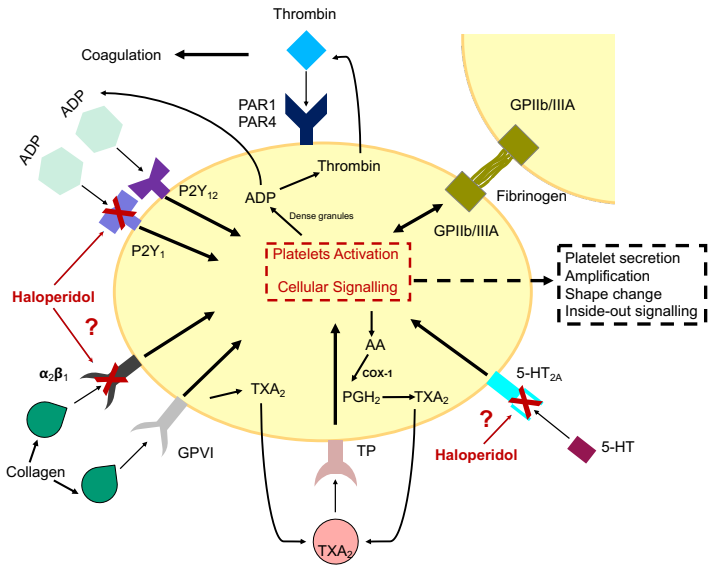

Figure 6.1. Established and suggested targets of haloperidol in platelets (adapted from[12–15]). **Abbreviations:** 5-HT: serotonin; 5-HT_{2A} receptors: serotonin receptors; $\alpha_2\beta_1$, GPVI receptors: collagen receptors; AA: arachidonic

acid; ADP: adenosine diphosphate; Cox1: cyclooxygenase1; GP IIb/IIIa: glycoprotein IIb/IIIa receptors; $P2Y_1$, $P2Y_{12}$: ADP receptors; PAR1, PAR4: protease-activated receptors; PGH2: prostaglandin H2; TP: thromboxane receptor; TXA_2: thromboxane A_2.

However, haloperidol-associated side effects in the CNS, due to the penetration of haloperidol through the blood-brain barrier (BBB), have formed the major limitation for its non-CNS applications, including its potential effects on platelets.[16]

We have recently developed a non-pro-drug macromolecule of haloperidol in which haloperidol was conjugated to poly(ethylene glycol) (PEG) through a non-bio-labile carbamate linker[17] (**Figure 6.2**).

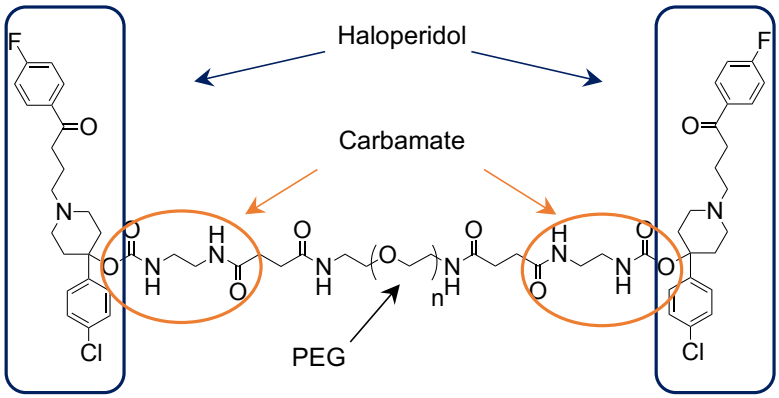

Figure 6.2. Chemical structure and components of PEG-haloperidol conjugate (adapted from[18]).

This system could prevent the diffusion of conjugated haloperidol through the BBB (*in vivo*) and, consequently prevent its side effects and preserve the antagonistic effects of the conjugate through D_2 receptors, however, at a lower level when compared with the free haloperidol (*in vitro*).[18]

Therefore, in the present study, we have focused on exploring the potential cardiovascular applications of the conjugated haloperidol where the effects of PEG-haloperidol to modulate the functions of platelets was assessed through an *in vitro* approach.

6.2. Materials and Methods

6.2.1. Materials

Alpha,omega-di-succinimidyl ester poly(ethylene glycol) (molecular weights of 6429 Da) were obtained from Iris Biotech GmbH, Germany. Haloperidol was obtained from (Sigma-Aldrich, UK). All other solvents and chemicals were purchased from (Fisher Scientific and Sigma-Aldrich, UK) and were used in experiments without any further steps of purification unless otherwise stated. Cross-linked collagen-related peptide (CRP-XL) was obtained from

Professor Richard Farndale at the University of Cambridge. Adenosine diphosphate (ADP) was purchased from Sigma-Aldrich, UK.

6.2.2. Methods

6.2.2.1. Synthesis and characterisation of PEG-haloperidol conjugate

The synthesis and characterisation of PEG-haloperidol conjugate was performed according to our previously reported protocols (Chapter 4).[18]

6.2.2.2. Human blood collection and test compounds preparation

The platelet-related experiments performed in this study were in agreement with the principles outlined in the Declaration of Helsinki. Human blood samples were collected in vacutainers containing 3.2% (v/v) citrate by venepuncture from aspirin-free and healthy volunteers. The blood collection was conducted after obtaining informed consent from volunteers. The consent forms and procedures used in this study were approved by the University of Reading Research Ethics Committee. The preparation of human platelets was performed according to standard protocols as previously reported.[5] The blood samples were centrifuged at 102 g at 20 °C for 20 minutes to obtain platelet-rich plasma (PRP) which was used in platelet aggregation assays. A stock solution of haloperidol was initially prepared in DMSO (100 %), and dilutions were further performed using modified Tyrodes-HEPES buffer (20 mM HEPES, 134 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.12H_2O$, pH 7.3) to the desired concentration for assays. Due to the hydrophilicity of PEG-haloperidol and PEG-COOH, their stock solutions were prepared by dissolving the materials in DMSO-containing Tyrode's-HEPES buffer. The final concentration of DMSO in platelets was maintained at ≤0.5 % (v/v) in all concentrations of all tested compounds.

6.2.2.3. Platelet aggregation assays

Platelet aggregation assays were carried out using optical aggregometry (Chrono-Log, USA) in the absence or presence of different concentrations of haloperidol, PEG-haloperidol or PEG-COOH (free PEG) along with a DMSO-containing vehicle control. 3 μL of the control or tested compounds (at haloperidol equivalent concentrations) were added to 267 μL of PRP and incubated for 5 min at 37 °C. The platelets were then stimulated with 30 μL of CRP-XL at 0.5 μg/mL, or ADP at 10 μM. The aggregation of the agonist-stimulated platelets was then

monitored for another 5 minutes. The data were normalised according to the level of aggregation achieved in the control which was considered as 100%.

6.2.2.4. Statistical analysis

Data were presented as mean ± SEM (standard error of mean, n=3-4) unless stated otherwise. Statistical analysis was performed using GraphPad Prism software (Version 8). Statistical significance was analysed using one-way Analysis of Variance (ANOVA) test followed by Bonferroni's *post hoc* test where appropriate; a $P < 0.05$ was set to be significant in the parameters.

6.3. Results

It has previously been reported that the antipsychotic haloperidol, a D_2 receptor antagonist, is able to inhibit platelet aggregation using different agonists (collagen and ADP) to induce the aggregation,[10,11] which may suggest possible cardiovascular therapeutic applications of haloperidol. However, haloperidol's free penetration through the BBB, and the associated side effects, has limited these clinical applications, which demonstrated the need to develop new drug delivery systems to localise the effects of haloperidol peripherally. We have recently shown that passive diffusion of haloperidol through the BBB was effectively blocked *via* conjugation to PEG, the conjugated haloperidol exhibited a retained activity towards D_2 and σ receptors.[18,19] Therefore, here, we carried out preliminary studies to explore the effects of PEG-haloperidol conjugate, prepared to localise haloperidol outside the CNS, on platelets aggregation induced by two agonists *in vitro*.

6.3.1. Effect of PEG-haloperidol on human platelets aggregation induced by CRP-XL

The ability of PEG-haloperidol to modulate platelet function was assessed using aggregation assays by optical aggregometry. Haloperidol was reported to inhibit platelets aggregation induced by collagen without identifying the exact receptor that mediates this effect.[10] Therefore, we first studied the ability of free and conjugated haloperidol to inhibit aggregation induced by CRP-XL (which act specifically *via* collagen GPVI receptors). Human PRP was incubated with a vehicle (DMSO) control or different concentrations of free haloperidol, PEG-haloperidol or free PEG at concentrations from 3.125 to 100 μM (haloperidol equivalent) for 5 minutes prior to the activation with CRP-XL (0.5 μg/mL) for a further 5 minutes. The results revealed that

neither of the tested compounds (at all tested concentrations) significantly inhibited CRP-XL-induced human platelet aggregation in PRP while the vehicle control displayed a clear aggregation of up to 100% (**Figure 6.3**).

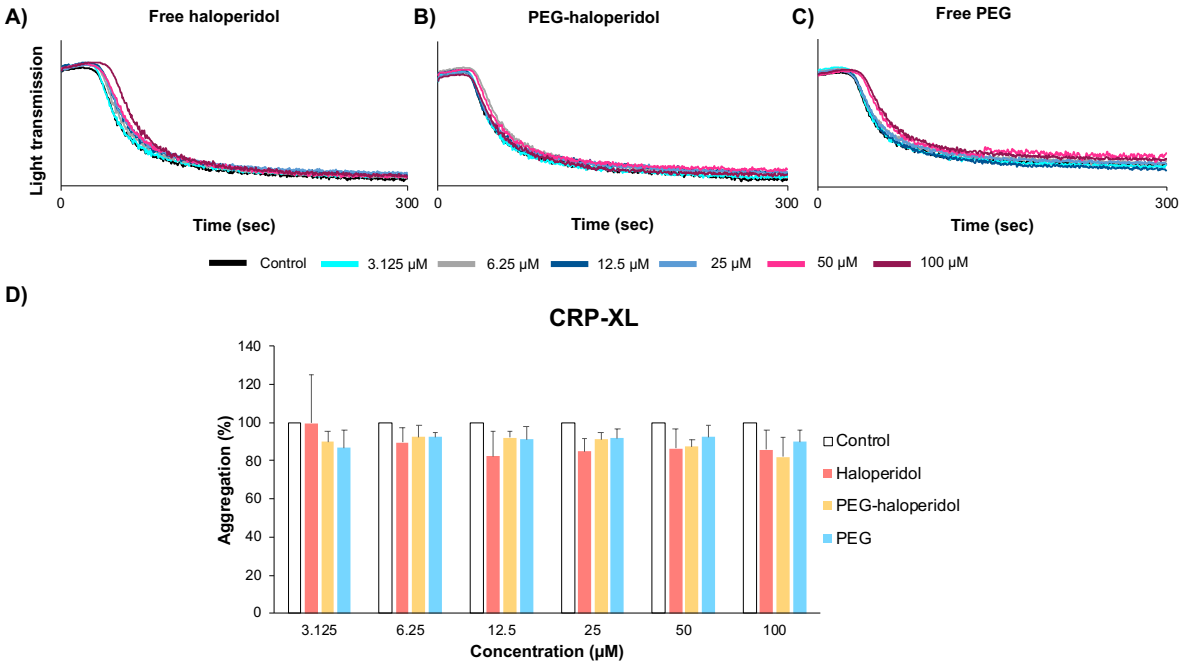

Figure 6.3. Effect of free haloperidol, PEG-haloperidol, and free PEG on CRP-XL-induced platelet aggregation. Human PRP was incubated with a DMSO- vehicle control or different concentrations of tested compounds for 5 minutes. Then, the platelets were stimulated with CRP-XL Platelet aggregation was then recorded for 5 minutes by optical aggregometry. The aggregation traces of the three different compounds are presented: (A) free haloperidol; (B) PEG-haloperidol; (C) Free PEG. (D) Percentage aggregation for treated compounds was calculated according data of the vehicle control which was considered as maximum aggregation at 100%. Data were represented mean ± SEM (n=4).

6.3.2. Effect of PEG-haloperidol on platelet aggregation induced by ADP

Given that haloperidol showed an ability to inhibit ADP induced aggregation,[11] we tested the effects of conjugated haloperidol on platelets aggregation using ADP as an agonist. When ADP was used to stimulate platelet aggregation in PRP at 10 µM, all tested compounds started to exhibit inhibitory effects. A concentration of 12.5 µM (haloperidol equivalent) and above showed clear effects in some donors, however, these were not significant (**Figure 6.4**). These results might indicate the impact of other factors such as the incubation time on the effects of the free and conjugated haloperidol, which requires further investigations.

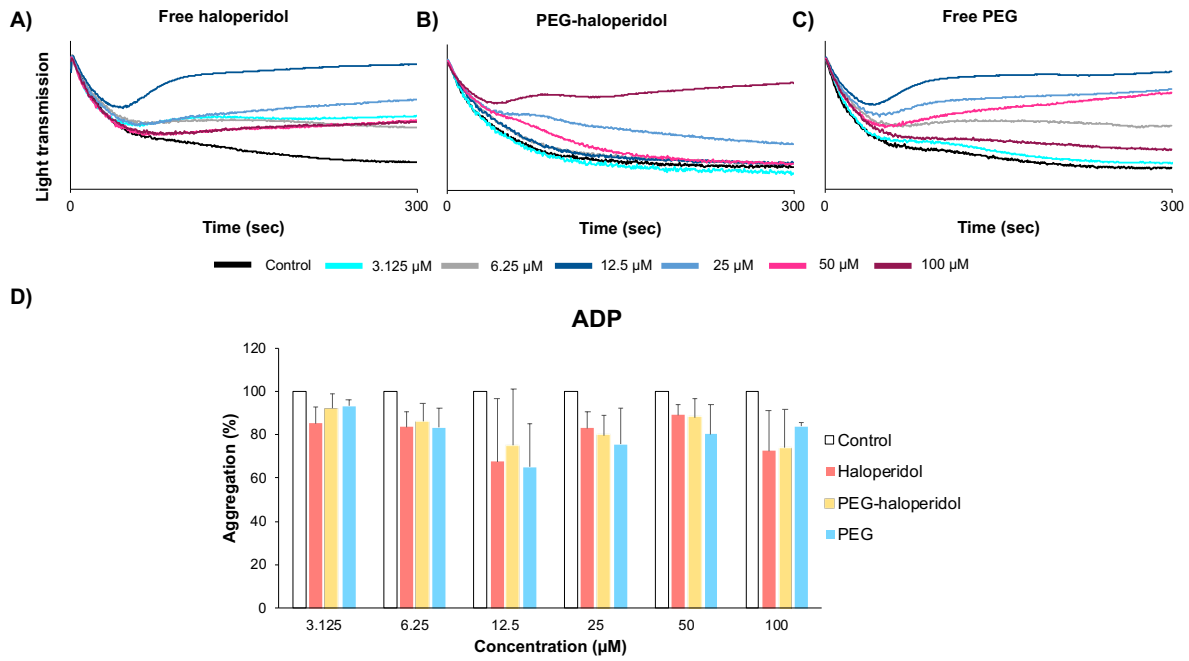

Figure 6.4. Effect of free haloperidol, PEG-haloperidol, and free PEG on ADP-induced platelet aggregation. Human PRP was incubated with a DMOS-containing vehicle control or different concentrations of tested compounds for 5 min. Then, the platelets were stimulated with ADP. Platelet aggregation was then recorded for 5 min by optical aggregometry. The aggregation traces of the three different compounds are presented: (A) free haloperidol; (B) PEG-haloperidol; (C) Free PEG. (D) Percentage aggregation for treated compounds was calculated according data of the vehicle control which was considered as maximum aggregation at 100%. Data were represented mean ± SEM (n=3).

