Clinical development of a poly(2-oxazoline) (POZ) polymer therapeutic for the treatment of Parkinson's disease – Proof of concept of POZ as a versatile polymer platform for drug development in multiple therapeutic indications

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Abstract

The potential of poly(2-oxazoline) or POZ to be a versatile and broad based platform for drug delivery with wide utility in multiple therapeutic areas has long been recognized by experts in the field. This feature article provides a case study which describes the chemistry and preclinical studies underlying the Investigational New Drug Application for SER-214, a POZ conjugate of rotigotine, for the treatment of Parkinson's disease. We report the chemistry, preclinical safety and pharmacology, and the early clinical safety, tolerability and pharmacokinetic data from the Phase I study in patients. SER-214 utilizes a POZ polymer and proprietary custom linker technology to deliver a sustained dose of rotigotine over a period of seven days following a single subcutaneous administration – a result not observed by any other polymer approach that we are aware of. As such, this candidate drug has the promise to be a major advancement in the treatment of Parkinson's disease. Furthermore, this feature article also highlights the versatility of the POZ polymer platform (POZ™) to deliver cancer drugs by actively targeting cancer cells. Preclinical data reported in this feature showcase the polymer's attributes in facilitating targeted delivery with folic acid and antibody targeting agents (ADCs). The ability of POZ to reliably delivery large payloads of anticancer drugs is of particular importance when pursuing low-receptor-density targets on the cancer cell. The data presented in this feature, much of it for the first time, establish the broad utility of the POZ polymer platform in drug development. Together with its ease of manufacture, ability to attach drugs to the polymer, and ability to administer an appropriate dose to patients, the results underscore the need to further explore and...
1. Introduction

The first report of a ring-opening polymerization reaction of 2-substituted 2-oxazolines was made in 1966 by Kagiya et al. at the Faculty of Engineering at Kyoto University in Japan [1]. In this report, a simple outline of the synthesis was provided with limited characterization data. Fifty years later, the basic polymerization principles from this report have not changed, but significant improvements have been made in the areas of monomer synthesis, polymerization process controls, polymer purity and physicochemical characterization. Industrial grade polyoxazolines were first prepared in the 1980s for use as an indirect food additive product [2]. Those polymers had a broad polydispersity index (D) and a high percentage of low molecular weight impurities, thereby preventing their use in pharmaceutical and medical device applications. The development of poly(2-oxazoline)s as a drug delivery vehicle for pharmaceuticals is much more recent. A number of studies reported by numerous groups show that POZ may have applications in multiple approaches for development of drugs, and a recent series of review publications show a growing interest in this polymer [3–5].

Cyclo imino ethers of the five, six and seven membered systems have the ability to polymerize through cationic ring opening mechanisms. The 2-oxazolines or five membered cyclic imino ethers are the most popular type and have generated significant interest in polymer therapeutics. Cationic ring-opening polymerization is carried out by the addition of an electrophilic initiator to the monomer dissolved in an appropriate solvent, followed by heating. During the reaction, the imino ether is converted to the amide. Finally, the polymerization process is terminated by the introduction of a nucleophile.

![Fig. 1. Chemistry of poly(2-oxazoline) synthesis.](image)

The R group comes from the initiator and it can be H, CH₃, an inert alkyl group, or a functional alkyl group [6,7]. X can be tosylate or triflate. The side chain R’ group is important in drug applications. When R’ constitutes methyl, ethyl or propyl alkyl groups, the polymer chain has inert amide side chains and can be referred to as a ‘pseudo-peptide’ [8]. But when R’ has carboxylic acid, amine, thiol, maleimide, alkyne or alkene groups, the polymer chain has functional side chains or pendants that can be used for drug attachment. The preparation of high purity functional monomers is of great value when designing and building pendant POZ polymers for conjugation to drugs. The R’ terminal group is also important in polymer synthesis. This group can contain, for example, a hydroxyl, carboxylic acid or an amine end group. This functional terminal group serves two purposes: (a) in the purification of the polymer by ion-exchange chromatography, and (b) in the attachment of drugs or targeting molecules to the end of the polymer chain.

A review of the literature shows that POZ polymers have been directly attached to a variety of classes of drugs, and as a result the polymer influences the properties of the newly conjugated molecule. They have also been used to enable other types of drug delivery systems such as liposomes, micelles and dendrimers where they provide stealth-like properties. One of the first studies with drug conjugation was reported by Verlander et al. [9], who showed that an activated peptide (HPCP) can be used to terminate and directly conjugate to a ‘living’ polymer of poly(oxazoline). They showed that the polymer-peptide retained its antibody binding activity when tested by ELISA. However, the POZ polymer was never purified, isolated, characterized and functionalized prior to peptide conjugation, so details on the polymer integrity and purity cannot be fully evaluated. Miyamoto et al. [10] prepared poly(2-ethyl-2-oxazoline) (PEOZ) and poly(2-methyl-2-oxazoline) (PMOZ) polymers terminated with a large excess of glutaric acid. They then converted the acid end groups on PEOZ to N-hydroxysuccinimide esters. These polymers were then reacted with bovine liver catalase enzyme containing 108 lysine residues, all potential sites for attachment. The conjugates prepared had variable enzymatic activity when tested in aqueous phosphate buffer (pH 7.0) and this may be due to the labile ester linkages between the POZ polymer chains and the lysine residues. Many years later, Mero et al. [11] prepared stable PEOZ conjugates of trypsin and Ara-C and showed that enzymatic activity of the trypsin conjugate and HeLa cell cytotoxicity activity of the Ara-C conjugate were comparable to their respective polyethylene glycol (PEG) conjugates. A more detailed study was later carried out by Viegas et al. with non-labile PEOZ conjugates of uricase, catalase, RNase and insulin using well characterized poly(oxazoline) polymers [12].

The ‘stealth properties’ of POZ can be used to enable other biomaterials. Woodle et al. prepared lipid (distearoylphosphatidylethanolamine or DSPE) conjugates with ~5000 Da PEOZ and PMOZ polymers [13]. They were able to demonstrate that these POZ conjugated liposomal materials had long circulation times with low hepato-splenic uptake in rats following one intravenous injection. Zalipsky et al. repeated the experiment in mice and showed that PEG, PMOZ and PEOZ injected...
lipoosomes evade the reticuloendothelial system [14]. The mean residence times of the PMOZ-DSPE composition was similar to that of the PEG-DSPE material after a single intravenous bolus injection of 500 nmol of liposomes. Comparatively, the PEOZ-DSPE composition was more hydrophobic and hence cleared the blood at a faster rate and was detected in relatively higher amounts in the liver and spleen. Kierstead et al. prepared liposomes using DSPE conjugates of ~2000 Da PEG, PMOZ, PVP, PDMA or HPMA polymers [15]. They compared the pharmacokinetics of the liposome and their uptake in key organs following two intravenous doses of formulation to rats, administered seven days apart. Their results showed that PEG and PMOZ liposomes cleared the blood rapidly after the second dose and had higher uptake levels in the liver and spleen when compared to the other polymers. The authors suggest an IgM response as a reason for accelerated blood clearance. POZ has also been grafted on poly (l-lysine) dendrimers to reduce the cytotoxicity of the dendrimer and to improve the drug loading of anticancer agents [16]. A number of micellar structures have been made with block co-polymers of POZ. A triblock polymer of PMOZ as the hydrophilic block and poly(2-buty1-2-oxazoline), poly(2-pentyl-2-oxazoline), poly(2-hexyl-2-oxazoline) or poly(2-ony1yl-2-oxazoline) as the hydrophobic blocks were prepared to create micelles with critical micellar concentrations (cmc) ranging from 1 to 10 μM. These 50 nm micelles are capable of loading up to 45% by weight of the anticancer drug paclitaxel [17].

The application of POZ polymers in the development of POZ-therapeutics has been ongoing in our laboratories at Serina Therapeutics, Inc. for almost a decade. SER-214, a once-weekly injectable that can be delivered in a standard insulin syringe, is a POEZ conjugate of the dopamine agonist rotigotine [18]. This appears to be the first POZ-therapeutic to enter human clinical studies, and is currently in clinical trials to treat Parkinson’s disease. Details on the chemistry, preclinical efficacy and summary of studies that supported the Investigational New Drug (IND) application for SER-214 are provided in subsequent sections.

2. Materials and methods

2.1. Commercial solvents and reagents

The methyl and ethyl oxazoline monomers are available in high purity from ACROS Organics, Geel, Belgium and Polymer Chemistry Innovation, Tuscon, Arizona. The functional monomers were prepared at Serina Therapeutics. Sigma-Aldrich in St. Louis, MO provided all the triflate and tosylate initiators and the amine and carboxylic acid terminating agents. These reagents were ACS grade or better. All the anhydrous solvents used in the synthesis and extraction of the polymer and polymer conjugates were acquired from EMD Chemicals.

2.2. Polymer synthesis and characterization

Anhydrous equipment, monomers, and solvents are required for the preparation of POZ. Even small amounts of water can prematurely terminate the propagation of the polymer chain. The monomers and organic solvent can be dried by refluxing over calcium hydride and then distilling and collecting in a dry flask containing molecular sieves. The propagation phase is usually conducted at temperatures between 80 °C and 110 °C. Propagation times can range from about 1 h to approximately 3 days depending on the size of POZ being synthesized. Polymerization can also be carried out with microwave energy to reduce the propagation times from days to hours [19,20]. In this case the monomer, solvent and initiator are premixed in a sealed vial before placement in a microwave chamber. Either process must be controlled, as uncontrolled exothermic reactions may occur, leading to highly polydisperse material with significant amounts of side chain by-products. The POZ polymer is finally isolated by extraction and precipitation in non-polar solvents such as ether or hexane.

2.3. Immunogenicity

The fifty-day immunogenicity study protocol was approved using the Institutional Animal Care and Use Committee (IACUC) at Cocalico Biologicals, Inc., Stevens, PA. Male New Zealand white rabbits (about 100 days old) were used in the study and were divided into two groups. On day 0, ten to fifteen milliliters of whole blood was drawn from each animal and centrifuged to collect a baseline serum sample. Each male adult rabbit then received their primary immunization dose of 2.67 mg/kg PEOZ as a subcutaneous injection. Group 1 (SETI 1–3) received booster doses of 2.67 mg/kg PEOZ on days 14, 28 and 42. Group 2 (SETI 4–6) received booster doses of 2.67 mg/kg PEOZ and the Incomplete Freund’s adjuvant (0.3 mL) on the same days. Whole blood (20 mL) was collected from each animal on days 35 and 50 and centrifuged to collect serum. The PEOZ polymer formulation was prepared in 5% dextrose solution and administered in a volume of 0.2 mL for both the primary and the booster injections. The collected serum was stored frozen until tested.