6.4. Discussion

The findings of our aggregation study suggest the need for further work to investigate the potential therapeutic indications of PEG-haloperidol on the cardiovascular system. It has been reported that haloperidol could have potential cardiovascular applications *via* inhibition of platelets aggregations.[20] Two studies have previously indicated that haloperidol has effects on human platelets *via* different mechanisms. In a study conducted by Dietrich-Muszalska *et al*, haloperidol significantly inhibited collagen-induced aggregation of human platelets by ~20%. These effects were observed when the human platelets were incubated with haloperidol at 20 ng/mL (therapeutic concentrations in the blood of patients with schizophrenia) for 30 min.[10] In another study by the same group, haloperidol (20 ng/mL, incubated for 30 min) did not significantly inhibit platelets aggregation induced by ADP (10 µM, stimulation for 2 min).[21] However, in contrast to these data, ChiehWu *et al*, have reported that the ADP-induced aggregation of human platelets (10 µM of ADP and monitored for 6 min) was abrogated in a dose-dependent manner when the PRP was pre-incubated with haloperidol (0.01-10 µM) for 1 h. In addition to that, pre-treatment of PRP with haloperidol (1µM) for 1 h magnified

clopidogrel (30 μM for 3 min) induced inhibition of PRP stimulated with ADP (10 μM for 6 min) by ~2-fold, which suggested that haloperidol effects on platelets are mediated *via* the $P2Y_1$ receptor pathway. Moreover, haloperidol at 1-10 μM for 1 h significantly inhibited thromboxane A2 production from platelets when stimulated with fibrinogen (3 μM for 3 min) or ADP (10 μM for 3 min). Haloperidol also significantly reduced the release of the arachidonic acid from platelets after their stimulation with ADP (10 μM for 3 min).[11]

However, these applications of free haloperidol are limited by the CNS-associated side effect of haloperidol, as a consequence of its penetration through the BBB.[22] To avoid the diffusion of haloperidol through the BBB, we developed the PEG-haloperidol conjugate as a non-pro-drug system which could prevent the penetration of conjugated haloperidol through the BBB.[18] Therefore, in the current study, we assessed whether PEG-haloperidol would exhibit potential inhibitory effects on human platelets aggregation, which might offer a new class of medications to treat cardiovascular diseases.

The platelet aggregation studies were carried out using two agonists to induce platelets activation: CRP-XL which acts through GPVI receptors, and ADP which induces platelet activation through $P2Y_1$ and $P2Y_{12}$ receptors.[23] Our results revealed that neither haloperidol nor PEG-haloperidol was able to significantly inhibit aggregation of platelets stimulated with CRP-XL, while the effect of free PEG was expected as an inert carrier. However, it has been previously reported that haloperidol could inhibit platelets aggregation induced by collagen (which act though two receptors: α2β1 and GPVI receptors, which are found on the membrane of the platelets).[10] This suggests that the inhibitory effects of the collagen-induced aggregation of haloperidol might be related to the effects on the pathway mediated *via* the α2β1. When CRP-XL (which acts through GPVI receptor only) was used, no significant effects of the free and conjugated haloperidol on platelets aggregation were recorded. Another possible explanation of our results is the short incubation time (5 min for pre-treatment with the tested compounds). Dietrich-Muszalska *et al* incubated the platelets with haloperidol for 30 min, which might indicate that free and PEG-haloperidol may need a further time to act.[10] To clearly identify these findings, further investigations are needed *via* testing the effects of haloperidol and PEG-haloperidol on the aggregability of platelets using CRP-XL (at low concentrations) and collagen as an agonist, and by employing longer time for incubation.

On the other hand, when the platelets were stimulated with ADP, the tested compounds started (not significantly) to exhibit potential inhibitory effects (~25-35% compared to the

control) at 12.5 μM (haloperidol equivalent). However, these data require further studies to determine the exact effects and the mechanism that haloperidol and PEG-haloperidol have as the inhibitory effects on platelets function were only observed in 1 donor (out of 3 tested), which may suggest carrying out the experiment for more replicates and studying the impact of incubation time on the activity of haloperidol and its conjugated form. Therefore, the next level of the study would continue testing free and conjugated haloperidol on platelets stimulated with ADP and other agonists such as collagen (acting through α2β1 receptors and GPVI receptors)[5,12] and measuring the levels of arachidonic acid and TXB2, the active metabolite of thromboxane A_2, produced after the stimulation with ADP. A longer incubation time could also be assessed as another parameter that influences the activity of haloperidol (free and conjugated forms) on the platelets. The effects of PEG-haloperidol on platelets aggregation could also be tested in the presence of serotonin (5-HT) which acts as an agonist through 5-HT_{2A} receptors on the membrane of the platelets.[24,25] In addition to its function in the process of platelets aggregation, 5-HT has been reported to contribute to the development of pulmonary hypertension and bronchopulmonary dysplasia in infants.[26] Haloperidol has been shown to have affinity towards the 5-HT_{2A} receptor.[27] Therefore, the potential cardiovascular applications of PEG-haloperidol through this receptor could be further studied. Furthermore, different assays could be applied to study the potential effects of conjugated haloperidol on human platelets, further reviewed by Paniccia *et al*.[28] This would enable us to explore the ability of free and conjugated haloperidol to modulate platelets function and determine through which receptors and mechanisms this is initiated. Moreover, after identifying possible pathways of PEG-haloperidol activity on modulating platelets function, molecular docking studies could be carried out to predict the impact of conjugation on the binding of haloperidol to the identified receptor, and, consequently, on the biological activity of conjugated haloperidol.

6.5. Conclusion

In this study, we assessed the possible cardiovascular applications of PEG-haloperidol as a non-prodrug macromolecule on modulating the function of human platelets *in vitro*. The study evaluated the potential inhibitory effects of the conjugated haloperidol on the aggregation of platelets stimulated with two different agonists, the CRP-XL and ADP which act *via* the GPVI and $P2Y_1$ and $P2Y_{12}$ receptors, respectively. The results revealed that no significant inhibition of platelet activation by haloperidol or PEG-haloperidol was observed when platelets were

stimulated with CRP-XL. On the other hand, when the ADP was used as a stimulator of the platelets, some inhibitory effects of haloperidol and PEG-haloperidol were indicated, however, the effects were not significant. These results suggest that haloperidol and its conjugated form might work *via* pathways signalled by other receptors or there are other factors influencing its effects such as the incubation time. However, the findings of this study are insufficient to understand the exact mechanism of action of the free and conjugated haloperidol on inhibiting platelet aggregation. Therefore, further studies must be carried out to identify possible receptors and targets that might conjugated haloperidol works through to modulate platelets function. Once the potential receptors are identified, molecular docking studies could be performed to understand how the conjugation strategy could affect the interaction of haloperidol with the receptors of the platelets at a molecular level.

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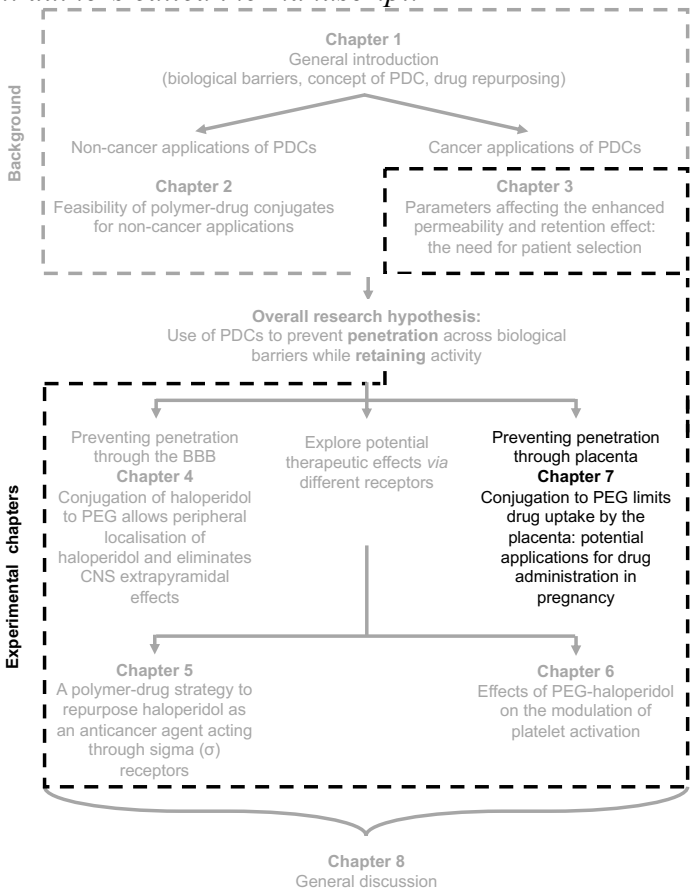
Chapter 7
Conjugation to PEG limits drug uptake by the placenta: potential applications for drug administration in pregnancy

Chapter summary:

In this chapter, we evaluate conjugation of drugs/dye to PEG as a feasible strategy to prepare non-prodrug PDCs that can reduce or prevent the transfer of conjugated drugs through the placenta. The explants treated with PEG conjugates showed limited uptake of the conjugates when compared to the free drugs (Cy5.5 dye or haloperidol) which indicated the suitability of the system to localise the effects of conjugated drugs at the maternal compartment. Moreover, no toxicity was observed in the placental explants when they were incubated with the free PEG.

*This chapter was written as a **manuscript** in consistency with thesis structure, paper in preparation.*

Author Contributions: *A.A.N, H.M.I.O. and F.G conceived of the research idea. G.P, H.M.I.O, L.K.H, and F.G designed, reviewed and supervised the study. A.G prepared and characterised the PEG-Cy5.5 conjugate. A.N.N and F.G designed the placental uptake studies of PEG conjugates. L.K.H and L.R designed, supervised and analysed A.D and A.E work. A.D and A.E performed the experiments of placenta-related cytotoxicity, IHC, and PEG-Cy5.5 uptake study. F.B performed determination of protein content and A.A.N analysed some data. A.A.N carried out uptake study of PEG-haloperidol. A.A.N analysed the data and prepared the original draft of the manuscript. All authors edited the manuscript.*



Conjugation to PEG limits drug uptake by the placenta: potential applications for drug administration in pregnancy

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Abstract

We have recently proposed that non-prodrug polymer-drug conjugates can be used to prevent crossing of biological barriers and, in turn, compartmentalise the effect of the conjugated haloperidol into one body compartment. Therefore, it was hypothesised that by applying the same strategy and conjugating drugs to a polymeric carrier, we could impair their uptake and transfer across the human placenta which could, in turn, be beneficial for the treatment of pregnant women without harming the fetus. The purpose of this study was therefore to evaluate, *ex vivo*, the feasibility of PEGylation to reduce or prevent the accumulation of drugs within the placental tissues without interfering with the normal function of the placenta. The preliminary results of the study indicated that PEG as a polymeric carrier was biocompatible with the human placenta. It did not significantly alter the basal rate of proliferation or apoptosis in term placental explants after incubation for 24 or 48 h, as confirmed *via* immunohistochemical staining. In addition, the release of lactate dehydrogenase, a marker of tissue necrosis, did not significantly vary after PEG treatment, nor did the levels of human chorionic gonadotropin (hCG) secreted. These finding indicated that normal placental function was not significantly affected by the free PEG. Interestingly, cellular uptake studies of PEGylated Cy5.5 using fluorescent microscopy revealed that no localisation of PEG-Cy5.5 conjugate (tested at a range of concentrations) could be detected in any region of the placenta after 6 or 24 h of incubation. In addition, the levels of PEG-haloperidol conjugates (6000 and

2000 Da, tested as model drugs) which accumulated in the placental explants were significantly lower than those of free haloperidol (with ~4- and 40-fold reduction of tissue uptake, $P < 0.05, 0.01$, respectively) as determined *via* RP-HLPC. These findings provide proof of principle data for designing novel polymer-drug conjugates as non-prodrug systems to reduce or even avoid the accumulation of conjugated drugs within the placenta. Moreover, the study highlights the potential of this approach for managing and treating maternal diseases during pregnancy, without impacting placental function and preventing exposure of the developing fetus to potentially teratogenic agents.

Keywords: Polymer-drug conjugate, PEG, pregnancy, placenta, transfer, drug delivery.

7.1. Introduction

Managing maternal health-related conditions during pregnancy can be particularly challenging, as many therapeutic agents that are routinely taken by patients who are not pregnant, are harmful to the fetus and must be avoided during pregnancy [1,2]. In many cases, it is possible to avoid these potentially unsafe medicines for the duration of pregnancy. However, there are conditions, such as (a) pre-existing chronic disease, (b) unexpected illnesses e.g. infections and (c) pregnancy complications e.g. gestational hypertension, that require treatment during pregnancy. This makes it necessary to administer drugs which could adversely affect placental function and/or the health of the developing fetus [3,4]. Therefore, designing new drug delivery systems that restrict the therapeutic effects of drugs to the maternal bloodstream would be beneficial for the expectant mother and could protect the fetus from the consequences associated with conventional systemic administration [5].

The primary functions of the placenta are to maintain a continuous supply of oxygen and nutrients to the fetus, to act as a potential barrier to infection and toxic substances, and to excrete waste products. In addition to that, it plays a vital role in synthesising steroids, peptides and hormones that are necessary for the successful continuation of pregnancy [6,7]. These physiological features make the placenta a unique organ that allows bidirectional transfer of substances to and from the fetus through various mechanisms. However, the most predominant mechanism is passive diffusion, and it is *via* this mechanism that most drugs can cross the placenta and reach the fetal bloodstream [8]. Therefore, manipulating the physicochemical properties of drugs to directly influence their mechanism of transplacental transfer, such as the size of the molecule and its lipophilicity, would offer opportunities to control or limit passive diffusion of drugs through the placenta.

The placenta has been shown to share similar features with the blood-brain barrier (BBB), where the penetration of small molecules (<400-600 Da) through this biological barrier is mostly mediated through passive diffusion and is dependent on their lipophilicity [9–11]. We have recently developed a novel drug delivery system that can prevent the diffusion of a drug through the BBB whilst retaining its therapeutic effects outside of the central nervous system (CNS) [12,13]. The polymer-drug conjugate (PDC) was designed to act as a non-prodrug macromolecule and used PEG as a polymeric carrier.

In this work, we propose the concept of polymer-drug conjugation *via* a non-degradable linker as a means to restrict drugs to the maternal bloodstream and impair or even prevent fetal transfer. Therefore, these systems could potentially protect the fetus from possible harmful side effects, whilst allowing simultaneous treatment of the expectant mother **(Figure 7.1).**

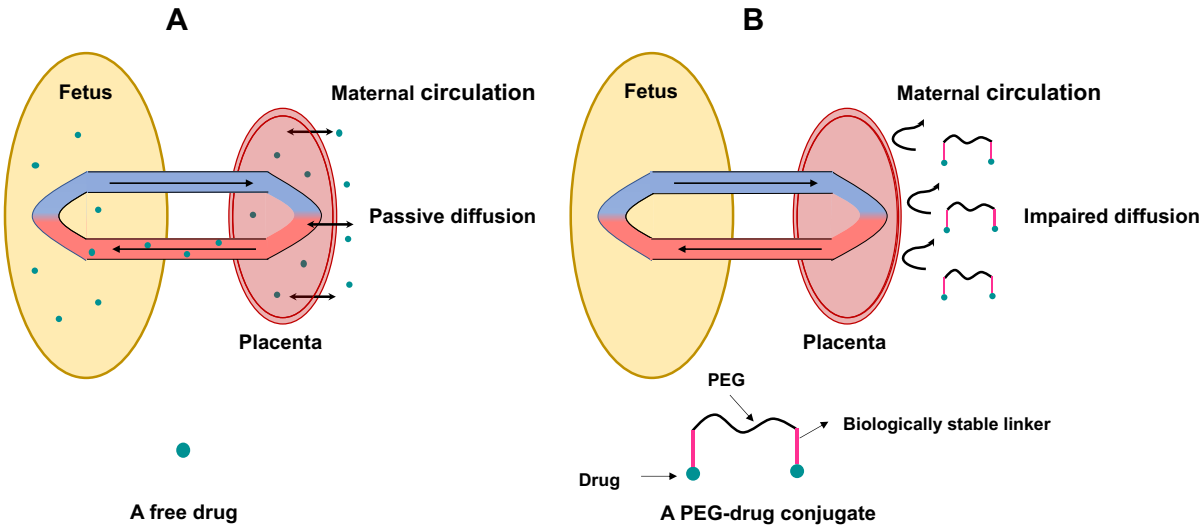

Figure 7.1. Panel **A**, Distribution of a free small molecule drug into the fetal compartment after systemic administration into the maternal circulation; Panel **B**, Restricted distribution of the PEG-conjugated drug within the maternal compartment, after its systemic administration in maternal circulation.

The concept of PDCs has previously proved successful for effective reduction/prevention of the diffusion of conjugated drugs through the BBB, by localising their effects peripherally [13]. However, to the best of our knowledge, there is only one *ex vivo* study reporting conjugation to large molecules as an effective strategy to reduce placental drug transfer. That system employed an ester linkage to conjugate an Alexa (488) dye to a PAMAM (G4) dendrimer. Using the "dually perfused, re-circulating term human placental lobule" *ex vivo* model, the study revealed negligible accumulation of the conjugate within the placental tissues. In addition to that, a very low transplacental transfer of the PAMAM-Alexa conjugate was measured by determining the concentrations of the conjugate in the fetal and maternal perfusates. The system was stable for 72 h in the perfusates used in the experiment *in vitro* [14].

In the current study, we demonstrate the feasibility of the PEGylation strategy, using biologically stable linkers, to develop a new (non-prodrug) drug delivery system that could limit drug transportation across the placenta.

Therefore, our hypothesis was tested using 2 different conjugates: a novel PEG-cyanine-5.5 (Cy5.5) (using PEG 5000 Da) and previously reported PEG-haloperidol conjugates (2000 and 6000 Da) [13,15] to explore for the first time, the effect of PEGylation on uptake of conjugated Cy5.5 and haloperidol by the placenta. First, the biocompatibility of free PEG with human term placental explants was assessed. The rates of proliferation and apoptosis within the placental explants, treated with free PEG, were quantified *via* immunostaining. In addition, the levels of necrosis and hCG secretions were determined *in vitro*. The impaired cellular uptake by the placenta was assessed *via* two methods. The localisation of the PEG-Cy5.5 conjugate within

the explants was determined qualitatively using fluorescent microscopy. This was further studied by quantifying the levels of placental uptake of PEG-haloperidol conjugates (used as a model drug with potential non-CNS applications) using RP-HPLC analysis.

7.2. Materials and Methods

7.2.1. Materials

Alpha,omega-di-succinimidyl ester poly(ethylene glycol) (molecular weights of 6429 and 1983 Da) and alpha-methoxy-omega-amino poly(ethylene glycol) (molecular weights 4933 Da) were obtained from Iris Biotech GmbH, Germany. Cy5.5-NHS ester was purchased from GE Healthcare (UK). Haloperidol was obtained from (Sigma-Aldrich, UK). All other solvents and chemicals were purchased from (Fisher Scientific and Sigma-Aldrich, UK) and were used without any further purification steps unless otherwise stated.

7.2.2. Methods

7.2.2.1. Synthesis and characterisation of PEG-Cy5.5 and PEG-haloperidol conjugates

The synthesis and characterisation of the novel PEG-Cy5.5 conjugate were carried out as follow. PEG-NH_2 (4.37 mg) was dissolved in 300 µl of 0.1 M Na_2HPO_4, NaCl 0.15 M, pH 8.0. 1 equivalent of dye, previously dissolved in anhydrous DMSO at a concentration of 10 mg/mL, was added. The reaction was stirred overnight at room temperature in the dark and were analysed by size exclusion chromatography (SEC)-HPLC with an analytical Zorbax GF-250 column (250 × 4.6 mm), eluted with 20 mM Na_2HPO_4, 130 mM NaCl (pH 7.2) containing 20% (v/v) of ACN, at a flow rate of 0.3 mL/min. The effluent was monitored by measuring the absorbance at 675 nm. PEG-Cy5.5 was purified from the unreacted dye through Pierce™ Dye Removal Columns and the purity of the products was confirmed by SEC-HPLC. After dialyzing the solution against water to remove DMSO, the product was lyophilized. The amount of Cy5.5 was determined spectrophotometrically at the maximum absorption of 694 nm (molar extinction coefficient: 250,000 M^{-1} cm^{-1}). The synthesis and characterisation of PEG-haloperidol conjugates (2000 and 6000 Da) were performed according to our previously reported studies [13,15].

7.2.2.2. Human tissue collection

Eleven healthy term placentas (37-42-week gestation) were collected from normal pregnancies within 30 min, following delivery by elective Caesarean section. The study was performed in

accordance with North West Local Research Ethical Committee approval (REC 15/NW/0829) and written informed consent was obtained from all women.

7.2.2.3. Placental explant tissue culture

After removal of the decidua and chorionic plate, samples of villous placental tissue were randomly collected, washed with serum-free medium and dissected into 3 mm^3 explants under sterile conditions. Placental explants were cultured in a 1:1 ratio of DMEM and Ham's F12 media (Lonza Biosciences, UK) supplemented with 10% (v/v) foetal bovine serum (Life Technologies), L-glutamine (2 mmol/L), streptomycin (100 µg/mL), penicillin (100 IU/mL) and amphotericin B (2.5 mg/mL) (Invitrogen), in 24 well culture plates pre-coated with agarose (1% (w/v); Sigma Aldrich). The explants were maintained at 37°C in 5% CO_2 for up to 48 hours, without changing the culture media [16].

7.2.2.4. Cytotoxicity assessment with free PEG

To assess any possible cytotoxic effects of free PEG, placental explants were incubated with PEG-COOH (6000 Da, called free PEG throughout the paper) (dissolved in 0.5% DMSO in sterile PBS) at a concentration range of 0-2 mg/mL for 24 or 48 h. At each time point, samples of culture media were collected in triplicate and stored at -20°C, to quantify lactate dehydrogenase (LDH) release and hCG secretion. The placental explants were treated with 0.3 M NaOH for protein quantification or subjected to fixation in 4% (v/v) neutral buffered formalin (pH 7.4) overnight. These explants were then washed in PBS, dehydrated and paraffin embedded.

7.2.2.5. Total protein assay

Placental tissue digestion was performed using either 0.3 M NaOH, or homogenisation in ice-cold 1.15% KCl solution. Then, total protein (mg) per explant was quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, UK) according to the manufacturer's instructions. 100 µL of the standard (bovine serum albumin) solutions or the unknown samples were added to a 96-well plate and mixed with an equivalent volume of the dye reagent. The plate was then incubated for 30 min at room temperature and the absorbance was measured at 595 nm. The amount of protein in the samples was then determined using the standard curve obtained.

7.2.2.6. Immunohistochemistry

Placental tissue sections (~5 μm) were deparaffinised in Histoclear and ethanol and then rehydrated in dd H_2O. For antigen retrieval, slides were microwaved using antigen retrieval buffer (sodium citrate (0.01 mol/L), supplemented with 0.05% (v/v) Tween 20 (pH 6.0)) for 10 min. After cooling for 10 min, tissue sections were incubated with 3% (w/v) hydrogen peroxidase for 10 min to block the activity of endogenous peroxides within the tissues, followed with (2 x 5 min) washing cycles in Tris-buffered saline (TBS). To block non-specific antibody binding, the tissue sections were incubated with a non-immune block solution consisting of 2% human serum (Dako) and 10% goat serum (Dako) in TBS-Tween (0.1%v/v) for 30 minutes at room temperature. Tissue slides were then incubated with the following primary antibodies: mouse anti-human M30 CytoDEATH (1:100; Roche Diagnostics), mouse anti-human Ki67 (1:500; MIB-1 clone, Dako) or control mouse IgG (matched concentration; Sigma Aldrich) overnight at 4 °C in a humidified chamber. Tissue sections were washed with TBS for 5 min, followed by 2 x 5 min wash cycles with TBS-Tween (0.6%) and finally with TBS for 5 min. Then, the sections were incubated with a biotinylated goat anti-mouse secondary antibody (1:200; Dako) for 30 min at room temperature. Tissue sections were then washed as described above and incubated with avidin peroxidase (5 μg/mL in TBS; Sigma Aldrich, UK) for 30 min at room temperature. Slides were then washed with TBS/TBS-Tween as described above and incubated for 2-3 min with chromogen diaminobenzidine (DAB; 0.05% (w/v); Sigma Aldrich, UK). Tissue sections were rinsed with dd H_2O, counterstained with filtered Harris' hematoxylin, and dehydrated in increasing concentrations of alcohol. The tissue sections were then passed in Histoclear and mounted in DPX mountant (Sigma Aldrich, UK).

Immunostained explants were imaged using an Olympus BX41 microscope to assess the numbers of M30-positive and Ki67-positive cells, as a measure of apoptosis and proliferation, respectively. Six random images of each explant were captured using the same exposure settings and analysed using HistoQuest® Analysis Software.

7.2.2.7. LDH cytotoxicity assay

LDH released into the explant culture medium after free PEG treatment was measured using a Cytotoxicity Detection Kit^{Plus} (Roche, UK) as per the manufacturer's instructions. Briefly, samples of the media were thawed on ice and added to a 96-well plate (100 μL/well). Then, the reaction solution (100 μL/well) was added and the plate was incubated for 15 min at room temperature. To quench the reaction, 50 μL of stop solution was added to each well. The

absorbance at 490 nm was measured using a plate reader (Versamax, Molecular Devices, Wokingham) and data were normalized to the total protein content (mg) of each explant.

7.2.2.8. hCG secretion assay

The levels of hCG in culture media were measured using a commercially available hCG ELISA kit (DRG Instruments GmbH, Germany) using appropriate standards and controls. 25 µL of the control, standard or unknown samples were added to individual wells of a microtitre plate, which had been pre-coated with a monoclonal antibody directed against the alpha-chain of hCG conjugated with horseradish peroxidase. 100 µL of the enzyme conjugate was then added to each well and mixed for 10 seconds. The plates were incubated for 30 min at room temperature. The solution was removed, and the wells were washed with dd H_2O (5 x 400 µL per well). Then, 100 µL of substrate solution was added to each well. The plates were further incubated for 10 min at room temperature. The enzymatic reaction was then quenched by adding 50 µL of the stop solution per well. Absorbance of each well was read at 450/620 nm using an Omega Fluostar plate reader. The hCG concentrations within media samples were determined using the standard curve obtained and normalised to total protein content of each explant.

7.2.2.9. PEG-Cy5.5 conjugate uptake study

In 24 well plates, placental explants were treated with the PEG-Cy5.5 conjugate at a range of concentrations from 10 µg/ml to 1 mg/ml. The placental homing peptide iRGD-rhodamine (CRGDKGPDC-Rho; 20 µM), which rapidly accumulates in the outer trophoblast layer of the explants was employed as a positive control [16]. The explants were incubated in the dark for 6 or 24 hours at 37 °C 5% CO_2, then embedded in OCT. Tissue sectioning was performed at 5µm; sections were fixed in ice-cold methanol for 10 minutes followed with washing with PBS (2 x 5 min). Slides were mounted using Vectashield mounting medium containing DAPI (Vectorlabs, UK). The tissue uptake of the conjugate was then assessed using the Zeiss Axio Observer fluorescence microscope. The PEG-Cy5.5 conjugate was excited at 650 nm and the emitted lighted was detected at 670 nm.