The ELISA method was co-developed by Serina Therapeutics, Inc and iXpressGenes, Inc. (see Section 6 below). Mouse anti-nitrotyrosine antibody standards (Invitrogen Cat# 32-1900, Lot# 1462447A) were diluted to 1–10 μg/mL in blocking solution. Rabbit serum from both groups of animals was diluted 1:20, 1:50, 1:100 or 1:1000 in blocking solution; the 1:100 dilution gave the most consistent results in the scinting reactions. Blocking Solution was 1% BSA commercially available as Thermo Blocker BSA 10% (Thermo Scientific Cat# 37525) diluted 10× with PBS (150 mM NaCl, 10 mM sodium phosphate pH 7.4, 0.01% Tween). Goat anti-Mouse IgG:HRP (Life Technologies Cat# A24524) was diluted to 0.4 μg/mL in blocking
solution at the time of assay. Goat anti-Rabbit IgG:HRP (Life Technologies Cat# A24531) was diluted to 0.4 μg/mL in blocking solution. TMB solution - 3,3′,5,5′-tetramethylbenzidine (Life Technologies Cat#00-2023) was prepared freshly at the time of each assay. Stop Solution (Life Technologies Cat# SS04) was added and allowed to incubate for 30 min. Streptavidin-coated 96-well plates (Thermo Scientific Cat# 15119) were used in all assays. The reaction was read by a plate reader: SpectraMax Plus 384. All assays were performed in triplicate.

2.4. Targeted therapies for cancer - POZ-polymer antibody-drug conjugates (ADCs) and small molecule targeting with folate

PEOZ-polymer ADC conjugates were prepared by loading the anti-mitotic agent MMAE to the pendants of the POZ polymer followed by its conjugation to a humanized mouse anti-human CD79b monoclonal antibody. The monomethyl auristatin E (MMAE) molecule was attached to a valine-citrulline (val-cit) dipeptide self-emolating linker and a short PEG–azide chain linker (prepared for Serina by Concortis Biosystems, San Diego, CA). This azido-PEG4-vc-PAB-MMAE molecule was next ‘clicked’ to a PEOZ 20K 10 pendant acetylene and ethylene diamine–Boc terminated polymer using the conditions described for SER-214 in Section 8.6 below. Boc deprotection was performed with trifluoroacetic acid treatment, followed by addition of succinimidyl iodoacetate and triethanolamine in a dry chloroform solvent. Two PEOZ (vc-PAB-MMAE)-lidoacetamide conjugates with different drug loading were prepared, one with an average of one and the other with an average of five MMAE molecules per polymer backbone. The PEOZ-MMAE or just MMAE was attached to a single selenocysteine site on the antibody chain to create a drug-antibody ratio (DAR) of 1 or 5. The CD79b scFv-Fc-Sec/POZ-MMAE conjugates were purified with Protein A (GE Healthcare) chromatography. The conjugates were resuspended at 1 mg/mL in PBS and stored at 4 °C for short term use and at −80 °C in aliquots for long term use.

Human B-cell non-Hodgkin lymphoma cell line Ramos was obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (all from Life Technologies) at 37 °C in an atmosphere of 5% CO2 and 100% humidity. Cells were plated in 96-well tissue culture plates at 2.5 × 104 cells per well. Serial dilutions of the conjugates and controls were added to the wells at final concentrations ranging from 0 to 300 nM. After incubation for 96 h, the cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following the manufacturer’s instructions and calculated as a percentage of untreated cells (≈100%). The results are shown in Section 7.1 below.

Folic acid-PEOZ-irinotecan compounds were prepared to evaluate small molecule targeted POZ-toxins for cancer indications. Folic acid NHS ester was prepared by treating folic acid with N-hydroxysuccinimide, triethylamine and dicyclohexylcarbodiimide (DCC) in dry DMSO. The solution was filtered to remove the dicyclohexylurea (DCU) and precipitated and washed several times in diethyl ether. The product was collected and dried under vacuum. The PEOZ 20K 10 pendant acetylene with ethylene diamine terminated polymer described above was used as the starting material. It was reacted with the folic acid-NHS to prepare the folic acid-POZ compound. The topoisomerase II inhibitor, irinotecan, was used as the anti-cancer compound. A proprietary azido-alkyl-carboxylic acid was first coupled to the 4-hydroxy group on the irinotecan molecule. This compound was next clicked to the folic acid-PEOZ 10 pendant acetylene molecule using the method described for SER-214 in Section 8.6. The resulting compound was purified, extracted, precipitated in diethyl ether, collected and dried under vacuum.

Female athymic (Ncr:Nu) mice were implanted subcutaneously with KB cervical carcinoma cancer cell tumor fragments, and the tumors were allowed to reach a weight range of 160–190 mg. The animals were divided into three groups of 10 mice each. In the first cycle of dosing (3 doses every fourth day), the control group received normal saline. The irinotecan group received two cycles of intravenous irinotecan in normal saline at a dose of 60 mg/kg; the second cycle of dosing was performed on day 28. The third group of mice received an initial cycle of intravenous irinotecan at 60 mg/kg, and on day 28 received a single cycle of folic acid-PEOZ-irinotecan solution in 5% dextrose injection at a dose of 60 mg/kg (based on irinotecan equivalents). The animals were weighed and the tumors measured twice weekly after administration of the first drug injection. All doses of folate-targeted POZ-irinotecan and irinotecan were well tolerated. The maximum loss in body weight for both oncolytic groups was 5%. The results are shown in Section 7.2 below.

2.5. Animal models of Parkinson’s disease

A number of rat and monkey models for Parkinson’s disease are available. The methods for assessment of behavior in rats that underwent hemi-lateral injection of 6-hydroxydopamine (6-OHDA) have been previously published [18]. Animals were placed in a harness device that measured the number of contraversive turns (contraversive to the side of the 6-OHDA lesion) every ten minutes over a 6-h period on days 1, 5 and 9. The rotational behavior in the rats following a single injection of vehicle, rotigotine, SER-212 (another POZ-rotigotine conjugate that releases rotigotine with a half-life of 2–3 h) or SER-214 were determined over seven days. A summary of the results are in Section 8.8 below.

In the second study, the sustained drug delivery and anti-Parkinsonian benefit of SER-214 was investigated in the MPTP-monkey model. In one group of animals, SER-214 was dosed subcutaneously once a week for several weeks at a dose of 1 mg/kg (based on rotigotine equivalents). In a second group, animals received oral doses of L-Dopa at 15 mg/kg b.i.d. In the last group, animals were dosed subcutaneously once a week with placebo polymer. Videos of animal mobility were recorded for six hours immediately following dosing and reviewed in a blinded fashion by an independent neurologist who is an expert in movement disorders. The results are discussed in Section 8.8 below.
2.6. Quantitative whole body autoradiography

Two studies were performed in rats with radioactively labeled SER-214 to determine the distribution of this molecule by Quantitative Whole Body Autoradiography (QWBA). The radiolabeled $^{[14C]}$ SER-214 compound was prepared by using the same procedure described for SER-214 in Section 8.6 below. In this case, a 3-bromopropionic acid (2-, 3-$^{14}$C) linker (ViTrax Co., Placentia, CA) was first used to couple to rotigotine and this conjugate was then clicked to the POZ 10 pendant acetylene molecule, purified, extracted, precipitated and washed with diethyl ether and dried under vacuum. The compound was $>99\%$ radiochemically pure and had a specific activity of about 13 Ci/mg.

The initial QWBA study was a dose-ranging study to evaluate the target organ distribution of SER-214 following a single subcutaneous administration. In this study, a relatively high dose of compound was given to evaluate microautoradiography (MARG) analysis. The second study employed a human equivalent dose (HED) and injection volume (0.2 mL) to determine how SER-214 would distribute when dosed similarly to humans. The second study utilized 5 male Sprague-Dawley rats, which were approximately 8 weeks of age, and weighed 262.4–278.2 g at the time of dosing. Rats were administered a single SC injection into the left hind leg in the thigh region of $^{[14C]}$ SER-214 at a target dose of 3.85 mg/40 $\mu$Ci/kg, 0.175 mL/kg (0.50 mg/kg rotigotine equivalents) in 5% dextrose for injection (D5 W). One rat was euthanized at 2 h, 24 h, 72 h, 168 h, and 336 h post-dose. They were deeply anesthetized via isoflurane anesthesia, a blood sample was collected by cardiac puncture (approximately 3 mL) into tubes containing K$_2$EDTA as the anticoagulant, and each rat was euthanized by being frozen in a hexane/solid carbon dioxide bath for at least 15 min at each scheduled time point. Each frozen carcass was embedded in an aqueous suspension of approximately 2% (w/v) carboxymethyl cellulose and frozen into a block. The blocks were stored at approximately -20°C prior to sectioning. Each block was mounted on the object stage of a cryomicrotome and a number of whole-body sections (approximately 40 $\mu$m thick) were taken in the sagittal plane at various levels of interest using a whole-body cryomicrotome set at -20°C. All of the major tissues, organs, and biological fluids were represented. The sections were collected on adhesive tape and dehydrated in the cryomicrotome for at least 48 h prior to removal for mounting and exposure. A set of whole-body sections for each rat was mounted on a cardboard backing, covered with a thin plastic wrap, and exposed along with calibration standards to a $^{14}$C-sensitive phosphor imaging plate (Fuji Biomedical, Stamford, CT). The imaging plate and sections were placed in light-tight exposure cassettes, in a copper-lined lead safe, for a 4-day exposure at room temperature. The imaging plate was scanned using the Typhoon 9410 image acquisition system (GE/Molecular Dynamics, Sunnyvale, CA, USA) and the resultant image quantified by image densitometry using MCID image analysis software (v. 6.0 & 7.0, Interfocus Imaging Ltd, Linton, Cambridge, UK) and a standard curve constructed from the integrated response and the nominal concentrations of the $^{14}$C calibration standards. The concentrations of radioactivity in each of the 51 target tissues were then expressed as the $\mu$g equivalents of $^{[14C]}$ SER-214 per gram of tissue sample ($\mu$g equiv/g).