7.2.2.10. PEG-haloperidol conjugates uptake study

Synthesis and characterisation of PEG-haloperidol conjugates using PEG (6000 and 2000 Da) were carried out according to our protocols [13,15]. In 24 well plates, placental explants were incubated either with free haloperidol or the PEG-haloperidol conjugates at a final

concentration of 20 μM (haloperidol equivalent) for 24 hours at 37°C and 5% CO_2. The extraction of haloperidol or PEG-haloperidol from the tissue was performed according to a previously reported protocol for extracting haloperidol from biological tissues [17]. Briefly, placental explants were washed 3 times with PBS and homogenised in ice-cold 1.15 % KCI solution (3 volumes) using Stuart-SHM1 homogeniser (10-15 seconds cycles). Samples for protein quantification were obtained and the homogenates were then, mixed and vortexed with 2% (v/v) acetic acid in methanol (2 volumes). The final homogenate mixture was centrifuged at 10k x g for 5 min. The supernatants were collected and subjected to RP-HPLC analysis to quantify the amount of free or conjugated haloperidol in the tissue.

To validate the extraction method, explant culture medium containing haloperidol (20 μM) was incubated without explants for 24 h under the same incubation conditions of the treated explants and was subjected to the same extraction procedure. Explant culture medium containing freshly added haloperidol (20 μM) was also subjected to the same extraction procedure. The recovery (%) was calculated using the following equation:

$$\frac{100 \times (\text{concentration of haloperidol extracted from treatment solution after incubation})}{(\text{concentration of haloperidol extracted from treatment solution before incubation})}$$

The mean recovery of haloperidol was 66%.

7.2.2.11. RP-HPLC analysis of extracted free haloperidol and PEG-haloperidol

The RP-HPLC analysis of haloperidol was performed according to our previously reported protocol [13]. The RP-HPLC analysis was carried out using a C18 column. A gradient system was applied using an aqueous (0.25% acetic acid) (A) gradient in acetonitrile (B) (10% of B increased to 70% of B over 25 min, then decreased to 10% of B for 10 min). The flow rate was adjusted at 1 mL/min and the injection volume was 20 μL. The system was run for 40 min and the eluant was detected using a UV detector at λ =245 nm. A stock solution of haloperidol (1 mg/mL) in 2% acetic acid in methanol was prepared and used to obtain a range of concentrations (0.025-30 μg/mL). A RP-HPLC calibration curve was obtained to determine the concentration of free haloperidol or PEG-haloperidol conjugates taken up by the placental explants.

7.2.2.12. Statistical analysis

Graphical representation and analysis of the data was generated using GraphPad Prism software (Version 7). Data were either expressed as medians (for non-parametric data) or presented as mean ± SEM (standard error of mean, n=3-5) unless stated otherwise. Statistical analysis was performed using Wilcoxon matched pairs and Friedmann test for (for non-

parametric data), or one-way Analysis of Variance (ANOVA) test followed by Bonferroni's *post hoc* test where appropriate; a P value < 0.05 was set to be significant in the parameters.

7.3. Results and discussion

The human placenta is a complex organ that controls the transfer of oxygen and nutrients to the developing fetus and forms a biological barrier to infection and xenobiotics to protect the fetus during all stages of pregnancy [18]. However, finding effective and safe treatments for managing health complications during pregnancy remains a challenge as several different classes of drugs, such as some antibiotics, antihypertensives, antiepileptics, freely cross the placenta and can be very harmful to the developing fetus. This leaves clinicians in the difficult position of having to weigh up the competing treatment requirements of both mother and child [8].

In the current study, we assessed the suitability of PEGylation to reduce or prevent the uptake of conjugated drugs by the placenta. We have recently proved the ability of PEGylation strategy to prevent the diffusion of conjugated haloperidol through the BBB, which shows some similarity to the placenta in terms of drug transportation [9–11]. Haloperidol was conjugated to PEG through a biologically stable carbamate linkage, therefore, the system exhibited excellent stability in biological fluids and the conjugated haloperidol retained its biological activity without being released [12,13]. Therefore, this already optimised system could provide a platform to design drug delivery systems that could limit the effects of conjugated drugs to the maternal circulation to treat complications during pregnancy.

In the present work, the placental uptake of non-prodrug PEG conjugates of i) Cy5.5 (a fluorophore) and ii) haloperidol (a model drug which is known to cross the placenta [19], and with potential non-CNS applications for the expectant mother) has been evaluated. The polymeric carrier PEG was first assessed for its biocompatibility with human term placental tissue. Moreover, the ability of the PEGylated system to impair the uptake of the conjugated drugs by the tissues was evaluated *ex vivo*.

7.3.1. Synthesis and characterisation of PEG-Cy5.5 conjugate

The labelling of PEG-NH_2 with Cy5.5 was obtained with a dye loading of 18.47% (w/w) and Cy5.5/PEG molar ratio was 0.99 as determined spectrophotometrically (**Figure 7.2**). The purity of the product was determined using SEC-HPLC. The Cy5.5 fluorescent probe was chosen as it has good solubility and a relatively high fluorescence quantum yield. Moreover, its excitation/emission spectra greatly minimise their overlap with the autofluorescence of

biomolecules. Therefore, it is widely used in biological applications including placenta-related research [20–23]. In addition to that, its chemical structure allows conjugation to PEG. The conjugation of Cy5.5 to PEG was achieved *via* an amide linkage, which is biologically stable for the intended study [24].

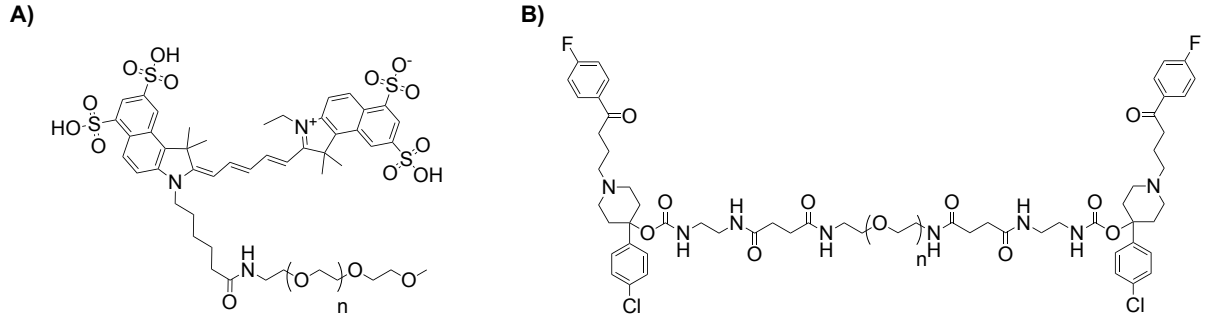

Figure 7.2. Chemical structures of A) the novel PEG-Cy5.5; B) the previously reported PEG-haloperidol conjugates used in this study [13,15].

7.3.2. Assessment of proliferation and apoptosis rates in term placental explants after incubation with free PEG

The first step was to determine any potential cytotoxic effects of PEG on the placental explants which might alter the normal placental function and affect drug uptake and transplacental transfer. This study is the first study, to the best of our knowledge, reporting the compatibility of PEG with the placental tissues using an *ex vivo* approach.

The biocompatibility of the polymeric carrier is a key factor influencing the design of the polymer-drug conjugates [25]. Therefore, it was important at the beginning of the study to evaluate the potential toxic effects of free PEG (6000 Da) on the placental explants.

As such, we assessed the cellular turnover post-treatment with free PEG. Explants of villous placental tissue were incubated with a range of concentrations of free PEG (6000 Da) for 24 h or 48 h, and then proliferation and apoptosis rates were determined by immunostaining with antibodies to Ki67 and M30, respectively. Treatment with free PEG did not significantly alter the basal rates of the proliferation or apoptosis at any concentration tested, when compared with the untreated controls (**Figure 7.3** and **Figure 7.4**). More importantly, the duration of incubation did not affect the basal proliferation rate; the median values for Ki67-positive cells after incubation with different concentrations of free PEG for 24 h were not significantly different from those observed after 48 h of incubation (**Figure 7.3M, N**).

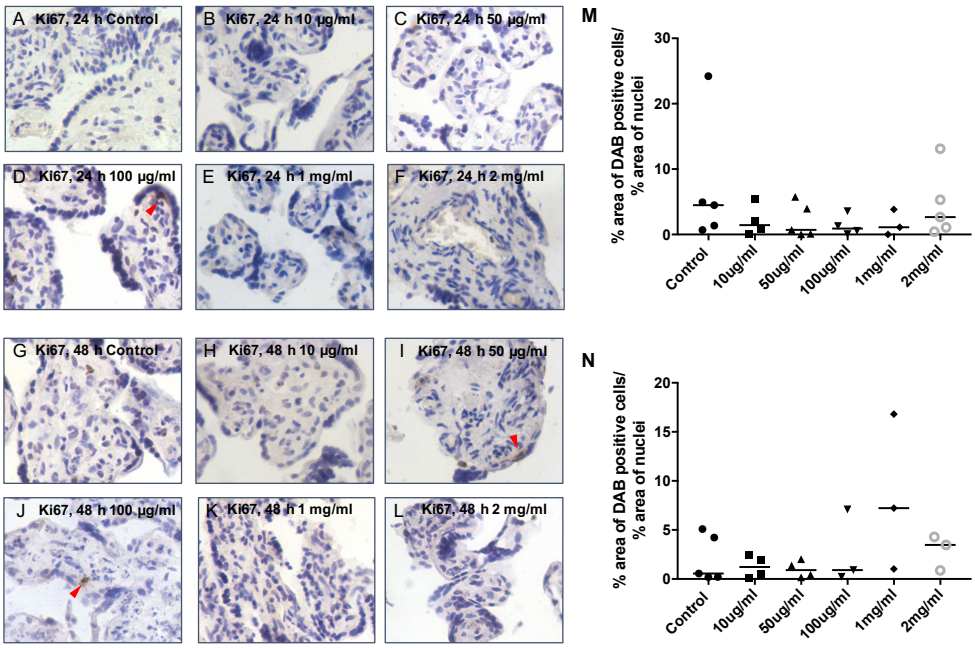

Figure 7.3. Evaluation of proliferation following incubation of placental explants with free PEG by immunostaining with an antibody to Ki67 (DAB; brown); nuclei were stained with hematoxylin (blue). **(A-L)** Representative images of Ki67-positive cells at 24 h and 48 h after explants were treated with different concentrations of free PEG. Red arrows indicate positive staining for Ki67. All images were captured at x20 magnification; **(M, N)** Quantification of Ki67-positive cells at 24 h and 48 h, respectively. Data presented as medians, n= 5 ($P > 0.05$, Friedman test).

Additionally, treatment with free PEG had no effects on the incidence of apoptosis. As indicated by M30-immunohistochemical staining, the number of positively stained cells was not significantly increased when the explants were treated with free PEG at any concentration, for 24 h or 48 h (**Figure 7.4M, N**).

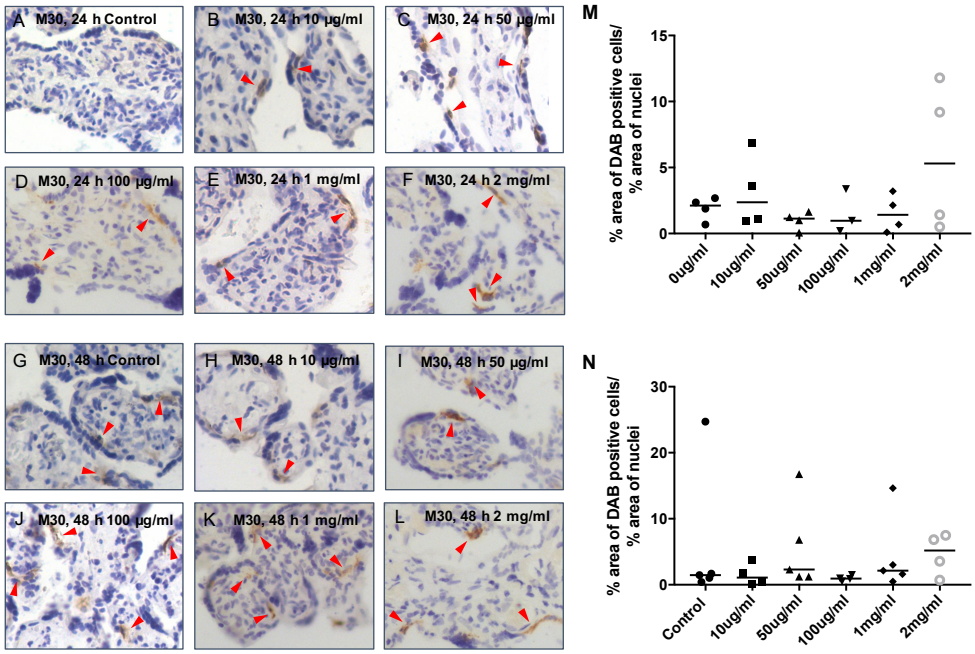

Figure 7.4. Evaluation of apoptosis following incubation of placental explants with free PEG by immunostaining with an antibody to M30 (DAB; brown); nuclei were stained with hematoxylin (blue). **((A-L)** Representative images of M30-positive cells at 24 h and 48 h for after explants were treated with different concentrations of free PEG. Red arrows indicate positive staining for M30. All images were captured at x20 magnification; **(M, N)** Quantification of M30-positive cells at 24 h and 48 h, respectively. Data presented as medians, n= 5 ($P > 0.05$, Friedman test).

7.3.3. Assessment of necrosis in placental explants treated with free PEG

The presence of tissue necrosis after exposure to a xenobiotic is indictive of its toxicity [26]. Given that exposure to free PEG did not alter the basal rate of proliferation or apoptosis in cultured explants, it was necessary to determine whether free PEG induced tissue necrosis.

Release of the cytoplasmic enzyme LDH into the culture medium was quantified, as an indirect indicator of necrosis after exposure to free PEG. Importantly, no concentration-dependent effects of free PEG were observed after 24 h or 48 of incubation, and LDH release did not significantly increase ($P > 0.05$), above levels observed in untreated control explants (**Figure 7.5A, B**).

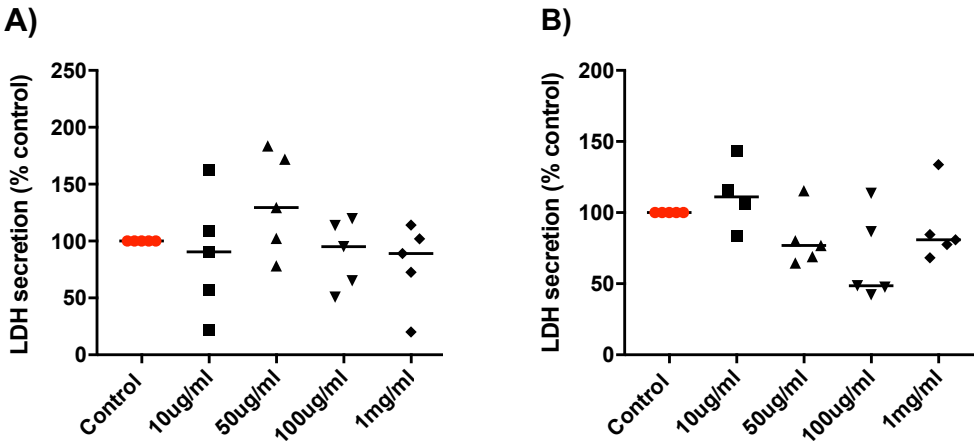

Figure 7.5. Measurement of LDH release in the medium after treating term placental explants with free PEG. **A)** Percentage change in LDH secretion after incubation of explants with free PEG for 24 h; **B)** Percentage change in LDH secretion after incubation of explants with free PEG for 48 h. Data presented as medians, n=5 ($P > 0.05$, Friedman test).

Measuring the amount of LDH released in the media by the explants is considered as an indicator of necrosis, as LDH is released from the cytoplasm when the cell membrane lyses [27]. Treatment with PEG, even at high doses (1 mg/mL) for 48 h, did not cause damage to the explants as no significant increase in LDH release was evident. Taken together, these results demonstrated the biocompatibility of PEG with placental tissues.

7.3.4. Evaluation of syncytiotrophoblast function in placental explants after treatment with free PEG

Another angle to investigate as a marker of biocompatibility is to evaluate different markers of the physiological function of placental explants exposed to free PEG. Potential alterations in placental endocrine function were assessed by measuring the amount of hCG released into the culture medium [28]. Interestingly, incubation with free PEG had no significant effects on hCG release at any tested concentration, either at 24 h or 48 h post-treatment (**Figure 7.6A,B**).

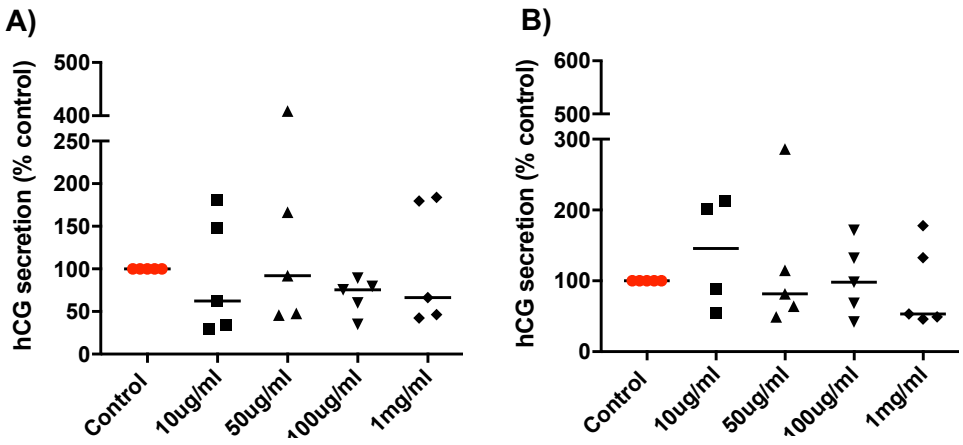

Figure 7.6. Quantification of hCG secretion following exposure of explants to free PEG. hCG was quantified relative to total protein content (mg) of each explant. **A)** Quantification of hCG release from individual explants at 24 h; **B)** Quantification of hCG release from individual explants at 48 h. Data presented as medians, n=5 ($P > 0.05$, Friedman test).

The results indicated that normal syncytiotrophoblast function was not modulated and normal endocrine function was retained after treatment with PEG. These findings are in line with previously reported data indicating the biocompatibility of PEG [29]. Therefore, these results indicated the suitability of the PEG polymer to design drug delivery systems for use in pregnancy, without inducing placental dysfunction.

7.3.5. Assessment of term placental villous tissue uptake of free Cy5.5 and PEG-Cy5.5 conjugate

The ability of the conjugation strategy to impair the cellular uptake of the conjugated system was firstly evaluated by assessing the accumulation of the PEG-Cy5.5 conjugate within term placental villous tissue. Explants were incubated either with the tumour-homing peptide iRGD-Rho (20 µM; positive control), which is known to freely accumulate within the outer syncytiotrophoblast layer of the placenta [16], the free Cy5.5 dye or with different concentrations of PEG-Cy5.5 conjugate for 6 or 24 h. As expected, the iRGD peptide was detected within the outer syncytiotrophoblast layer of the placental villi after 6 h and 24 h of incubation (**Figure 7.7A**). The free Cy5.5 dye was also able to accumulate within the explants, even after only 4 h of incubation at 1 µg/mL (**Figure 7.7B**).

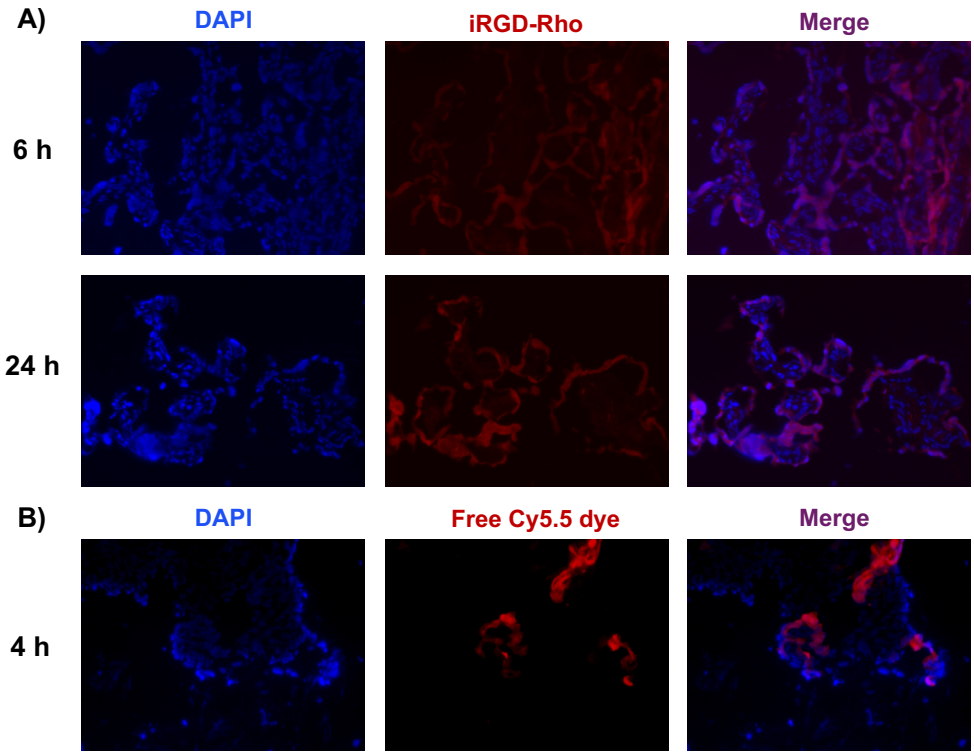

Figure 7.7. A) iRGD-Rho tumour-homing peptide (red) accumulation in the outer syncytiotrophoblast layer of term placental explants, after incubation for 6 or 24 h. B) Free Cy5.5 dye (red) accumulation in term placental explants after incubation for 4 h. Individual explants were OCT embedded, frozen and sectioned then counterstained with DAPI, a nuclear marker (blue). Placental uptake was assessed by fluorescence microscopy. All images were captured at x20 magnification. Representative images of n=3 experiments.

In contrast to the free Cy5.5 and the tumour homing peptide iRGD, the PEG-Cy5.5 conjugate did not show any accumulation in any region of the placental explants after 6 h of incubation at different concentrations (ranging from 10- to 1000-fold higher than that of free Cy5.5) (**Figure 7.8A)** Therefore, additional explants were incubated with PEG-Cy5.5 for 24 h to allow more time for uptake and accumulation. The results showed that, even when the explants were incubated for 24 h, no fluorescence of the PEG-Cy5.5 conjugate was detected at all tested concentrations (**Figure 7.8B**).

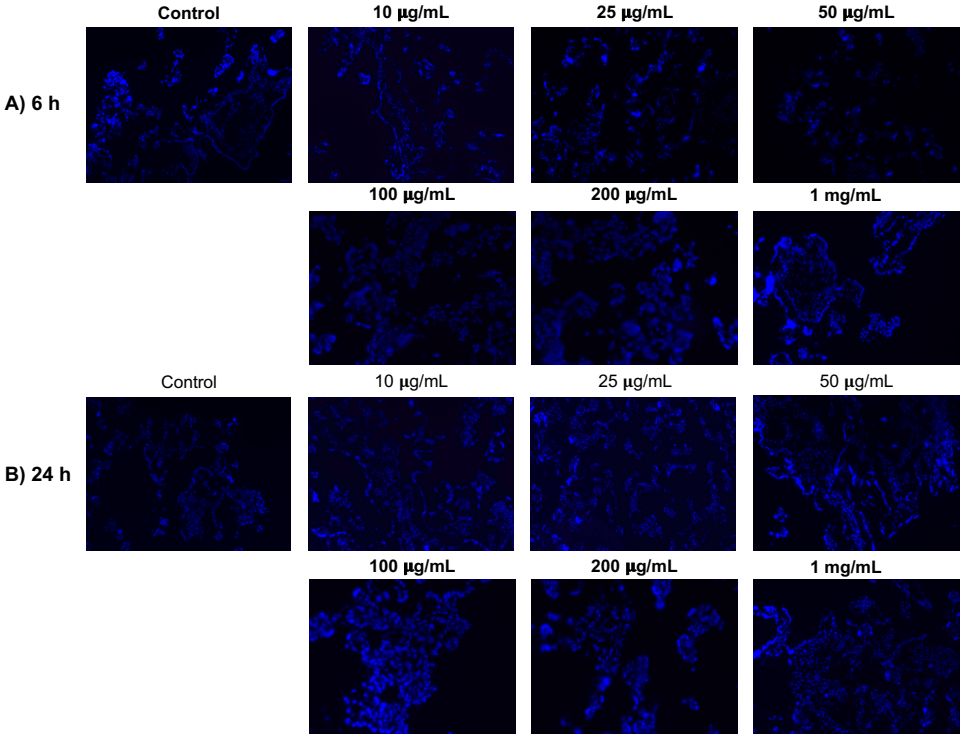

Figure 7.8. PEG-Cy5.5 conjugate showed no placental accumulation after treatment at a range of concentrations for 6h or 24h. Individual explants were OCT embedded, frozen and sectioned then counterstained with DAPI, a nuclear marker (blue). Placental uptake was assessed by fluorescence microscopy. Pictures are presented as merged. All images were captured at x20 magnification. Representative images of n=3 experiments.

The results of the fluorescent uptake study revealed that while free Cy5.5 readily accumulates in placental tissue, the PEG-Cy5.5 conjugate does not. Accumulation of similar conjugates in other tissues is reportedly highly dependent on the size of the molecule and the mechanism of its transportation [30]; the mechanisms regulating PEG uptake by the placenta are currently unknown.

7.3.6. Assessment of term placental villous tissue uptake of a PEG-haloperidol conjugate

Having proven that free PEG is biocompatible with human term placental tissue, and that conjugation of Cy5.5 to PEG did indeed prevent the fluorophore from being taken up by the placenta, we repeated these experiments using a clinically relevant drug, to assess its uptake by the placenta. Specifically, we studied the uptake of PEG-haloperidol conjugates (using haloperidol as a model drug that can cross the placenta and cause potential teratogenic effects [19]). Haloperidol, a D_2 receptor antagonist used to treat psychosis, has been reported to be repurposed for potential non-CNS applications, including antifungal and anti-inflammatory effects [31,32]. These applications could be of significant importance during pregnancy when other medications are not appropriate. However, the side effects and potential teratogenicity of haloperidol, due to its passive accumulation within various tissues including the central nervous

system, and its ability to cross the placenta, have limited these potential clinical applications [33,34]. Therefore, we assessed whether conjugating haloperidol to PEG, to develop a non-prodrug macromolecule, would affect its uptake levels by the placenta. We have recently tested the safety and activity of haloperidol and two PEG-haloperidol conjugates prepared using PEGs (6000 and 2000 Da) at 20 µM (haloperidol equivalent) on the human umbilical vein endothelial cells [15]. We also prepared a conjugate with PEG (2000 Da), in order to increase the loading of the conjugated haloperidol, which was indicated by a 3-fold increase in the total content of haloperidol. This would counterbalance any potential loss of the biological activity of haloperidol due to conjugation [13,15].

Therefore, the uptake of these two PEG conjugates and the free form of haloperidol was compared in the experiment (**Figure 7.9**). The results revealed that free haloperidol freely diffused into the explants and accumulated at 1.8 ± 0.51 µg/mg protein after 24 h of incubation. This was expected, due to the physicochemical properties (i.e. lipid solubility (log P= 4.3) [13] and small size MW (375.86 Da)) of haloperidol, which allows its accumulation within the tissues, most probably *via* passive diffusion mechanism [35].

As hypothesised, as a consequence of conjugation of haloperidol to the hydrophilic PEG, the PEG-haloperidol conjugates dramatically reduced penetration of haloperidol into the placental tissues; uptake was reduced by up to 98% compared to explants exposed to free haloperidol. The placental levels of haloperidol in explants incubated with the PEG-haloperidol conjugates (6000 and 2000 Da) were 0.43 ± 0.13 and 0.04 ± 0.03 µg/mg protein ($P < 0.05, 0.01$), respectively. However, further work using other experimental models is required to investigate whether the PEG-haloperidol conjugates taken up by the placenta reach the fetal circulation.

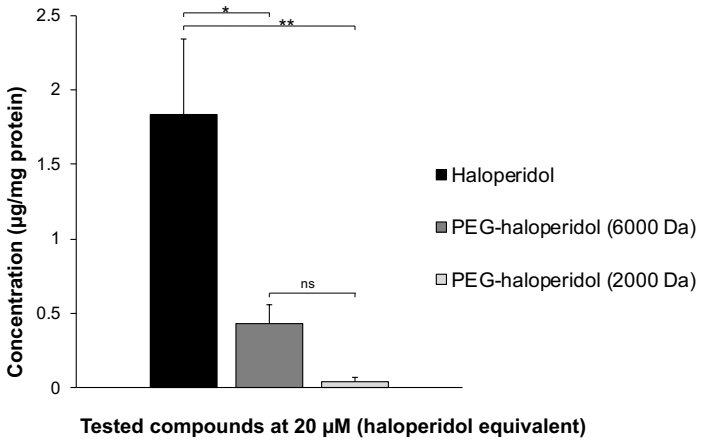

Figure 7.9. Uptake of free and conjugated haloperidol by placental explants after 24 h of incubation. Data were normalised to protein contents within the explants. Data were represented as mean ± SEM (n=4), (*: $P < 0.05$, **: $P < 0.01$, ns: not significant, One-way ANOVA).