3. Chemistry and synthesis

A number of polymerization techniques have been reported in the literature for the synthesis of POZ (unless otherwise stated in subsequent sections, the abbreviation POZ in this manuscript refers to poly(2-ethyl-2-oxazoline). Some papers focus on the types of monomers (inert or functional), solvents, initiators and terminating agents that should be used, while others stress the need for a good heat transfer process to control the polymerization rate. The two oxazine monomers frequently used are the 2-methyl and 2-ethyl types, but other oxazine monomers with amine [21], aldehyde [22], alkene [23] and alkyne [24] side chains have been successfully prepared. Various organic solvents such as acetonitrile, chloroform, chlorobenzene and dimethylacetamide have been successfully used to prepare POZ. The choice of these solvents depends on their boiling points and their heat transfer capacity. A stoichiometric amount of initiator must be used to initiate the polymerization reaction. In general, the molar ratio of monomer (M$_0$) to Initiator (I$_0$) defines the molecular weight of the polymer being synthesized. When a low ratio of initiator to monomer is used, the molecular weight of the POZ polymer produced is high, and vice versa. Examples of initiators used in POZ synthesis are alkyl tosylates [25], alkyl triflates, triflic acid [26], alkyl sulfonates [27], pluritriflates [28], benzyl bromide and methyl iodide [29]. Finally, termination of the reaction occurs by the introduction of a nucleophile such as OH or R$_2$NH, R$^-$COO$^-$ or R$^-$S$^-$ to the living cation mixture. When termination is conducted with an aqueous solution of sodium carbonate or sodium hydroxide, an OH terminal group is formed [30]. Termination with a secondary amine such as morpholine or piperidine can give a terminal tertiary amine or termination with ammonia gas gives a primary NH$_2$ group [31]. Low molecular weight di-carboxylic acids [32] and mercapto-acids [33] can also be used to give a terminal COOH group. When terminal —NH$_2$ and —COOH groups are present, POZ can be purified by ion-exchange chromatography to produce a pure product with a narrow polydispersity. This step is especially important for high molecular weight polymers. A limited number of studies discuss in length the need to purify and isolate the POZ polymer.

The important steps required for POZ polymer synthesis are summarized in Table 1 below.

The synthetic processes described above appear to be straightforward, but in reality a number of process and scale-up difficulties are possible, especially for large scale or high molecular weight polymers. The various side reactions that occur lead to impurities that are difficult to remove, thereby creating products that are yellow in colour and with D of $>1.3$ that...
would be problematic for drug delivery and reproducible synthesis. In addition, the termination with the weak nucleophile water does not always give the desired product of 5-position attack (the “thermodynamic” product), but rather gives an attack in the 2-position (the “kinetic” product). This kinetic product is not stable and can rearrange to give an ester product or undergo reversal to the cation [34]. Most polymerization processes will yield polymers with high-MW and low–MW polymer species which can be detected by gel permeation chromatography. It is generally stated in the literature that this broadening of the MW distribution is due to chain transfer process through an elimination-dimerization mechanism, although structural details have not been described [35]. Park and Kataoka reported this observation as coupling and repolymerization [36]. Wyffels et al. have proposed a two-step polymerization process. The first sacrificial polymerization step generates high molecular weights impurities. When this reaction mixture is distilled, the distillate with unreacted monomers and solvent can be collected and re-polymerized in a second step to give polymers with improved D values [37].

4. Characterization

The architecture of POZ polymers for the target product profile of the intended drug defines the level of characterization required by the FDA or other regulatory agency. If POZ is attached to a dendrimer or a lipid, it can be considered as a raw material. But if it is attached directly to a pharmacologically active molecule, it is a key starting material which requires proper characterization using qualified methodologies. Purified POZ polymers can be characterized by a number of methods to assess purity, molecular weight and functionality. The molecular weight of the polymer is determined by matrix assisted laser desorption/ionization (MALDI) and gel filtration chromatography (GFC). Ion-exchange (IEX) chromatography is used to determine the % of acid or amine terminated polymer. Proton nuclear magnetic resonance (NMR) experiments are useful as they can show the groups associated with the attachment of pendent chains and terminal end groups.

4.1. Matrix Assisted Laser Desorption Ionization (MALDI)

Mass spectrometry measurements of molecular weight distribution are performed on a MALDI time-of-flight instrument. A stock solution of the polymer in 0.1% TFA in water or acetonitrile is made and spotted on a MALDI target along with matrix (a-cyano-4-hydroxycinnamic acid) and salt (sodium iodide) solutions. Fig. 2 shows a PEOZ 10K acid terminated polymer with a D of less than 1.05. Each repeating mass unit is of 99 Da.

4.2. Proton nuclear magnetic resonance (1H NMR) spectroscopy

1H NMR spectroscopy is a useful tool in measuring the pendent and terminal end groups on the polymer chain i.e. R and R’ (as shown in Fig. 1). The polymer sample is dissolved in deuterated chloroform or deuterated dimethyl sulfoxide and transferred to NMR tubes. The sample is typically scanned a minimum of 128 times with a relaxation time of 1 s and a pulse angle of 45°. Fig. 3 is an example of the 1H NMR spectrum of a linear PEOZ polymer with ten pendents showing the peaks associated with the ethyl and the pentynyl side chains.

Fig. 2. MALDI TOF mass spectrum of a PEOZ 10K linear polymer. The calculated average mass unit (ΔM) was 98.1 Da which is in good agreement with the monomer unit of 99.13 Da. The Na⁺ adduct appears as a shoulder on each peak and calculates as a mass unit of +23.

Fig. 3. ¹H NMR spectrum of a PEOZ polymer with ten pentynyl pendants (CDCl₃ as solvent).
4.3. Gel filtration chromatography (GFC)

The molecular weight and polydispersity of a PEOZ polymer is determined by size exclusion chromatography. Since PEOZ polymers have a UV absorbance with a $\lambda_{\text{max}}$ of around 228 nm, an HPLC instrument with an ultraviolet (UV) detector and gel filtration chromatography (GFC) software is suitable for this method. Highly purified PEOZ standards with molecular weights ranging from 1 kDa to 40 kDa are typically employed, and the molecular weight and polydispersity of the sample is derived from the standard curve. The polydispersity index (Ð), an indicator of the degree of molecular weight uniformity in a polymer sample, can also be determined. Fig. 4 shows two chromatograms. The first panel is an example of a chromatogram showing a crude PEOZ polymer sample with high and low molecular weight impurities. Ion-exchange chromatography removes the impurities resulting in a low Ð (<1.10) sharp peak as seen on the second chromatogram.

5. POZ polymer attributes desirable for drug delivery

Poly(2-oxazoline) polymers have a number of desirable properties that make them suitable for drug delivery. They are stable under ambient conditions and in the presence of light. These polymers may be stored as a lyophile in vials and kept at room temperature and in a dry atmosphere for at least three years without detectable changes. Higher temperatures and humidity are not recommended because the glass transition temperature of POZ has been reported to be between 35 and 45 °C; they adsorb water and turn from amorphous or crystalline free-flowing powders into hard crystalline adherent materials. PEOZ polymers are stable under mild acid and alkaline conditions. Under very harsh conditions the alkyl side chains can be removed by boiling the polymer for 5–10 h in strong acid such as 10% v/v hydrochloric acid. The resultant product is the charged polyethyleneimine (PEI) polymer, which is known to have serious adverse effects in vivo. PEOZ polymers are stable in vivo and do not undergo any biotransformation to PEI [38, Serina unpublished observations]. Doses of up to 2 g/kg have been safely dosed to rodents [12]. Another property of PEOZ and other polymers is lower critical solution temperature (LCST), represented by cloud-point temperature. It is the temperature at which phase separation starts and the polymer solution becomes turbid due to the formation of polymer-rich emulsion droplets. This behavior is temperature dependent and is attributed to the concentration of polymer in solution, the molecular weight of the polymer, and the hydrophilic-hydrophobic nature of the polymer backbone. All polymer formulations are prone to cloud point behavior and careful attention needs to be paid to formulations that turn cloudy at ambient and at body temperatures. PEOZ polymers can be synthesized so that their formulations have a high cloud point temperature. This can be accomplished by using a higher ratio of hydrophilic to hydrophobic oxazoline monomers in the copolymer chain, by creating a random distribution of hydrophobic oxazoline monomers as opposed to a block distribution in the copolymer chain, and by not overloading the amount of hydrophobic drug on the polymer chain [39,40].

Table 2 compares the physicochemical properties of PEOZ and some other polymers that have advanced into clinical development such as polyethylene glycol (PEG), polydextrans and poly N-(2-hydroxypropyl)-methacrylamide copolymers (pHPMA).
Properties of POZ, PEG, polydextran and poly-HPMA.

<table>
<thead>
<tr>
<th>Property</th>
<th>POZ</th>
<th>PEG</th>
<th>Polydextrans (Polyacetals)</th>
<th>Poly-HPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis</td>
<td>One “pot” synthesis with safe starting materials which are readily available Limited manufacturers of quality POZ (Serina, USA)</td>
<td>Multi-step synthesis with explosive starting materials Limited suppliers of quality PEG</td>
<td>Semisynthetic acyclic polyacetal prepared from dextran B-512 from bacterial cell walls of <em>Leuconostoc mesenteroides</em> strain</td>
<td>Living radical, atomic transfer radical and RAFT polymerization processes</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>Poly (2-methyl) and poly (2-ethyl) oxazolines are soluble in water and alcohol</td>
<td>PEG is soluble in water and alcohol, but high MW PEGs are sparingly soluble in alcohol</td>
<td>Soluble in water</td>
<td>Soluble in water</td>
</tr>
<tr>
<td>Stability</td>
<td>Stable at ~20 °C, refrigerator and at room temperature Does not form peroxides No antioxidants needed</td>
<td>Stable at ~20 °C Forms peroxides and BHT is added as an antioxidant</td>
<td>Not stable in water when the pH is less than 6.0</td>
<td>Stable in water</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Relatively low viscosity in aqueous solution and can be dosed subcutaneously</td>
<td>Relatively high viscosity in aqueous solution</td>
<td>Relatively low viscosity with low MW polymers</td>
<td>Relatively low viscosity</td>
</tr>
<tr>
<td>Drug attachment</td>
<td>High drug loading and active targeting when pendent POZ is used</td>
<td>Low drug loading</td>
<td>High drug loading and active targeting</td>
<td>High drug loading and active targeting</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Stable in vivo Excreted unchanged in urine with no accumulation Non-immunogenic in animal models No known anti-POZ antibodies in human population</td>
<td>Stable in vivo Excreted unchanged and can accumulate in some organs (kidney, liver, spleen, lymph nodes, others) Immunogenic in animal models Anti-PEG antibodies present in 25% of human population</td>
<td>Not stable in vivo Biodegradable and the metabolites are excreted in urine with no accumulation Known to cause hypersensitivity reactions No known anti-dextran antibodies in human population</td>
<td>Stable in vivo Excreted unchanged and can accumulate in some organs Immunogenicity reported with some co-polymers of HPMA No known anti-HPMA antibodies in human population</td>
</tr>
</tbody>
</table>

6. POZ polymers are non-immunogenic

The immunogenicity of polymers can lead to unpredictable safety and efficacy in clinical results, especially when the polymer is administered repeatedly. Of the polymers used in drug development, polyethylene glycol (PEG) is the most well characterized in terms of immunogenicity. Approximately 25% of the US population has pre-existing antibodies to PEG [41], and this is postulated to play a role in whether patients respond effectively to some PEGylated products. One such product, PEGloticase (Krystexxa®), is a PEGylated recombinant uricase that is infused every two weeks in patients (with refractory gout) is associated with acute anaphylaxis in approximately 7% of patients. These reactions are associated with high titer antibodies to the PEG component of the PEGylated protein, and >90% of patients develop antibodies to PEG during chronic administration of PEGloticase [42]. In approximately 5% of patients very high titers (greater than 1:2400) may prevent an adequate response in lowering serum uric acid levels.