The conjugation of haloperidol to PEG significantly reduced placental uptake by ~4-fold in the case of PEG-haloperidol (6000 Da) and by more than ~40-fold when PEG-haloperidol (2000 Da) was tested, which indicates a potential size-dependent uptake in the placental explants [36]. More in-depth investigations are still required to study the effects of the length of PEG chains on the placental uptake of the conjugates. It is important to highlight that there was no significant difference in the uptake levels of the two PEGs.

These findings are in good agreement with a previous *ex vivo* study, which reported the use of PAMAM (G4-OH) dendrimers as a nanocarrier to reduce the transplacental transport of the PAMAM-Alexa (488) conjugate [14]. The results revealed that the PAMAM-Alexa (488) conjugate (16700 Da) exhibited a low rate of transfer across the placenta using the "dually perfused, re-circulating term human placental lobule" *ex vivo* model. Despite the absence of the PAMAM-Alexa (488) from capillaries of the villi, the immunohistochemical studies revealed the presence of PAMAM-Alexa at sparse levels in the outer rim of villous branches and in the intervillous spaces. Moreover, the PAMAM-conjugate was occasionally detected in the cytoplasm and nuclei of the syncytiotrophoblast, which was not the case for our PEG-Cy5.5 conjugate, which was completely absent from the tissue. However, concerns regarding the safety profiles of dendritic PAMAMs (based on their generation, charge and concertation) may limit the therapeutic applications of these systems [37]. Although PAMAM G4-OH has shown to be well tolerated *in vivo* [38], their unpredictable pharmacokinetics and variable characterisation profiles (fluctuating purity and reproducibility) make them less attractive to design and develop such systems for treating complications during pregnancy [39,40].

It is important to highlight that for effective treatment of the expectant mother with drug delivery systems prepared from PEG-drug conjugates, it is essential to retain the therapeutic activity of the conjugated drug when a non-prodrug polymer-drug conjugate is developed. This is because the activity of the system does not depend on the release of the conjugated drug. We have previously proven that PEG-conjugated haloperidol maintained its therapeutic effects *via* different receptors *in vitro*, however, at a lower extent when compared to the free haloperidol [13,15]. These results indicate the suitability of our PEG-drug conjugates as non-prodrug macromolecules to design and develop nano-seized drug delivery systems that could provide compartmentalised therapeutic effects to pregnant women without affecting the developing fetus.

7.4. Conclusion

In this study, we have used in *ex vivo* tissue culture to demonstrate the utility of PEG-drug conjugates, as a non-prodrug system, to reduce uptake of drugs by the placenta. The approach evaluated (a) the biocompatibility of PEG (as a carrier) with the placental tissues and (b) the ability of PEGylated systems to reduce the uptake of conjugated drugs into placental tissue. The study revealed that PEG did not significantly influence the basal rate of proliferation, apoptosis, or necrosis in human placental explants, even when incubated for extended periods at high concentrations. Moreover, no modulation of hCG secretion from the placental explants was recorded after exposure to free PEG, which suggesting that PEG does not negatively affect the normal tissue function. With regards to the impaired uptake into the placenta, in contrast to the free Cy5.5, no conjugated Cy5.5 was detected within the placental explants at any of the tested concentrations. This impairment was further evidenced by measuring the placental accumulation of PEG-haloperidol conjugates, where the conjugation of haloperidol to PEG (6000 and 2000 Da) reduced the tissue concentration of haloperidol by 4 and 40-fold, respectively. Together, these findings indicate that the non-prodrug design of PEG conjugates is a feasible approach to reduce or even prevent the transfer of drugs across the placenta. Therefore, careful selection of drugs and proper design of the linker would allow the localisation of therapeutic effects of the conjugated drugs within the maternal circulation, while protecting the fetus from their harmful effects.

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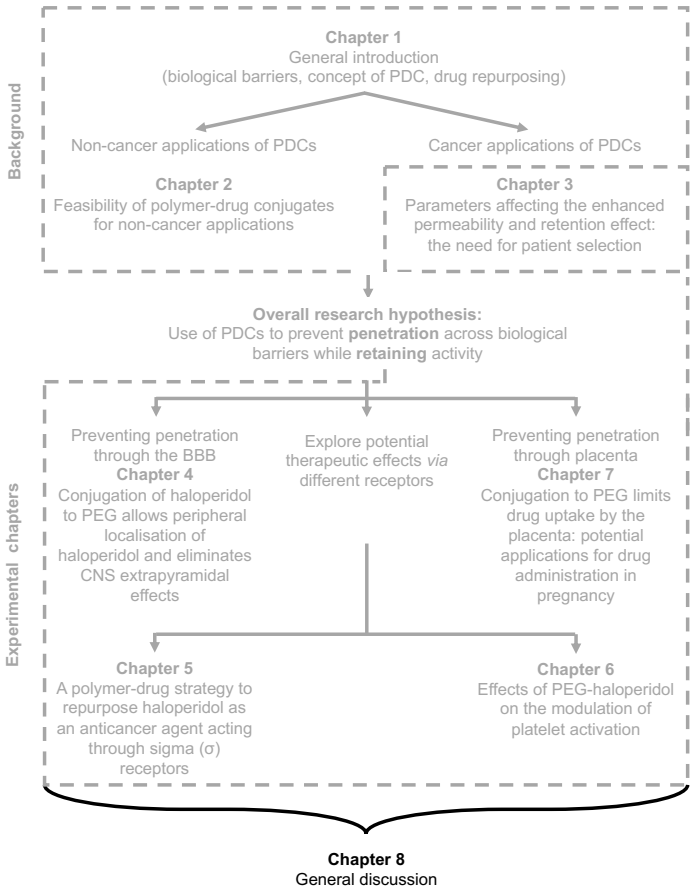
Chapter 8

General discussion

Chapter summary:

In this chapter, the key findings of the work presented in this thesis are summarised and critically evaluated. Potential future research of PEG-haloperidol is also suggested.



8.1. Introduction

The global market value of nanomedicines (PDCs, polymer-protein conjugates, antibody-drug conjugates, liposomes, nanoparticles, micelles, etc.) has been anticipated to be of more than $330 billion in 2025 [1–5]. The applications of these nano-sized DDSs have varied widely to cover the diagnosis of and treating of several health-related problems ranging from enzyme deficiency-related diseases to cancers of different origins [6,7]. The development of these systems is usually rationalised with the need for having more cost-effective therapeutic agents with fewer side effects in comparison to the traditional therapy [8]. However, translation of nanomedicines from the bench into the market remains challenging with only 100 nanomedicine-related products have been approved by the FDA [9].

The vast majority of these systems have been employed to improve cancer treatment. However, the complexity of the disease led to challenges translating the promise of their clinical outcomes. In addition to cancers, nanomedicines have also been explored to treat diseases other than cancers where they might form the base to develop new effective therapies [10].

In this chapter, a critical evaluation of the body of work presented in this thesis is carried out. In section **8.2**, the key findings of the current project are summarised. This is followed by a critical evaluation of some aspects related to the thesis, section **8.3**. Finally, section **8.4** represents a discussion of what, in my opinion, are the meaningful and possible future development of this project.

8.2. Key findings of the current research project

This work primarily aimed to assess the feasibility of utilising PDCs (as non-prodrugs) to design a new DDS using PEG as a polymeric carrier to achieve two main outcomes: a) to compartmentalise the effects of the conjugated drugs at the intended site of action and avoid unwanted diffusion across the biological barriers; b) to retain the biological activity of the conjugated drug without needing to be released from the conjugate.

Factors influencing the magnitude of the EPR effects (Chapter 3) [3]

The type of tumours was one of the factors that governed the passive accumulation of nanomedicines within the tumour tissues. The highest levels of nanomedicines accumulation were observed in the tumour tissues of the pancreas, colon, breast, and stomach. The stage and the tumour size also influenced the EPR effects, with high accumulation levels observed in large tumours, while small and very large tumours showed levels of accumulation that were

similar to each other (the categorisation of the size and the stage of tumours is discussed in **Chapter 3**). Perfusion levels, the presence of angiogenesis, and inflammation in tumour tissues were identified as other parameters that impacted the accumulation of nanomedicines within tumour tissues. Our findings indicated that the EPR effect is a very complex phenomenon affecting the clinical outcomes of using nanomedicines for treating cancer. Other studies have also highlighted the high degree of complexity and heterogeneity the EPR effect has among tumours originate from the same tissues and among tumours and their metastasis occurred within the same patients. This complexity and heterogeneity of the EPR effect were identified as key challenges to predict the clinical outcomes of the EPR-based therapeutics [11,12].

PEGylation as a strategy to avoid the penetration of haloperidol across the BBB while antagonising the dopaminergic effects (Chapter 4) [13]

The *in vitro* [^{35}S]GTPγS-binding assay revealed that the PEG-haloperidol conjugate retained the antagonistic effects of haloperidol on D_2 receptors, albeit at a lower level compared to haloperidol. Recording catalepsy in rats showed an absence of cataleptogenic effects of PEG-haloperidol compared to the free haloperidol which might indicate prevented penetration across the BBB of PEG-haloperidol. These findings are in agreement with data reported for Movantik® (the PEGylate naloxol) which retained its antagonistic effects on the opioid receptors in the intestine without releasing naloxol and did not cross the BBB [14]. Moreover, another study documented similar outcomes of conjugating a μ-opioid agonist to a PEG oligomer (named as NKTR-181) which reduced the penetration of the μ-opioid agonist across the BBB. NKTR-181 exhibited significantly reduced penetration across the BBB and retained its analgesic activity similar to that of oxycodone [15]. Therefore, PEG-drug conjugates as non-prodrug systems form an effective DDS to localise the effects of drugs at the desirable compartment.

Potential anticancer applications of PEG-haloperidol conjugates (Chapter 5) [16]

Two PEG-haloperidol conjugates using PEG of two MWs (2000 and 6000 Da) and were tested for their possible effects in the field of cancer. The use of PEG (2000 Da) enhanced haloperidol's loading in the conjugate approximately 3-fold compared with the loading of haloperidol in the PEG-haloperidol conjugate (6000 Da). The cytotoxicity studies using the conjugates on breast cancer cell lines (MCF-7 and MDA-MB 231) revealed the limited antiproliferative applications of conjugated haloperidol due to the high IC_{50} values of the conjugates on both cell lines (>100 μM) (see section **8.3.2**). The *in vitro* cell migration assay showed a potential anti-migratory activity of the PEG-haloperidol conjugates against VEGF-

induced cell migration of HUVECs *in vitro*. Both conjugates significantly inhibited cell migration although at lower levels compared to the free haloperidol, which may suggest their applications in angiogenesis-related conditions. However, further studies are required to investigate the inhibitory effects of PEG-haloperidol conjugates on other components of the angiogenesis process (see section **8.4**).

Evaluation of potential cardiovascular applications of PEG-haloperidol conjugate (Chapter 6)

The effects of PEG-haloperidol on the aggregability of human platelets after stimulation with two agonists were assessed on PRP *in vitro*. The preliminary data indicated no significant inhibitory effects of PEG-haloperidol on CRP-XL (*via* GPVI receptors) or ADP (*via* $P2Y_1$ receptors) induced aggregation of the platelets. It has been reported that haloperidol has shown antiplatelet effects when collagen and ADP were used as agonists to stimulate platelets aggregation [17,18]. However, in these experiments, different methodologies (including different concentrations and incubation times) were used which may lead drawing incomplete conclusions about the efficacy of haloperidol and its PEGylated form to inhibit platelet aggregation. Therefore, further studies are required to identify the exact mechanism the haloperidol or its PEG-conjugate could exert their effects on the platelets. This could include studying the inhibitory effects of free and conjugated haloperidol on platelets aggregation by modifying the incubation time with platelets, testing different agonists and using different assays to measure these effects (see **Chapter 6** for further details).

PEGylation as a strategy to reduce drugs transfer across the human placenta (Chapter 7)

The non-prodrug PDC strategy, using PEG as a polymeric carrier, proved viable for preventing the penetration of conjugated drugs through the human placenta *ex vivo*. Free PEG did not significantly modulate the proliferation or significantly affect the apoptosis rates after incubation with placental explants. Treatment of the explants with free PEG did not significantly induce any necrosis or alter the normal function of the placental tissues. The cellular uptake studies using fluorescent microscopy showed no localisation of the PEGylated Cy5.5 (at all tested concentrations) in any region of the placental tissue. Moreover, conjugating haloperidol to PEG significantly reduced its uptake levels by placental tissues when compared to the free form. The conjugation strategy was also applied in another study where the transfer rate through the human placenta of PAMAM-dye conjugate was significantly lower than that

of the free dye [19]. Therefore, these findings suggest the feasibility of developing PDC systems to treat diseases during pregnancy without affecting the development of the fetus.

8.3. Critical evaluation of the presented work

8.3.1. Suitability of PEG as a polymer for conjugation

Since the introduction of the concept of PDCs, several polymers have been utilised to design these systems [20]. The selection of the appropriate polymer is initially based on the rationales behind the design of these PDCs and the type of developed macromolecules (pro-drugs (in most cases) or non-prodrugs (in few cases)) (see **Chapter 1** and **2**). The design of our system was based on developing a non-prodrug PDC of haloperidol, therefore, a limited range of polymers could be selected (mainly the non-biodegradable polymers) to conjugate haloperidol. The main rationale for using such polymers is to avoid the degradation of the polymeric chain into small fragments. In addition to PEGs, polyglycerol, poly(2-oxazoline)s, poly(acrylamide), poly(vinylpyrrolidone) and poly(*N*-(2-hydroxypropyl)methacrylamide) are other classes of non-biodegradable polymers that have been explored to develop PDCs (mostly used in designing macromolecular pro-drugs). However, the pharmacokinetics and safety profiles of most of these polymers require further identifications and investigations, which limited their application in our system [21].

In the 1970s, two key papers were reported by Davies and Abuchowsky indicating the modification of albumin and catalase enzyme using PEG to extend their half-life, enhance their solubility and mask their immunogenicity [22]. Since that date, the PEGylation strategy was further improved and employed to develop conjugates for various therapeutic agents (antibodies, proteins, enzymes, and small drugs) with 18 PEG-based products available on the market in 2020 [23–25]. Additionally, PEGylation has been reported to improve the *in vivo* stability of nanosized DDSs such as liposomes, micelles, dendrimers and nanoparticles [26].

Researchers have identified several reasons for the superiority of PEG over other polymers. This included the characterises that PEGs have such as availability in a wide range of molar masses (0.4-50 kDa) and its very low chemical reactivity (almost inert) [21]. PEGs have a low polydispersity index (PDI) (<1.1) which makes PEGylation a reproducible process in terms of safety and the residence time in the body after administration [27]. The solubility of PEG in several organic solvents has made the modification of PEG with different functionalities relatively simple and feasible. Furthermore, its hydrophilicity can increase the solubility of the conjugated drug [22]. PEGs are generally considered non-toxic and they show reduced interaction with blood components and body organs. Therefore, PEGylated therapeutics are less

haemolytic with decreased risk of emboli formation, less antigenic and immunogenic [28]. PEGs are biologically stable in mammalians due to a lack of etherase enzymes in turn allowing the design of PDCs as non-prodrug macromolecules [29]. Finally, PEGs are well studied and have been approved by the FDA for use in humans *via* different routes (internal, oral and topical) [30] and the majority of the FDA-approved polymer conjugates are developed using PEG, **Table 8.1**. All of these above-mentioned advantages make PEG one of the most promising polymers for designing PDCs with potential clinical applications, therefore, it was the polymer of choice to prepare our conjugates in this study.

However, there are further points related to PEG that should be considered when PEGylation is considered as the proposed strategy for developing PDCs of various therapeutic agents. The non-biodegradability of PEG could be considered a limitation for its therapeutic applications. The biostability of PEG has been considered as an advantage of this polymer over other polymers especially in terms of small fragments of oligomers formed after degradation (see **Chapter 2** and **4** [13]). However, the fate of this polymer in the body has been a critical issue. It has been reported that small PEGs <400 kDa underwent metabolic degradation *via* several enzymes: cytochrome P-450, alcohol and aldehyde dehydrogenases [31–33]. This led to the formation of toxic hydroxy acid and diacid metabolites [21]. Therefore, PEGs of MW >400 Da should be used when PEGylation is required for pharmaceutical applications. On the other hand, PEGs of MWs higher than the renal threshold (i.e. 40 kDa) should not be used to avoid their accumulation in the liver. It has been reported that PEGs with MW < 20 kD are mainly excreted *via* the kidney. However, the renal clearance has been noticed to be slow for PEGs with MW >20 kDa where the hepatic clearance is predominant for their elimination [34]. Therefore, branched or multi-armed forms of PEGs could be used to offer high MW for targeted PEGylated therapeutics while allowing their renal excretion after degradation [35].

With consideration of this point, our system was developed using PEGs of relatively small MWs (2000 and 6000 Da). PEGs of these MWs allowed reasonable loading for conjugated haloperidol (~8-25%, for PEG 6000 and 2000 Da, respectively). In addition to that, conjugated haloperidol would not accumulate in the body and be eliminated *via* renal clearance as their MWs are below the renal threshold (which in turn reduces risks upon short and long-term treatments) while fulfilling other essential properties (hydrophilicity and MWs high enough to prevent the penetration across the biological barriers) (see **Chapters 4** [13] and **7**).

Table 8.1. Summary of PEG conjugates available on the market or in clinical trials (http://clinicaltrials.gov) [23,24].

PEG conjugates	Product	Company	Indication	Used PEG	Linker	ROA	Rationale for PEGylation	Status	Ref
PEG-drug conjugates									
PEG-naloxol	Movantik®	AstraZeneca	Opioid-induced constipation	PEG (7 monomers)	Ether	p.o	Limit distribution to the CNS	Marketed	[36]
PEG-μ-opioid agonist	NKTR-181 (Oxycodegol)	Nektar	Chronic low back pain/ long term treatment	PEG (6 monomers)	Ether	p.o	Limit distribution to the CNS	Stopped	[15]
PEG-irinotecan	NKTR-102	Nektar	Metastatic breast cancer	4-arm PEG 20 kDa	Ester linker	*i.v.*	Improve tumour uptake and reduce renal clearance	Phase 3	[37]
PEGylated TLR7/8 agonist	NKTR-262	Nektar	Locally Advanced or metastatic Solid Tumour Malignancies	4-arm PEG	No data available	*i.v.*	Improve tumour uptake and pharmacokinetics	Phase 1/2	[38]
PEG-camptothecan analogue SN-38	PEG-SN38	Nektar	Metastatic colorectal carcinoma	4-arm PEG 40 kDa	Amide	*i.v.*	Improve solubility, tumour uptake and increase half-life	Phase 1B Discontinued	[39]
PEG-camptothecin analogue SN-38	PEG-SN38	Prolynx	Small cell lung cancer	4-arm PEG 40 kDa	Amide	*i.v.*	Improve solubility, tumour uptake and increase half-life	Phase 1B	[40]
PEG-protein conjugates									
PEG-exenatide	NLY01	Neuraly, Inc	Early untreated Parkinson's disease	Trimer PEG 50 kDa	Disulfide	*s.c*	Improve stability and reduce renal clearance	Phase 2	[41]
PEG-exenatide	PB-119	PegBio	Type 2 diabetes mellitus	PEG 23 kDa	Disulfide	*s.c*	Improve stability and reduce renal clearance	Phase 1	[41]
PEGylated oxyntomodulin analogue	TT401 (LY2944876)	Transition Therapeutics/ Eli Lilly	Obesity and type 2 diabetes mellitus	No data available	No data available	*s.c*	Improve pharmacokinetics and modulate activity*	Phase 2	[42]
PEG-interferon α-2a	Pegasys®	Hoffmann–LaRoche	Hepatitis C	Branched PEG 40 kDa	Amide	*s.c*	Reduce renal clearance	Marketed	[43]
PEG-interferon α-2b	Pegintron®	Schering-Plough	Hepatitis C	PEG 12 kDa	Urethane	*s.c*	Reduce renal clearance and reduce immunogenicity	Marketed	[44]
PEG-epoetin β	Mircera®	Hoffmann–LaRoche	Renal anaemia	PEG 30 kDa	Amide	*i.v/s.c*	Reduce renal clearance	Marketed	[45]
PEG-interferon α-2b	Sylatron®	Merck	Melanoma	PEG12 kDa	Urethane	*s.c*	Reduce renal clearance and reduce immunogenicity	Marketed	[46]

Table 8.1. (Continued)

Pegvisomant	Somavert®	Pfizer	Acromegaly	PEG 4-6 x 5 kDa	Amide	*s.c*	Reduce renal clearance, improve stability and reduce immunogenicity	Marketed	[47]
PEGylated erythropoietin-mimetic homodimeric-peptide (Peginesatide)	Omonty®	Affymax and Takeda	Anaemia due to chronic kidney disease (CKD) in adult patients on dialysis	Branched PEG 40 kDa	Urethane	*s.c*	Reduce renal clearance and reduce immunogenicity	Withdrawn from the market	[48]
PEGylated coagulation factor VIII	Adynovate®	Takeda	Haemophilia A	PEG 20 kDa	Amide	*i.v.*	Reduce renal clearance	Marketed	[49]
PEGylated coagulation factor IX	Rebinyn®	Novo Nordisk	Haemophilia B	PEG 40 kDa	Amide	*i.v.*	Reduce renal clearance	Marketed	[50]
PEGylated coagulation factor VIII	Jivi®	Bayer	Haemophilia A	PEG 60 kDa	Disulfide	*i.v.*	Reduce renal clearance	Marketed	[51]
PEGylated β-interferon 1a	Plegridy®	Biogen Idec	Relapsing multiple sclerosis	PEG 20 kDa	Amine	*s.c*	Reduce renal clearance	Marketed	[52]
PEG-enzyme conjugates									
PEG-ADA	Adagen®	Enzon	SCID	PEG 5 kDA	Amide	i.m	Reduce renal clearance, reduce proteolytic degradation and reduce immunogenicity	Marketed	[53]
PEG-Aspargase	Oncaspar®	Enzon	All	PEG 5 kDA	Amide	i.m	Reduce elimination and reduce immunogenicity	Marketed	[54]
PEGylated phenylalanine ammonialyase	Palynziq®	BioMarin	Phenylketonuria	PEG 20 kDa	Amide	*s.c*	Reduce immunogenicity	Marketed	[55]
PEGylated uricase	Krystexxa®	Savient Pharmaceuticals	Gout	PEG 10 kDa	Urethane	*i.v*	Increase solubility, reduce renal clearance and reduce immunogenicity	Marketed	[56,57]
PEG-antibody conjugate									
Certolizumab pegol	Cimzia®	UCB Pharma SA)	Rheumatoid arthritis and Crohn's disease	Branched PEG 40 kDa	Thioether	*s.c*	Reduce renal clearance and reduce immunogenicity	Marketed	[58]

Table 8.1. (Continued)

PEGylated aptamer									
PEGylated anti-von Willebrand factor	ARC 1779	Archemix	Thrombotic Thrombocytopenic	PEG 20 kDa	Amide	i.vitre	Increase half-life	Phase 2 (completed)	[59]
PEGylated anti-C5 Aptamer (Anticomplement component 5)	ARC 1905	Ophthotech	Neurovascular AMD	Branched PEG 40 kDa	Amide	i.vitre	Reduce renal clearance	Phase 2b	
PEGylated anti-TFPI aptamer	BAX 499	Baxalta	Hemophilia	Branched PEG 40 kDa	Amine	*s.c*	Reduce renal clearance and reduce nuclease degradation	Phase 1 terminated	[60]
PEGylated anti-VEGF aptamer	Macugen® (Pegaptanib)	Eyetech Inc. and Pfizer Inc	AMD	Branched PEG 40 kDa	Phosphodiester	i.vitre	Reduce renal clearance and reduce nuclease degradation	Marketed	[61]
PEGylated antichemokine ligand 12	NOX-A12 (Olaptesed pegol)	Noxxon pharma	Glioblastoma, multiple myeloma, CLL	Branched PEG 40 kDa	Amide	*i.v*	Reduce renal clearance and reduce nuclease degradation	Phase 1/2 completed or active (recruiting)	[62]
PEGylated antichemokine ligand 2	NOX-E36 (Emapticap pegol)	Noxxon pharma	Type 2 diabetes mellitus, renal impairment, chronic inflammatory diseases	Branched PEG 40 kDa	Amide	*s.c*	Reduce renal clearance and reduce nuclease degradation	Phase 2 completed	[63]
PEGylated anti-hepcidin	NOX-H94 (Lexaptepid pegol)		Anaemia, end stage renal disease, chronic diseases, inflammation	Branched PEG 40 kDa	Amide	*i.v*	Reduce renal clearance and reduce nuclease degradation	Phase 2 completed	[64]
Pegfilgrastim	Neulasta®	Amgen	Neutropenia	PEG 20 kDa	Amine	*s.c*	Reduce renal clearance	Marketed	[65]
PEGylated anti-factor IXa	Pegnivacogin (REG1)	Regado Biosciences	Anticoagulant	Branched PEG 40 kDa	Amide	*i.v*	Increase half-life	Phase 3 terminated	[66]
PEGylated antiplatelets derived growth factor	Pegpleranib (E10030)	Archemix Corporation	AMD	Branched PEG 40 kDa	Amide	*i.v*	Increase half-life	Phase 3 terminated	[67]

*: to imbalance the biological activity in favour of glucagon-like peptide-1 receptor to glucagon receptor.
Abbreviations: ADA: adenosine deaminase; AMD: age-related molecular degeneration; CAD: coronary artery disease; CLL: chronic lymphocytic leukaemia; ROA: route of administration; SCID: severe combined immunodeficiency disease; TFPI: tissue factor pathway inhibitor.

Additionally, although PEGylation is a well-known strategy to mask the immunogenicity of proteins, several studies have reported immunogenic reactions and the presence of PEG antibodies in some patients treated with PEG products [68]. This phenomenon was observed in cases where methoxy PEGs were used [69,70]. This immunogenicity of PEG has been considered responsible for the enhanced clearance of PEGylated agents from the blood *via* the accelerated blood clearance (ABC) phenomenon, and, consequently, their reduced efficacy [71]. However, a critical review of the literature that included *in vivo* and clinical studies using PEGylated therapeutics has revealed that the vast majority of assays applied to detect anti-PEG antibodies are imperfect and not specific. Therefore, it would be premature to draw any conclusions regarding PEG-induced immunogenicity [72]. In order to reduce or even avoid any potential immunogenic reactions associate with the administration of our PEG-haloperidol conjugates, PEGs with carboxyl groups were utilised in the preparation of the conjugates [73]. This was considered as an extra step to improve the safety of our conjugates.

Moreover, cellular vacuolation has been articulated as another factor to consider when developing PEGylated therapeutics. Several *in vivo* and clinical studies have reported the formation of vacuolation of high MW PEGs in certain cell types and tissues. PEG vacuolation was detected in macrophages of lymph node, spleen and the bone marrow, as well as in the renal tubule and the liver [35]. Nonclinical toxicological studies of marketed PEGylated biotherapeutics revealed that vacuolation is a biological response of cells towards high concentrations of foreign large PEGs [74]. A recent study of collaboration across 14 companies, laboratories and institutes has assessed toxicology studies of PEGs and summarised their findings [75]. It has been found that vacuolation is associated with PEG MW >20 kDa as the cellular vacuolation was not detected when PEG MW <20 kDa was used (this was considered in our systems). More importantly, the study revealed that no changes of tissues functionality were observed when cellular vacuolations were detected and not associated with pathological effects such as inflammation, necrosis, tissue degeneration and distortion of cells. The PEG related cellular vacuolations were reversible when sufficient time for recovery was given [75]. However, it is still necessary to perform to toxicological studies to identify potential long-term safety of PEGylated therapeutics at an individual level.

8.3.2. Evaluation of physicochemical properties of the PEG-haloperidol conjugates

The systems presented in this thesis were thoroughly characterised with respect to the total content of conjugated haloperidol (%w/w) and the residual free drug. However, for PDCs, which are hydrophilic systems containing in most cases lipophilic drugs, additional

characterisation of their behaviour in aqueous solutions is still required. These systems have the ability to form supermolecular structures such as micelles and their physiochemical characterisation is considered a crucial step in their translation into clinical applications. The ability to form micelles (or intramolecular aggregate), their size distribution and stability are other important characteristics to be determined as they influence the biological activity of the conjugated drugs [76].