We sought to determine if PEOZ is capable of generating antibodies in rabbits. A PEOZ pendent polymer 20 kDa (PEOZ 10p 20 kDa with ~10 pendent propionic acid groups) was administered subcutaneously to two groups of New Zealand White rabbits. All animals received 2.67 mg/kg every 2 weeks for a total of 4 doses on days 0, 14, 28, and 42. One group of animals (n = 3) also received subcutaneous doses of Incomplete Freund’s adjuvant as an immune booster along with the polymer. Blood was collected for serum from these animals on days 0, 35 and 50 and stored frozen until analysis.

In order to develop an ELISA method that would detect possible anti-PEOZ antibodies, a 96-well in vitro assay was developed using relevant controls. The PEOZ 20K 10 pendent acetylene with ethylene diamine terminated polymer described in Section 2.4 was used as the starting material to make the positive control. Azidopropionic acid was first attached to the acetylene pendents to create PEOZ 20K polymer with approximately nine pendent propionic acid groups (this would represent the polymer left behind if all of the rotigotine molecules were released from SER-214). We also ‘clicked’ one single biotin molecule on the remaining one pendent side chain of the polymer backbone. Finally, the N-hydroxy succinimide ester of 4-hydroxy-3-nitrophenylacetic acid (a nitrotyrosine analogue) was made and reacted with the terminal ethylene diamine end of the polymer chain. This bi-functional polymer served as the positive control for PEOZ 20K 10p propionic acid polymer where nitrotyrosine is the immunogenic moiety on the polymer chain and biotin is the anchor for its attachment to

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Fig. 5. Schematic representation of the assay system developed for the detection of mouse anti-nitrotyrosine antibody (positive control panel A), and whether rabbits make antibodies to POZ (panel B). The bifunctional polymer (panel A) contains biotin "clicked" to the polymer to promote binding to the streptavidin plates (green), and nitrotyrosine at the other end (red) for specific recognition by the mouse anti-nitrotyrosine antibody. The second polymer (panel B) does not contain nitrotyrosine but does contain biotin, thus it would bind to the streptavidin plates but would not be detected by mouse anti-nitrotyrosine antibodies. Both polymers would allow binding of rabbit antibodies to any component of POZ if they are present in the serum of rabbits immunized with POZ (± Incomplete Freund’s). The mouse antibody to nitrotyrosine is blue, and goat anti-mouse IgG:HRP is beige. The rabbit antibodies are illustrated as purple and dark green.
commercially available streptavidin plates (Thermo Scientific). There is a commercially available mouse anti-nitrotyrosine monoclonal antibody (Invitrogen) that binds specifically to the nitrotyrosine moiety and not to any other amino acid group. The structure of this polymer and a cartoon of the detection system is illustrated in Fig. 5A below. A second control polymer was synthesized that lacked the nitrotyrosine moiety, but retained the biotin moiety so it would still bind to the streptavidin plates as shown in Fig. 5B. If rabbits (or humans) develop antibodies to any component of the polymer, it should be detectable in this system. The two control polymers have several potential immunogenic moieties, including the polymer backbone with inert ethyloxazoline units, the pendant propionic acid groups, and the triazole ring and if any specific antibodies are present to them or any combination thereof, they would be detected in this assay. All of the other reagents employed in development of this assay were commercially available from Thermoscientific and Life Technologies.

Numerous ELISA experiments were performed to optimize the levels of detection of mouse anti-nitrotyrosine antibody levels in the presence of rabbit pre-immune serum. The mouse anti-nitrotyrosine antibody levels were optimally detected at levels of ~130 ng in dilutions of 1:100 rabbit serum. Once optimized, the assay was used to determine if immunized rabbits (±Incomplete Freund’s adjuvant) contained antibodies that would bind to the polymer and be detected by goat anti-rabbit IgG:HRP (horseradish peroxidase). The first experiment was to determine the limits of detection of the mouse anti-nitrotyrosine antibody in the presence of pre-immune serum. This is indicated in the green line in Fig. 6, panel A and panel B (± Incomplete Freund’s, respectively). The data show that there is nothing in rabbit pre-immune serum that interferes with the detection of mouse anti-nitrotyrosine antibody down to ~130 ng/mL. PEOZ alone as immunogen or PEOZ plus Incomplete Freund’s adjuvant did not interfere with the detection of mouse anti-nitrotyrosine antibody either, as the standard curves were virtually superimposable (Fig. 6, panel A and panel B – red and blue lines). When goat anti-rabbit

![Fig. 6. Assay for detection of antibodies in a rabbit from the group that received no Freund’s adjuvant (left panel) and from the group that received Freund’s adjuvants (right panel). Assay conditions were optimized for detection of mouse anti-nitrotyrosine monoclonal antibody in the absence of immunization (Day 0, green line). The same conditions were then employed to look for an increase in the absorbance when post-immune antisera were used (Day 35, blue line and Day 50, red line). An increase in absorbance prior to addition of mouse anti-nitrotyrosine antibody would indicate that rabbits were making an antibody that bound to the POZ attached to the plate; no increase in absorbance was seen – the lines are superimposable in the presence of goat anti-rabbit IgG. Identical results were observed when anti-sera from rabbits immunized with Incomplete Freund’s adjuvant were tested.](image6)

![Fig. 7. Assay for detection of antibodies in a rabbit from the group that received no Freund’s adjuvants (left panel) and from the group that received Freund’s adjuvants (right panel), when the polymer lacking nitrotyrosine was used in the ELISA detection assay. Assay conditions were identical to those used previously for detection of mouse anti-nitrotyrosine monoclonal antibody, except in this experiment we used the polymer that did not contain nitrotyrosine. The same conditions were then employed to look for an increase in the absorbance when post-immune or pre-immune antisera were used (Day 0 and Day 35, respectively). An increase in absorbance would indicate that rabbits were making an antibody that bound to the POZ attached to the plate. No increase in absorbance was seen with either mouse anti-nitrotyrosine antibody (negative control) in the presence of goat anti-mouse IgG or any of the goat anti-rabbit antiserum. The lines are horizontal and at baseline levels of the assay.](image7)
IgG:HRP was added alone to the assay (no added mouse anti-nitrotyrosine antibody) there was no absorbance increase (data not shown). When goat anti-rabbit IgG:HRP was added to the same assay well as the mouse anti-nitrotyrosine antibody, there was no shift in the absorbance for either of the immune sera and the detection of mouse anti-nitrotyrosine antibody was identical to adding it alone - demonstrating the absence of any detectable rabbit antibodies to PEOZ that would have been detected by the goat anti-rabbit IgG.

In order to verify these results were due entirely to the binding of mouse anti-nitrotyrosine antibody, and an absence of production of antibodies in rabbits immunized with PEOZ that would be detected by goat anti-rabbit IgG, the second control polymer (illustrated in Fig. 5B) was used and the assay repeated exactly as above. Rabbit serum (Day 0 pre-immune, and Day 35 post-immune, ± Incomplete Freund’s adjuvant) from animals SETI3 and SETI5 were tested. Fig. 7 shows no apparent increase in absorbance in representative rabbits from either group.

We conclude from these carefully controlled experiments (both a positive control, panel A, and negative control, panel B, Fig. 5), which would have detected antibody down to a level of ~130 ng in the presence of rabbit antisera (less than ~1 nM antibody), that rabbits do not make detectable antibodies to any component of the PEOZ polymer employed as an immunogen in this experiment.

**7. Applications of POZ in oncology**

**7.1. POZ-polymer antibody targeting of cancer cells**

One of the most promising areas of drug development for cancer is antibody-drug conjugates (ADCs). After nearly twenty years of effort, in particular the efforts of scientists at Seattle Genetics and Immunogen, the first approved ADCs are now available for patients with cancer. The field has exploded in the last five years, with many new products entering the clinic. At this time there are over 100 clinical trials worldwide evaluating novel antibodies and toxins.

The “first-generation” ADCs employed similar approaches. All of the approved ADCs (Kadcyla® and Adcetris®), and over 90% of those in early stages of clinical development, employ antibodies with a drug attached directly to the antibody to create a drug antibody ratio (DAR) of between 2 and 4. These approaches take advantage of reduced sulfhydryls in the hinge and Fc region (Adcetris®), or modified lysines (Kadcyla®), that do not destroy the antibody binding or enhance its clearance. Thus approximately 4 sites on the reduced antibody are available for conjugation of toxins. While this approach is suitable for development of ADCs that target high density antigens (both Kadcyla® and Adcetris® target cell surface receptors that exceed 300,000 per cell), this ADC approach may not be suitable for cancer cells that express much lower cell surface receptors.

Polymers of PEOZ are perfectly suited to accomplish high DAR antibody-drug conjugates. We chose the humanized mouse anti-human CD79b monoclonal antibody SN8v28 engineered with a C-terminal selenocysteine (Sec) and expressed as single-chain variable fragment (scFv)-Fc format [43]. The procedures for cloning, expression, purification, and conjugation of the scFv-Fc-Sec followed previously established protocols [44].

Fig. 8 shows the effect of concentration of the CD79b-PEOZ conjugates on the viability (%) of Ramos cells in vitro. The data shows that PEOZ val-cit-PAB conjugates with one MMAE molecule attached had an IC50 of about 20nM when compared to the conjugate with an average of five MMAE molecules which had an IC50 of about 2nM. The non-PEOZ conjugate of CD79b-MMAE conjugate had an IC50 of about 10nM. This data shows that when the number of MMAE molecules per POZ conjugate is increased, the IC50 concentration can be lowered by as much as a factor of 10, showing that higher drug-to-antibody ratios (DAR) can improve on the activity and potency of the drug conjugate. While this shift in the IC50 might seem modest, this might readily translate into a substantial widening of the therapeutic index - allowing one to give less POZ-polymer ADC with a much higher toxin load in order to avoid potential toxicity issues. In addition, there are several known properties...
of POZ that should improve the synthesis of ADCs and target engagement of cancers – (a) enhanced plasma pharmacokinetics and stability of the ADCs, and (b) increased tumoricidal activity through a tumor enhanced permeation and retention (EPR) effect, and (c) PEOZ polymers may mask the hydrophobicity of some toxins, thus preventing aggregation of the armed antibodies during scale-up and manufacturing.