Our system was designed to conjugate haloperidol (a highly lipophilic drug with a log P value of 4.3 [13]) to a highly hydrophilic PEG. Therefore, the resultant molecule might have a tendency to self-assemble and form micelles or large aggregates in aqueous solutions and biological fluids. This phenomenon has been previously reported for PEG-based conjugates of doxorubicin (an anticancer agent with a log P value of 1.3 [77]) which formed micelles [78]. Several forms of micellar aggregates were observed with aggregation numbers ranging from 3 to 20 depending on the linker used. In another study, Ashok *et al* have documented that the length of the PEG chain influenced the formation of micelles of PEG-phospholipid conjugates, and the critical micelle concentration (CMC) depended directly on the length of the hydrophilic chain of PEG used [79]. It is important to note that, PDCs have shown a tendency to form micelles of several types (i.e. unimolecular and multimolecular micelles). This property has potentials to affect the therapeutic activity of these macromolecules by influencing the accessibility of conjugated drugs to their biological targets (e.g. enzymes, receptors etc..) [78,80].

Given that haloperidol is more lipophilic than doxorubicin (log P= 4.3 vs 1.3, respectively), the PEG-haloperidol conjugates might behave similarly and form unimolecular micelles. This might explain the difference in the cytotoxic effects of PEG-haloperidol (6000 and 2000 Da) conjugates studied in **Chapter 6** [16]. PEG-haloperidol (6000 Da) was more potent than the 2000 Da conjugate. The difference in the IC_{50} between the conjugates might be a result of less conjugated haloperidol (in the PEG-haloperidol 2000 Da) being available to bind to its target, as a consequence of the micelles' formation of this conjugate. Therefore, the formation of micelles and the CMC of the PEG-haloperidol conjugates could be determined, which could provide a further understanding of the behaviour of the conjugates in the biological fluids, and, as a consequence, their therapeutic effects. Fluorescent probes (8-anilionaphthalene-1-sulfonic acid (ANS) and pyrene) or methyl orange dye could be employed to determine the CMC of the conjugate at the physiological pH [81–83]. The formed micelles could be further studied using the dynamic light scattering, cryo-transmission electron microscopy (TEM) and small-angle x-ray scattering (SAXS) [84–86].

8.3.3. Evaluation of the pharmacokinetics of the PEG-haloperidol conjugates

PEG-haloperidol (as a conjugate) is a new chemical compound with a new chemical entity. After *i.v* administration, free haloperidol has been shown to rapidly distribute throughout the body with a volume of distribution of 1260 L. Its half-life and clearance ranges are 14.1-26.2 h and 6.5-11.8 mL/min/kg, respectively. Haloperidol has high plasma protein binding with more than 88% of the administered dose. The elimination of haloperidol is mainly *via* the hepatic route with less than 1% excreted unchanged in the urine [87]. However, conjugating haloperidol to PEG with a biologically stable linkage will change the pharmacokinetic profile of the conjugate haloperidol. Extended half-life, elimination *via* kidneys (MW <40 kDa), altered distribution profile and reduced plasma protein binding would all be expected when haloperidol is conjugated to PEG [88,89]. The improved pharmacokinetic profiles of the PEGylated small drugs were documented for different drugs such as PEG-zidovudine (antiviral) [90], PEG-doxorubicin (anticancer), PEG-vancomycin (antibiotic) [91]. This included increased AUC and improved $t_{1/2}$ with reduced toxicity when compared with the parent drugs [92].

Therefore, determining the pharmacokinetics of PEG-haloperidol conjugates is necessary. This would provide information about the biological activity and the kinetic profile of the conjugate in terms of the half-life, distribution within the body and the routes of elimination of the conjugate.

8.4. Possible future work

The work presented in this thesis confirmed the feasibility of employing the PEGylation strategy to prepare PDCs of haloperidol as non-prodrug systems offering compartmentalisation of haloperidol's activity at the intended site of action. The PEG-haloperidol conjugates exhibited activity towards D_2 and σ receptors, while their activity *via* other receptors (in the human platelets) still requires investigations. However, clinical translation of our findings needs further research. This would include carrying out some studies related to the physicochemical properties and the pharmacokinetics of the conjugate (see sections **8.3.2** and **8.3.3**). However, haloperidol has been shown to have potential therapeutic applications apart from its main application for treating schizophrenia [93–95]. Given the financial and the health-related outcomes of repurposing drugs (See **Chapter 1** for more details) by exploring old drugs for new applications, this would be highly beneficial in situations such as the current COVID-19 pandemic where time is costing lives and money (haloperidol, in addition to other medications, has shown potential application against SARS-CoV-2) [96].

Further antiangiogenic assessment of PEG-haloperidol conjugates

PEG-haloperidol conjugates significantly inhibited VEGF-stimulated migration of HUVECs (see **Chapter 6** [16]). These findings were in agreement with other studies that have reported the ability of haloperidol to inhibit the migration of cells (cancer cells, endothelial cells and fibroblasts) of different origins [97–99]. However, cell migration is one of many steps that cells exhibit for angiogenesis including matrix degeneration, proliferation and morphogenesis [100]. Secretion of matrix metalloproteinases (MMPs) has been reported to regulate the process of matrix degeneration during the formation of new blood vessels [101–103]. Interestingly, haloperidol has been shown to abrogate the secretion of MMPs which might suggest potential applications of haloperidol to inhibit this step of angiogenesis [104]. Therefore, the potential modulatory effects of PEG-haloperidol on matrix degeneration could be assessed using Zymogen assays, further reviewed in [105]. In addition, to that, the ability of the conjugated haloperidol to alter the morphogenesis of the endothelial cells could be further explored *via* performing *in vitro* tube formation assay [106]. Moreover, the proposed antiangiogenic effects of conjugated haloperidol can be assessed *in vivo via* the chicken chorioallantoic membrane assay [107]. Given the potential targeted effects of the conjugated haloperidol, this would form a promising strategy for treating angiogenesis-related problems (such as cancers, diabetic retinopathy, etc.) when the use of other agents is limited [108,109].

Further evaluation of PEG-haloperidol conjugates in the field of cancer

Although neither conjugates of haloperidol (i.e. PEG-haloperidol 2000 and 6000 Da) showed antiproliferative effects on two breast cancer cell lines (see **Chapter 5** [16]), the application of PEG-haloperidol for treating tumours may still be worthy of future exploration. It has been reported that free haloperidol effectively inhibited the P-glycoprotein efflux system in multidrug-resistant tumour cells which potentiated the pharmacological effects of chemotherapeutic agents by reducing their efflux outside the tumour cells which enhanced their cytotoxic activity [110].

It has been reported that haloperidol treatment with a range of concentrations (1, 6, 10 and 30 μM) improved the chemosensitivity of vinblastine-resistant human leukaemia (K562/VBL) cells towards vinblastine [111]. This resulted in improved cytotoxicity of vinblastine by 20-fold (this IC_{50} of vinblastine was shifted from 4 μM to 0.19 μM) at 30 μM of haloperidol. The findings of this study were in agreement with another study in which the co-treatment of doxorubicin-resistant uterine sarcoma (MES-SA/Dx5) cells with doxorubicin (2, 4, 8 μM) and haloperidol (1, 10, 20 μM) resulted in a dose-dependent inhibition of growth of the cells when

compared to the control with maximum inhibitory effects of 72% observed at 20 µM of haloperidol compared to the control (32%). The authors indicated that this potential application of haloperidol is limited by its safety as an antipsychotic agent at this concentration [112].

The possible side effects of free haloperidol related to its unfavourable distribution through the body (to the CNS and the placenta) still forms an obstacle for its non-CNS applications. Therefore, the chemo-sensitising effects of applying haloperidol as PEG-conjugates (to compartmentalise its effects peripherally) with other anticancer agents could be further investigated. This application could be evaluated using several possible strategies; A) PEG-haloperidol could be co-administered with chemotherapeutic agents (small molecules); B) PEG-haloperidol could be co-administered with a PDC of the chemotherapeutic agent. This PDC would be designed to liberate the chemotherapeutic agents within the tumour tissues using a biologically labile linker to allow the selective release of the anticancer agent within the tumour tissues; C) a single PDC could be designed offering a conjugation of haloperidol and a chemotherapeutic agent to the same PEG (**Figure 8.1**), these strategies were reviewed in [113].

In the latter system, a multi-arm or branched PEG could be used as a polymeric carrier [114]. A non-prodrug system could be applied to conjugate haloperidol utilising a carbamate linker to avoid the release of haloperidol, therefore, conjugated haloperidol would not cross the biological barrier and its effects would be compartmentalised (see **Chapters 4** [13]). The anticancer drug would be conjugated *via* a biologically labile linker to work as a macromolecular pro-drug releasing the conjugated chemotherapeutic agent within the tumour tissues. This protocol would benefit from ensuring that both drugs could be delivered to the tumour tissues simultaneously as their distribution in the body would be the same.

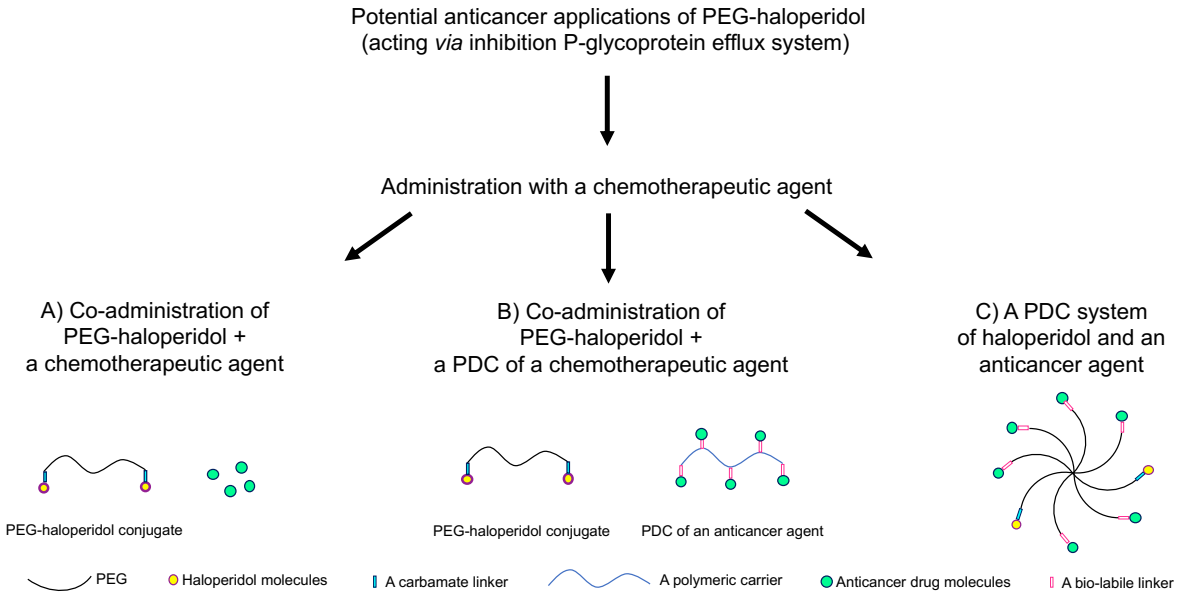


Figure 8.1. Potential strategies to use PEG-haloperidol with other chemotherapeutic agents adapted from [113].

Further evaluation of PEG-haloperidol conjugates in the field of infections

Further studies could be performed to identify other potential therapeutic applications of PEG-haloperidol. Haloperidol exhibited potential antifungal activity against *Candida albicans* with MIC (minimal concentration of drug inhibiting ≥ 50% of microbe's growth) of 0.38 μg/mL which was comparable to the standard antifungal drug fluconazole with MIC of 0.3 μg/mL [95]. This application could be significant when other antifungal agents are not efficient in treating *Candida albicans* related candidemia in cancer and immunocompromised patients [115]. In addition to that, haloperidol showed potential antibiotic-sensitising activity when administered with isoniazid and rifampicin against intracellular drug-resistant *M. tuberculosis,* as it could act as an efflux inhibitor. Haloperidol was able to reduce the resistance levels against isoniazid from a high to a low level in 4 out of 8 resistant strains and against rifampicin in 1 out of 5 resistant strains when tested at nontoxic concentrations (1.25 μg/mL) [116]. These results suggest exploring PEG-haloperidol, to avoid any side effects associated with the unfavourable distribution of the free haloperidol (e.g. into CNS), against microbial infections (a short-term application) either as an antifungal or in a combination treatment with other antimicrobial agents to overcome potential antimicrobial resistance.

Further evaluation of PEG-haloperidol conjugates in the field of inflammatory diseases

Another possible therapeutic application of haloperidol is its ability to alleviate inflammation. It has been reported that patients (with acute mania) treated with haloperidol (5 mg twice a day) showed marked improvement in their rheumatoid arthritis. Therefore, an *in vitro study* was carried out to evaluate these potential applications. The results showed that haloperidol was able to inhibit lipopolysaccharide-induced production of both tumour necrosis factor α and interleukin 1β cytokines in a dose-dependent manner (0.5-50 μg/mL) in whole blood culture [117]. It is important to highlight that, in this study, the patient was initially admitted with acute mania, therefore, the crossing of the BBB by haloperidol was required to treat their condition. Therefore, in other (non-manic cases), avoiding penetration of haloperidol across the BBB would be crucial to avoid any CNS side effects associated with the administration of free haloperidol. These data are in good agreement with another recent *in vitro* study by Yamamoto *et al* [118]. Haloperidol (10 μM) significantly suppressed CD80 expression induced by lipopolysaccharides in bone marrow-derived primary macrophages. Additionally, haloperidol also showed a dose-depended inhibitory effect of the activity of the nuclear factor NF-κB (which plays an important role in inflammation [119]) in RAW-Blue cells. The authors suggested that the anti-inflammatory effects of haloperidol are mediated by

its antagonistic effects on D_2 receptors. These findings suggest studying the potential effects of PEG-haloperidol to regulate inflammation in diseases such as rheumatoid arthritis.

In conclusion, the PEG-drug conjugates as non-prodrug systems represent a newly developed class of therapeutic agents, which they exert their pharmacological effects without releasing the conjugated drugs. The work presented in this thesis, using PEG-haloperidol as a model, has shown the feasibility of this strategy to localise the conjugated haloperidol at the intended site. In addition to that, potential non-CNS applications of PEG-haloperidol were explored and suggested. Further work is still required to study the potential applications of such non-prodrug PEGylated macromolecules to treat different diseases which could make their translation into the clinic more feasible.

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