7.2. PEOZ-polymer small molecule targeting of cancer cells

Folate receptor α (FR-α) is a membrane-bound protein with high affinity for binding and transporting physiologic levels of folic acid into cells. The overexpression of folate receptors on a number of types of cancer cells has led to considerable research on folate targeting of drugs [45]. These receptors (primarily the α isoform) are overexpressed on as many as 40% of human cancers and mediate internalization of their attached drugs by receptor-mediated endocytosis. Folate has been conjugated directly to drugs through linking groups [46], on to drug-loaded liposomes [47], and to polymers [48]. The conjugation has involved linkages to carboxylic acid groups on folic acid. Folic acid bears two such groups, labeled α and γ, and in our example we attach the POZ polymer to the γ group. Our proof of concept cytotoxic molecule is a topoisomerase-I inhibitor, irinotecan, and in our example we have attached between 8 and 10 molecules per polymer backbone employing ‘click’ chemistry.

Changes in KB tumor weight (relative to control) following administration of two courses of irinotecan, or a single course of Folate-PEOZ-irinotecan following an initial course of irinotecan, are shown in Fig. 9. KB carcinomas express high levels of folate receptor α and grow very aggressively in this model, reaching such a large volume by day 50 the animals must be humanely sacrificed. In the irinotecan-only cohort of mice, there is an initial suppression of tumor growth until approximately day 18, when there is an “escape” of the tumor and it enters an exponential growth phase again. We allowed the tumors to reach an ~10-fold increase in volume before initiating a second cycle of treatment. In the irinotecan group (red line), tumor growth suppression and growth delay was again observed for ~18 days followed by a similar aggressive growth curve at day 45. In the example where we dosed initially with irinotecan, followed by a single course of Folate-PEOZ-irinotecan, complete tumor regression was observed in 6 of the 10 animals by day 48 with minimal residual tumors in the remaining animals.

This study demonstrates that small-molecule targeting of PEOZ polymers to a high affinity receptor on the surface of solid cancers can have a dramatic affect on the growth of these tumor xenografts. While this study was limited to folate targeting, we believe that other small molecules might also be candidates for this approach.

8. Parkinson’s disease: rationale for development of SER-214

8.1. Parkinson’s disease: an overview

Approximately 1,000,000 people in the US and perhaps as many as 10 million people worldwide suffer from Parkinson’s disease (PD). The average age of onset is approximately 62 years. It is the second most common neurodegenerative disease after Alzheimer’s. There is no cure or therapy that slows disease progression. Parkinson’s disease is characterized clinically by both motor and non-motor symptoms. Motor symptoms include tremor, rigidity, slow movement (bradykinesia), and loss of the ability to initiate movement (akinesia) (collectively, the “off” state). Pathologically, the disease is characterized by degeneration of dopamine-producing nerve cells in the substantia nigra pars compacta region of the brain.

8.2. Parkinson's disease: a dopamine deficiency state

In the mid 1960s, it was discovered that Parkinson's disease was caused by a deficit of dopamine in the brain. Equally important was the discovery that levodopa (L-dopa), an amino acid precursor to dopamine, was able to replenish the depleted neural dopamine and greatly ameliorate the symptoms of Parkinson's disease. This L-dopa therapy (in combination with a dopamine decarboxylase inhibitor) revolutionized the clinical management of Parkinson's disease and was regarded as one of the most important therapeutic advances in neurology.

8.3. Limitations of L-dopa therapy

Despite the substantial benefits associated with its administration, chronic L-dopa therapy is associated with the development of adverse effects in more than 50% of treated patients. These include motor fluctuations, dyskinesias (involuntary muscle movements), and neuropsychiatric problems. Gradually, after several years of L-dopa therapy, the duration of therapeutic benefit ("on period") from L-dopa progressively shortens and the lack of therapeutic benefit ("off-period") is prolonged. The incidence and severity of the dyskinesias increase with the duration of L-dopa therapy and are usually worsened when high doses of L-dopa are administered [49]. Current evidence suggests that levodopa-induced motor complications result from the non-physiologic administration of levodopa resulting in pathological fluctuations in brain dopamine levels leading to molecular changes, neurophysiologic alterations, and ultimately motor complications [50] that may contribute to the pathogenesis of the disease [51].

Dopaminergic neurons in the basal ganglia fire in a continuous manner in normal individuals, thus striatal dopamine concentrations are maintained at a constant level. A therapeutic strategy that would deliver continuous dopaminergic stimulation (CDS) would represent a significant advance in the treatment of PD. It follows that a preparation of L-dopa (or other dopaminergic agent) offering continuous stimulation of the dopamine receptors might be useful in minimizing the occurrence of the motor complications associated with L-dopa therapy. This has proven difficult to achieve and physicians tend to utilize low doses of levodopa combined with additional medications to provide anti-Parkinsonian benefits with a reduced risk of motor complications [52], and as a consequence patients are often under-treated.

8.4. Dopamine agonists

Results from a five-year multinational trial showed that early PD patients could be managed successfully with an agonist alone, or an agonist/low dose L-dopa adjunct added later. When compared to L-dopa monotherapy, subjects treated with an agonist from the onset had a lower risk of developing dyskinesias [53]. The anti-Parkinsonian efficacy of dopamine agonist (DA) therapy is not dependent on the integrity of the degenerating dopaminergic terminals and thus, full activity of the drug may be maintained at a more advanced stage of disease. This approach to dopamine replacement appears to diminish the risk of basal ganglionic changes that contribute to the appearance of motor response complications [54,55]. Moreover, agonists selectively interact with certain dopamine receptor subtypes and thus possibly achieve a higher therapeutic index than L-dopa, which when decarboxylated to dopamine interacts with all dopamine receptor subtypes [56]. Finally, all dopamine agonists presumably avoid the risk of accelerating free radical induced neuronal damage due to excessive dopamine production [57].

More than two dozen dopaminergic agonists with various dopamine receptor subtype binding profiles [49] have undergone clinical trials [51]. Dopaminergic agonists are often used in advanced PD for lessening motor fluctuations such as wearing-off, dystonic spasms, start hesitancy, and unpredictable "off" states. While most of the marketed dopamine agonists have similar clinical efficacy and adverse effect profiles, several have ergot or ergoline chemical structures which have been associated with systemic fibrotic disorders and cardiac valve disease that develop idiosyncratically [53].

Rotigotine, whose structure is (–)[5,6,7,8-tetrahydro-6-[propyl-2-(2-thienyl-ethyl)amino-1-naphthalenol HCl], is a DA with greatest potency (pKi < 10^-9) at the D3 dopamine receptor [53]. It is also a full DA at D3, D2, and D1 receptors, and a partial D4 DA. Rotigotine effectively reverses motor impairments in animal models of Parkinsonism. Previously designated as N-0923 [50], rotigotine has been approved for the treatment of PD and for restless legs syndrome. Rapid clearance with transdermal delivery has made rotigotine ideal for achieving desired plasma concentrations over a 24 h period [53], but side effects including skin irritation, acute hypersensitivity due to the meta-bisulfites in the excipients, and patient compliance limit its usefulness. In previous clinical studies, rotigotine was well-tolerated and yielded dose related anti-Parkinsonian effects as monotherapy [54–56] and combined with levodopa [58].

Studies in MPTP monkeys have shown that despite the relatively long half-life of rotigotine, intermittent doses of the drug may be associated with an increased level of dyskinesia in comparison to continuous infusion of the drug [59]. We sought to develop a POZ-rotigotine conjugate, designated SER-214, that would provide continuous delivery of rotigotine over one week and thereby provide sustained anti-Parkinsonian benefits. In addition, a once a week therapy should be associated with reduced liability for dyskinesia, would necessitate lower doses of levodopa, provide enhanced patient compliance, and afford more convenience for PD patients. This therapy would represent a significant advance in the treatment of PD.
8.5. Rationale for early treatment with dopamine agonists

In pre-clinical primate studies, L-dopa (short-acting, pulsatile) and dopaminergic agonists (long-acting; non pulsatile) provide comparable clinical benefit, but with agonists inducing significantly less dyskinesia. Based on pre-clinical and clinical study results, treatment of Parkinson’s patients with long-acting levodopa or dopamine agonists should provide symptomatic benefit, and significantly delay the onset of motor complications [60]. This can be accomplished with duodopa or continuous subcutaneous apomorphine but these treatments are associated with significant side effects. To date, no practical method of providing continuous drug delivery using a dopaminergic agent for therapy of patients with early PD to prevent the development of motor complications has been approved.

8.6. Chemical synthesis of SER-214

SER-214 is a PEOZ conjugate of rotigotine that is being developed by Serina Therapeutics for symptomatic treatment of Parkinson’s disease and restless leg syndrome (RLS). It is a once-per-week subcutaneous injection that provides continuous drug delivery of rotigotine. In the synthesis of SER-214, rotigotine (active S enantiomer) is first coupled to an azido alkyl carboxylic acid linker through a covalent ester bond. The azide on the coupled drug is next attached to the acetylene pendant side chains of the PEOZ polymer backbone using a copper(I) catalyzed click reaction [61] that yields a chemically stable 1,4-disubstituted 1,2,3 triazole ring structure, as shown in Fig. 10. A 20 kDa PEOZ polymer with approximately ten pendent 2-pentynyl-oxazoline groups is used in the synthesis, resulting in quantitative conversion of the pendent groups to a PEOZ-conjugate that is 12% loaded with rotigotine by weight.

The release of the attached rotigotine molecule occurs by hydrolysis of the ester linkage. We believe this is mediated by pH and soluble carboxylesterases that are part of the plasma component of blood, or by other proteins with arylesterase activity. During the preclinical characterization of this drug candidate, SER-214 was evaluated in multiple animals in vivo to evaluate the pharmacokinetics before advancing to efficacy using established animal models.

8.7. Pharmacokinetics of SER-214

The plasma levels of rotigotine and polymer conjugate were measured in rodent and non-rodent animals following single and multiple subcutaneous injections of SER-214. In general, we observed a prolonged PK profile following subcutaneous

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Fig. 11. Plasma levels of released rotigotine (●) and total releasable rotigotine (□) in MPTP-lesioned monkeys (n = 3, ±SD) following four weekly subcutaneous injections of SER-214 at a dose of 1 mg/kg (rotigotine equivalents).

Fig. 12. Time course (on Day 5) of rotational behavior in 6-OHDA lesioned rats following single injections of vehicle, rotigotine, SER-212 or SER-214 (n = 8, ±SEM).

Fig. 13. Good “On-Time” in monkeys who receive either vehicle, L-DOPA twice daily or SER-214 as a single weekly injection.
administration in rat, dog and monkey that persisted for ~1 week. In one study, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-treated cynomolgus macaques (n = 3) received weekly doses of SER-214 at doses of 1 mg/kg (based on rotigotine equivalents). Plasma levels of released rotigotine and total rotigotine (SER-214 pro-drug levels) were measured. Fig. 11 shows the plasma levels of released drug mirrors the total releasable rotigotine levels over the four week period. Plasma levels of both released and total drug rise steadily within the first 3–4 days, and reach an apparent steady-state by Day 7. Upon repeat dosing there is a continued steady-state release of rotigotine from the polymer conjugate that achieves levels of plasma rotigotine of 1–2 ng/mL. Upon cessation of dosing, both the released and total rotigotine

Fig. 14. Development of dyskinesia in MPTP-treated monkeys that received a weekly injection of vehicle, oral L-DOPA (15 mg/kg b.i.d.) or SER-214 (1 mg/kg rotigotine equivalents).

Fig. 15. Quantitative whole body autoradiography of radiolabeled SER-214 at 24 h.
concentrations fall below the levels of detection after approximately one week of the wash-out period. This study demonstrates the potential of SER-214 for continuous delivery of rotigotine.

8.8. Animal models of Parkinson’s disease

A number of rat and monkey models for Parkinson’s disease are available. The rat 6-hydroxydopamine (6-OHDA) model was first used to study the effect of a single injection of SER-214. Animals were placed in a harness device that measured the number of contraversive turns (contraversive to the side of the 6-OHDA lesion) every ten minutes over a 6-hour period on days 1, 5 and 9. The rotational behavior in the rats following a single injection of vehicle, rotigotine, SER-212 (another POZ-rotigotine conjugate that releases rotigotine with a half-life of 2–3 h) or SER-214 were determined over seven days. Fig. 12 shows rotational measurements at Day 5 of the study. SER-214 demonstrates significant prolonged activity at the high dose of 6.4 mg/kg. The rat forelimb symmetry test was also performed with the same animals. Similar observations were also noted on Day 5 of the study [18].

In the second study, the sustained drug delivery and anti-Parkinsonian benefit of SER-214 was investigated in the MPTP-monkey model. MPTP is itself not neurotoxic, but it readily passes the blood brain barrier where monoamine oxidase-B in glial cells convert it to MPP⁺ - a potent neurotoxin that has selectivity for the dopaminergic neurons in the substantia nigra. Over a period of several months, monkeys dosed with MPTP daily will develop severe Parkinsonism that is dependent on L-dopa for daily activity. The MPTP model of Parkinson’s disease has been used in the approval of every drug currently used clinically to treat Parkinson’s disease. We sought to determine if SER-214 would show both safety and efficacy in this model. SER-214 was dosed subcutaneously once a week for several weeks at a dose of 1 mg/kg (based on rotigotine equivalents) to one group of MPTP animals. Oral doses of L-dopa at 15 mg/kg b.i.d. were administered to the second group of animals. The last group of animals received placebo polymer dosed subcutaneously once a week. The animal mobility data was recorded and scored by a blinded neurologist from the videos. Fig. 13 shows that SER-214 (but not placebo polymer) resulted in good “on-time” (accumulated time with a bradykinesia score of zero, without dyskinesia) that was comparable to an oral dose of L-dopa. Importantly, SER-214 did not induce dyskinesia, whereas three of the six monkeys in the L-dopa cohort had

![Fig. 16. Quantitative whole body autoradiography of radiolabelled SER-214 at 72 h.](http://dx.doi.org/10.1016/j.eurpolymj.2016.09.052)
developed dyskinesia by week two (Fig. 14). By week four of dosing, all of the monkeys in the L-dopa cohort had developed dyskinesia; none of the monkeys in the SER-214 cohort developed any of these involuntary movements at any point in the study.

In summary, four weekly subcutaneous injections of 1.0 mg/kg SER-214 (rotigotine equivalents) produced dyskinesia-free, anti-Parkinsonian effects in otherwise treatment-naïve MPTP-lesioned monkeys. In contrast, robust anti-Parkinsonian benefits of L-dopa (15 mg/kg) were observed throughout the 4 week period, but were compromised by dyskinesia. While the L-dopa anti-Parkinsonian locomotor effects were maintained throughout a 13 week treatment, the locomotor activity mediated by SER-214 seemed to occur only within the first 4 weeks of treatment. This was perceived as a "wearing off" or "drowsiness" effect in the animals even though they remained responsive to external stimuli and had no signs of dyskinesia. This observation was also observed in another non-human primate model where rotigotine was delivered continuously via a subcutaneous pump in MPTP-treated marmosets (Callithrix jacchus) [62].

The PK study indicated that a dose of 1.0 mg/kg produced steady-state levels of plasma rotigotine in the pharmacologically active range of 1–2 ng/mL. The estimated SER-214 levels (measured following alkaline hydrolysis of the attached rotigotine) precisely mirrored the plasma levels of released rotigotine, suggesting that a single weekly administration of SER-214 would provide continuous delivery of rotigotine in the therapeutic window for control of motor fluctuations. Overall, these studies provided demonstration that a weekly administration of subcutaneous SER-214 was associated with anti-Parkinsonian benefits in a robust monkey model of the disease.

8.9. Quantitative Whole Body Autoradiography (QWBA) with microautoradiography (MARG)

Autoradiography is a powerful, high resolution, quantitative molecular imaging technique used to study the tissue distribution of radiolabeled xenobiotics in biologic models. The imaging of organs, organ systems and/or whole-body sections (WBA) and microautoradiography (MARG), which provides localization of radioactivity at a cellular level, has been used to support drug discovery and development efforts for many years [63].

In order to further characterize the pharmacokinetics and distribution of SER-214 we radiolabelled the polymer backbone with $^{14}$C. The QWBA scintigraphic analyses at 2 h post-dose showed that most of the radioactivity was in the blood.
(42.6 μg equiv/g) and in the urinary bladder contents (76.6 μg equiv/g) and to a lesser extent in the tissue (data not shown). After 24 h, the radioactivity was predominantly in the kidney cortex (28.8 μg equiv/g), kidney medulla (20.1 μg equiv/g), liver (5.2 μg equiv/g), spleen red pulp (3.5 μg equiv/g), inguinal lymphatic region (66.5 μg equiv/g), sublumbar lymphatic region (72.5 μg equiv/g), urinary bladder contents (12.7 μg equiv/g), cecum (6.9 μg equiv/g), small intestine (2.5 μg equiv/g), and large intestine (3.5 μg equiv/g) as shown in Fig. 15. These tissue levels peaked between 24 and 72 h and steadily declined thereafter (Figs. 16 and 17). Based on these observations, the urine was confirmed to be the major route of elimination for 14C-SER-214.

At 168 h post-dose there was substantial “wash-out” of the radiolabelled SER-214, with residual radioactivity at a low level (<1% of the administered dose). Renal excretion is the primary route of elimination as suggested by the high concentrations observed in the kidney and urine compared to concentrations in liver and bile. However, the liver and bile should be considered as additional but minor excretion pathways, as radioactivity was detected in the liver, bile and gastrointestinal content at the earlier time points. Microautoradiography (MARG) analysis of the kidney tissue at 24 h showed significant presence in the renal tubule collecting ducts, the glomerulus and the interstitial spaces (Fig. 18).

In summary, the QWBA of radiolabelled SER-214 showed that kidney was the major route of elimination (consistent with other internal studies at Serina, unpublished observations). As early as two hours after administration the glomerulus showed significant uptake, followed by renal filtration and appearance in collecting ducts and bladder. Regional lymphatics also appeared to take up the polymer conjugate, and this would result eventually in uptake in the vascular compartment. Peak radioactivity in most tissues occurred at 24–72 h, which was consistent with the Cmax levels of released rotigotine in the blood plasma in single dose pharmacokinetic studies in rats [18]. After Day 3, radioactivity levels appear to decline rapidly due to renal clearance with appearance of high levels of radioactivity in the bladder and collecting tubules of the kidney. At day 7 and day 14 there was a small amount of radioactivity still present in the liver with some radioactivity in the bile and intestine. It is possible that biliary uptake and excretion may contribute to clearance of the polymer conjugate but this represents a small fraction of the renal clearance (<1–2%). The organs that were identified for close observation in the pivotal toxicology studies were the kidney, liver, spleen and bone marrow. In neither tissue did histologic findings suggest an adverse consequence of chronic dosing (see Sections 9.1 and 9.2). The distribution of SER-214 appeared to be entirely extracellular and no tissues showed a clear and consistent intracellular accumulation of the conjugate by MARG.

In a recent elegant study from the Hoogenboom laboratory [37] the comparison of POZ versus PEG showed similar results – a high rate of clearance by the kidney. Surprisingly, for a relatively low molecular weight 20 kDa POZ versus 20 kDa PEG
there was a striking difference in clearance – POZ cleared much faster than PEG. Estimates of the renal threshold for filtration of POZ approximated 60–70 kDa (consistent with internal observations at Serina). Finally, there was also a high degree of uptake of both POZ and PEG polymers by the spleen but the subcellular localization of those accumulations was not elucidated.

9. IND-enabling toxicology

Regulatory compliance requires that two species be evaluated in formal toxicology studies to support dosing in humans (US FDA Good Laboratory Practice Regulations 21 CFR 58, effective June 20, 1979 and subsequent amendments). The first species chosen for SER-214 was the Sprague-Dawley rat, and the second species chosen was cynomolgus macaque monkey. In both instances chronic dose studies were undertaken with 90 days of dosing followed by a four-week recovery period.

9.1. 90-day repeated toxicity and toxicokinetics of SER-214 in Sprague-Dawley rats

The purpose of this study was to determine the toxicity and toxicokinetics of SER-214 following repeated weekly subcutaneous dosing for 3 months (90 days) in male and female Sprague-Dawley rats. The study included three groups of animals, the main toxicity group, the recovery group and the toxicokinetic group. Table 3 shows the breakdown of each group by number, sex of animal, treatment and dose. Assessment of toxicity was based on mortality, clinical signs, ophthalmic observations, body weights, food consumption, clinical and anatomic pathology and toxicokinetic evaluations.

All animals survived to the scheduled termination interval. Some animals in all the groups were observed to have some fur shedding on the forelimbs, hindlimbs or abdominal regions. This observation was not considered to be test article related because it was also observed in the vehicle and placebo groups and there was no apparent dose-relationship.

During the dosing and recovery period, the body weight of the animals in both sexes in all groups was stable and increased gradually, except for the changes in the high dose cohort 5.0 mg equivalent rotigotine/kg for both sexes. Significant statistical differences were noted between the Group 4 females and Group 5 males and females, and the vehicle control group during the dosing period. The body weight changes were typically less than 10%, and completely resolved during the recovery period. The loss in body weight gain is likely related to the release of rotigotine, which is known to suppress appetite and result in body weight loss (EMA Approval Document for Neupro, 2006).

For the hematology parameters, during the dosing period, the female rats receiving ≥0.5 mg/kg of SER-214 had a dose-dependent decrease in total leukocytes when compared to the vehicle control animals. This observation was not made in male rats, and the decrease was apparent at the Days 29, 57 and 85 intervals. These changes were attributed to decreases in neutrophils and lymphocytes, but were also contributed to by occasional decreases in eosinophils and monocytes. The changes among leukocytes in female rats were considered test article related and had resolved by the first three days into the recovery period (Day 88). The significance of this observation is not clear, as it was not seen in male rats, did not occur with the polymer control and resolved within three days of cessation of dosing. There were no pathological changes in the bone marrow noted in female rats.

There were also some occasional mild decreases in MCHC, MCV, MCH, PLT and/or reticulocytes in both sexes in treatment groups. Although some changes were statistically significant, the values remained well within the clinical range, with no physiological significance. These findings had completely resolved by the recovery interval in both sexes and were not considered test article-related.

| Table 3 | Groups of male and female Sprague-Dawley rats in 90-day toxicity study. |
| --- | --- | --- | --- |
| **Group** | **Subgroup** | **Dose** (mg/kg) | **Number of animals & sex** |
|  |  |  | **M** | **F** |
| Main | 1 | 0 (vehicle control) | 10 | 10 |
|  | 2 | 0 (placebo polymer 36 mg/kg) | 10 | 10 |
|  | 3 | 0.5 | 10 | 10 |
|  | 4 | 1.25 | 10 | 10 |
|  | 5 | 5.0 | 10 | 10 |
| Recovery | 1 A | 0 (vehicle control) | 5 | 5 |
|  | 2 A | 0 (placebo polymer 36 mg/kg) | 5 | 5 |
|  | 3 A | 0.5 | 5 | 5 |
|  | 4 A | 1.25 | 5 | 5 |
|  | 5 A | 5.0 | 5 | 5 |
| Toxicokinetics | 6 | 0 (placebo polymer 36 mg/kg) | 6 | 6 |
|  | 7 | 0.5 | 9 | 9 |
|  | 8 | 1.25 | 9 | 9 |
|  | 9 | 5.0 | 9 | 9 |

* Dose based on rotigotine equivalents. Subcutaneous dose volume was 0.4 mL/kg on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78 and 85.

No test article-related effects were identified in food consumption, ophthalmic observations, coagulation, clinical chemistry, urinalysis, bone marrow smear observations, organ weight, organ weight ratio, macroscopic and microscopic observations.

Plasma rotigotine was readily detected following weekly subcutaneous injections of SER-214 for 13 weeks. The systemic exposure (AUC$_{0-t}$) for males had a 1.64-fold increase (0.5 mg/kg), 2.02-fold increase (1.25 mg/kg) and a 1.84-fold increase (5.0 mg/kg) when comparing week 1 and week 13 data. The systemic exposure (AUC$_{0-t}$) for females had a 2.02-fold increase (0.5 mg/kg), 2.10-fold increase (1.25 mg/kg) and a 2.1-fold increase (5.0 mg/kg) when comparing week 1 versus week 13 data. These AUC increases were attributed to higher Cmax values in some animals between 3 and 24 h post-dose as shown in Fig. 19 for the mid-dose group. There was no significant increase or decrease in the MRT and t½ values between weeks 1, 4, 7, 10 and 13 for male and female rats, suggesting that the drug was being cleared at similar rates each week.

Following 13 weeks of once weekly repeated subcutaneous administration of SER-214 at 0.5, 1.25 and 5.0 mg/kg in Sprague-Dawley Rats, each dose was well tolerated. The maximum tolerated dose (MTD) was 5.0 mg/kg/week, the highest dose tested. There was no NOAEL (No Observed Adverse Effect Level) determined, as the changes in either loss in body weight gain (expected for the active ingredient rotigotine) or the decrease in WBC in female rats only completely resolved. No evidence of histologic abnormalities was noted, specifically no evidence to suggest accumulation (vacuoles) or clearance of the polymer by tissues that are known to clear other polymers such as PEG (liver, kidney, lymphatics, spleen, bone marrow, or other tissues).

9.2. 90-day repeated toxicity and toxicokinetics of SER-214 in cynomolgus macaque monkeys

The second species chosen for formal toxicology to support chronic dosing in humans was the monkey, cynomolgus macaque. The purpose of this study was to determine potential toxicity of SER-214 (and polymer placebo) when administered by subcutaneous injection once weekly for a total of 13 doses (90 days). The study was designed to assess the reversibility, persistence, or delayed occurrence of toxic effects following a 4-week recovery period. In addition, the toxicokinetics of SER 214 were determined in a separate cohort.

A total of 25 female and 25 male cynomolgus macaque monkeys, approximately 1.5–3.5 years old and weighing 2.4–3.2 kg and 2.3–2.8 kg for males and females, respectively, at start of study, were social housed in steel cages in environmentally controlled, HEPA-filtered rooms with a 12-h light cycle. The monkeys received qualified feed and reverse-osmosis purified and chlorinated water. Feed was withdrawn overnight prior to ophthalmologic examination, scheduled blood collections for hematological and serum biochemical determinations, during urine collections, and overnight prior to scheduled necropsies. Feed and water were available ad libitum. Table 4 shows the breakdown of each group by number, sex of animal, treatment, dose and main and recovery. Three SER 214 treatment groups of 5 female and 5 male monkeys each received 0.5, 1.5, or 7 mg equivalents rotigotine/kg/week of SER 214 and the vehicle was 2.5% w/v of Dextrose anhydrous USP and 2.5% w/v of mannitol powder USP in water for injection. The vehicle control animals (5 females and 5 males) received the vehicle only. The placebo polymer control animals (5 females and 5 males) received the placebo polymer, which was comprised of a 20 kDa PEOZ polymer with pendent propionic acid groups without active drug, at a dose of 50 mg/kg. The dosing volume for all animals was 0.2 mL/kg.

Criteria for evaluation included survival, toxicokinetics, clinical observations, body weights, food consumption, ophthalmologic examinations, electrocardiography, clinical pathology evaluations (hematology, coagulation, serum chemistry, and urinalysis), serum prolactin levels, organ weights, and gross and microscopic pathology analyses. Terminal necropsy was on day 88 for 3 males and 3 females per group, while recovery necropsy was on day 115 for 2 males and 2 females per group.

![Fig. 19. Plasma concentrations of rotigotine following weekly subcutaneous injections of SER-214 for 13 weeks in Sprague-Dawley male rats measured at week 1, week 4, week 7, week 10 and week 13 (n = 3; ±SD).](image-url)
For plasma drug concentration analyses (plasma rotigotine and total releasable rotigotine), nonfasted blood samples were collected from all animals. On Day 1, 29, 57 and 78 of dosing phase, blood samples were collected from all available animals at 3, 6 h postdose, and 1, 2, 3, 4, 5, and 7 days postdose. On Day 88, 92, 95, 99, 102, 106, 109, 113 and 115, blood samples were collected once from the recovery animals.

As shown in Fig. 20, weekly injections of SER-214 resulted in steady-state levels of released rotigotine over a 13-week period. In week one, there is a slow but steady rise to a Cmax on about Day 3–4, and in each subsequent week of administration there is a steady-state level of released rotigotine in the range 8–10 ng/mL. This is quite extraordinary, as it would be virtually impossible to achieve such steady-state levels even by continuous intravenous administration of free rotigotine, which has a half-life of 2–3 h in both monkey and human. We believe this steady-state concentrations in blood levels is achieved by a near perfect balance between the exit of the drug from the subcutaneous compartment, prolonged circulation of SER-214 without tissue uptake, release of rotigotine in blood, and rapid clearance by the kidney. Within 8–9 days following the last weekly dose, the remaining polymer conjugate in the subcutaneous depot appears to clear rapidly as levels of both polymer conjugate and released rotigotine fall below levels of detection (Fig. 21). The apparent half-life of clearance of both polymer conjugate and released rotigotine parallel one another exactly, indicating no apparent accumulation of conjugate or released drug.

All monkeys survived to scheduled termination. There were no test-article-related changes in clinical observation, body weight, food consumption, ophthalmology examinations, electrocardiography, clinical pathology, prolactin, gross necropsy or organ weight.

In general, most microscopic findings following the dosing phase in animals given placebo polymer and animals given ≥1.5 mg/kg/dose were similar in incidence and severity, consisting of minimal or mild renal tubular vacuolation in the 7.0 mg/kg/dose group only, and minimal to moderate accumulations of foamy macrophages at the injection site and in several lymph nodes. The only exceptions were a higher incidence and severity of mononuclear cell infiltration at the injection site for animals given ≥0.5 mg/kg/dose SER 214 compared to placebo polymer, and a higher incidence and severity of subcutaneous edema at the injection site for animals given ≥1.5 mg/kg/dose SER 214 compared to vehicle control. This may be attributable to the release of rotigotine in the injection site itself, since it was not seen with the placebo polymer.

### Table 4
Groups of male and female cynomolgus macaque monkeys in 90-day toxicology study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose* (mg/kg)</th>
<th>Number of animals &amp; sex</th>
<th>Sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M F Recovery</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 (vehicle control)</td>
<td>3 3 2 2</td>
<td>Blood for Hematology and Clinical Chemistry taken just prior to dosing on days 29, 57, 85 and sacrifice day</td>
</tr>
<tr>
<td>2</td>
<td>0 (placebo polymer 50 mg/kg)</td>
<td>3 3 2 2</td>
<td>88 and recovery day 115 Plasma collected at regular intervals during dosing on days 1–85 and during recovery on days 86–115</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>3 3 2 2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>3 3 2 2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>3 3 2 2</td>
<td></td>
</tr>
</tbody>
</table>

Subcutaneous dose volume was 0.2 mL/kg on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78 and 85.

* Dose based on rotigotine equivalents.

For plasma drug concentration analyses (plasma rotigotine and total releasable rotigotine), nonfasted blood samples were collected from all animals. On Day 1, 29, 57 and 78 of dosing phase, blood samples were collected from all available animals at 3, 6 h postdose, and 1, 2, 3, 4, 5, and 7 days postdose. On Day 88, 92, 95, 99, 102, 106, 109, 113 and 115, blood samples were collected once from the recovery animals.

As shown in Fig. 20, weekly injections of SER-214 resulted in steady-state levels of released rotigotine over a 13-week period. In week one, there is a slow but steady rise to a Cmax on about Day 3–4, and in each subsequent week of administration there is a steady-state level of released rotigotine in the range 8–10 ng/mL. This is quite extraordinary, as it would be virtually impossible to achieve such steady-state levels even by continuous intravenous administration of free rotigotine, which has a half-life of 2–3 h in both monkey and human. We believe this steady-state concentrations in blood levels is achieved by a near perfect balance between the exit of the drug from the subcutaneous compartment, prolonged circulation of SER-214 without tissue uptake, release of rotigotine in blood, and rapid clearance by the kidney. Within 8–9 days following the last weekly dose, the remaining polymer conjugate in the subcutaneous depot appears to clear rapidly as levels of both polymer conjugate and released rotigotine fall below levels of detection (Fig. 21). The apparent half-life of clearance of both polymer conjugate and released rotigotine parallel one another exactly, indicating no apparent accumulation of conjugate or released drug.

All monkeys survived to scheduled termination. There were no test-article-related changes in clinical observation, body weight, food consumption, ophthalmology examinations, electrocardiography, clinical pathology, prolactin, gross necropsy or organ weight.

In general, most microscopic findings following the dosing phase in animals given placebo polymer and animals given ≥1.5 mg/kg/dose were similar in incidence and severity, consisting of minimal or mild renal tubular vacuolation in the 7.0 mg/kg/dose group only, and minimal to moderate accumulations of foamy macrophages at the injection site and in several lymph nodes. The only exceptions were a higher incidence and severity of mononuclear cell infiltration at the injection site for animals given ≥0.5 mg/kg/dose SER 214 compared to placebo polymer, and a higher incidence and severity of subcutaneous edema at the injection site for animals given ≥1.5 mg/kg/dose SER 214 compared to vehicle control. This may be attributable to the release of rotigotine in the injection site itself, since it was not seen with the placebo polymer.

**Fig. 20.** Daily plasma concentration of released rotigotine following weekly subcutaneous injections in normal cynomolgus macaque monkeys at Week 1 (initial dose ●), Week 5 (▲), Week 9 (▼) and Week 12 ( ●). Dose was 58 mg/kg of SER-214 (7 mg/kg equivalents of rotigotine).
Following the recovery phase, renal tubular vacuolation in the high dose group and accumulation of foamy macrophages were reversible, and edema and mononuclear cell infiltration at the injection site were completely resolved. There were no delayed effects.

All of the microscopic findings in animals given placebo polymer or >0.5 mg/kg/dose SER 214 were considered non-adverse, based on lack of evidence that the changes affected organ function or the overall health and well-being of the animals.

For the toxicokinetics, the concentration of released and total rotigotine (ng/mL) increase each day in Week 1, and then plateau and level out between Weeks 2 and 13. The increase in Week 1 was dose-dependent. $T_{\text{max}}$ values for released rotigotine were observed at 6.0 and 168.0 hours post-dose in Week 1 and between 0.0 and 120.0 h post-dose in Weeks 5, 9 and 12. $T_{\text{max}}$ values for total rotigotine were observed at 96.0 h at 0.5 mg/kg/week and at 168.0 h at 1.5 and 7.0 mg/kg/week in Week 1, and between 0.0 and 6.0 h post-dose in Weeks 5, 9 and 12. No marked sex differences in systemic exposure to released and total rotigotine was observed at any dose levels in Weeks 1, 5, 9 or 12. As the dosage increased from 0.5 to 7.0 mg/kg/week, the systemic exposure to released and total rotigotine increased dose proportionally in both sexes from Weeks 1 to 12. Generally, no significant change was observed in systemic exposure to released and total rotigotine during Weeks 2–12.

The no observed adverse effect level (NOAEL) for this study was 7 mg equivalent rotigotine/kg/week (58 mg SER-214/kg/week), which was also the maximum tolerated dose (MTD) as it was the highest dose evaluated. The plasma exposures of released rotigotine at Week 12 revealed a $C_{\text{max}}$ of 9.54 ng/mL and $AUC_{\text{all}}$ of 947 h ng/mL in the males, and $C_{\text{max}}$ of 11.4 ng/mL and $AUC_{\text{all}}$ of 1190 h ng/mL in the females. The plasma exposures of total rotigotine in Week 12 were $C_{\text{max}}$ of 9560 ng/mL and $AUC_{\text{all}}$ of 944,000 h ng/mL in the males and $C_{\text{max}}$ of 11,700 ng/mL and $AUC_{\text{all}}$ of 951,000 h ng/mL in the females.

In summary, the 90-day repeat dose evaluation of SER-214 in adult cynomolgus macaque monkeys revealed a NOAEL of 7 mg equivalents rotigotine/week. This was also the maximum tolerated dose (MTD) as it was the highest dose evaluated. Unlike the rat, where loss in body weight gain is known to occur with high doses of rotigotine (EMA Approval document for Neupro, 2006), monkeys do not lose body weight upon repeated doses of rotigotine. The PK profile of released rotigotine (Fig. 20) revealed no evidence of accumulation of rotigotine at any dose evaluated, nor was there any evidence of accumulation of polymer conjugate (Fig. 21). Indeed, the week-to-week variation in the plasma concentration of rotigotine strongly suggests that SER-214 will provide continuous drug delivery of rotigotine. It should be pointed out that the dose administered in the high dose group of 7.0 mg/kg/week is approximately fifteen times the anticipated human equivalent dose predicted for efficacy. Finally, upon cessation of dosing, plasma levels of both SER-214 and released rotigotine fall within three days to levels that would not be expected to provide symptomatic relief of motor fluctuations.

### 9.3. Additional IND-enabling toxicology studies

A number of safety pharmacology and genotoxicity studies were conducted with SER-214 and the placebo polymer as summarized in Table 5.
Two separate studies were conducted to assess the acute effects of SER-214 on the respiratory system and the central nervous system in Sprague-Dawley rats. Male and female rats (5/sex/group) were administered a single subcutaneous injection of vehicle or SER-214 at doses of 0.5, 1.25 or 5 mg/kg (dosed based on rotigotine equivalents). Tidal volume, respiratory rate, and derived minute volume were measured at pre-dose and at 2, 24, and 168 h post-dose for 30 min. Animals were placed in ‘head-out’ plethysmographs and allowed to acclimate to environmental conditions for at least 15 min prior to each data collection period. In the behavioral test, a functional observation battery (FOB) assessment (consisting of home cage observation, removal from home cage, open field observation, manipulative test and hand-hold observation and quantitative parameters) was conducted prior to dose administration and once at 24 and 168 h post-dose. A single subcutaneous injection of SER-214 to Sprague Dawley rats at dose levels of 1.25 and 5 mg/kg SER-214 (dosed based on rotigotine equivalents) resulted in an increase in respiratory rate, tidal volume and minute volume 2–2.5 h post-dose. In the low dose group respiratory parameters returned to control levels by the next time point tested (24 h post-dose). In the 5 mg/kg dose group, the parameters remained increased, but by 168 h post-dose all parameters were similar to control and/or pre-dose levels, indicating a full recovery. A single subcutaneous injection of SER-214 at dose levels of 1.25 and 5 mg/kg (rotigotine equivalents) was associated with a dose-dependent increase in stereotypical sniffing 2 h following dose administration. These effects resolved at later time points.

In the cardiovascular study, 5 male and 5 female cynomolgus macaques from the toxicology study were administered vehicle, placebo (50 mg/kg) or SER-214 at dosages of 0.5, 1.5 or 7.0 mg/kg (based on rotigotine equivalents) by subcutaneous injection once weekly for 13 weeks. Electrocardiograms (ECG) were obtained from all animals twice at pre-study, once during Week 13 and at the end of the 4-week recovery period. ECG parameters evaluated were heart rate, respiratory rate (RR), PR, QRS and QT interval durations and description of waveform morphology for P, QRS and T waves. QTc interval was calculated according to Bazett’s formula. There were no test article-related effects on any of the qualitative or quantitative ECG parameters after 13 weekly subcutaneous injections of SER-214 or placebo POZ polymer.

The in vitro genotoxicity assays performed on SER-214 and the POZ polymer were the Bacterial Reverse Mutation Assay (Ames) and the Chromosome Aberration Assay. In the Ames assay, Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 and Escherichia coli WP2 uvrA were incubated with test article at twelve dose levels (1.0, 2.5, 5.0, 10, 25, 50, 100, 500, 1000, 2500 and 5000 µg/plate) with or without the Aroclor-induced rat liver microsome preparation (S9 fraction). Mutagenicity and cytotoxicity were assessed by counting the number of revertants (his+1) colonies appearing on the minimal agar medium after 48 h of incubation at 37 °C. In the chromosome aberration assay, Chinese Hamster Ovary (CHO) cells were incubated with ten concentrations of test article (0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 µg/mL) dissolved in DMSO in the absence and presence of the S9 metabolic activation system. After a predetermined period of time of incubation, the cells were washed free of test article and cultured for an additional 17 h. Colcemid® was added 2 h prior to harvesting in order to arrest the cells in the metaphase stage. The viable cell counts were used to determine cell growth inhibition relative to the concurrent solvent control. The remainder of the cells were centrifuged and resuspended in cold fixative. Slides were prepared for microscopic evaluation of chromosomal aberrations. Results from both tests show that SER-214 and POZ polymer with pendent propionic acid were negative in the Ames test for mutagenicity and were not clastogenic.

10. First-in-man Phase I clinical trial

Serina Therapeutics filed the Investigational New Drug (IND) application with the FDA in July 2015, and the first subjects were enrolled in the study in the first quarter of 2016. The study protocol is entitled “A multi-center, open-label, multiple...
ascending dosage-ranging cohort (MAD) study in early, untreated or stably treated subjects with Parkinson’s disease (PD), to determine the safety, tolerability and pharmacokinetics (PK) of injections of SER-214 administered subcutaneously once a week for two weeks after 0–2 weeks of dose titration. The specifics of the trial can be found on the ClinicalTrials.gov website at https://clinicaltrials.gov/show/NCT02579473. Briefly, Parkinson’s disease (PD) patients who are on a stable regimen of drugs and who are not experiencing motor fluctuations, or those who have been recently diagnosed with PD but have not yet been started on medications and are naïve to dopamine agonists, are eligible for participation in the trial if they meet all of the inclusion criteria and do not meet any of the exclusion criteria. The first cohort of subjects (designated as Cohort 0) has completed dosing and the multi-dose phase of the study is in-progress.

10.1. Results from Cohort 0

Cohort 0 was a single-dose cohort where four subjects received a single 20 mg dose of SER-214 (equivalent to ~2.4 mg of rotigotine) administered subcutaneously by the study site nurse. Extensive safety and tolerability information prior to dosing, and during follow-up, have been incorporated into the trial. A complete list of these safety and tolerability assessments can be found on the ClinicalTrials.gov posting. Subjects had an indwelling venous catheter for collection of blood samples for pharmacokinetic determination of total releasable rotigotine (TRR) and released plasma rotigotine (PR) at time 0, 1 h, 2 h, 4 h and 8 h.

All four subjects completed dosing without any adverse events of note. None of the subjects developed any changes in blood pressure (baseline or orthostatic), blood chemistries, liver panels, hematology, ECG or changes from baseline in any of the assessments of Parkinsonian endpoints or other safety assessments.

The plasma levels of total releasable rotigotine (TRR) and plasma rotigotine (PR) are shown in Fig. 22. The plot shows that there is a slow and steady rise in plasma levels for both PR and TRR, that reaches an approximate C_max on about Day 3–4. There is no “burst” release of the rotigotine in the first 8 h, which could have been a safety concern. These profiles are similar to the observations seen with the monkey plasma levels at the low dose (Fig. 11), indicating quite likely that the exit from the subcutaneous depot and entry into the blood compartment in humans will closely parallel the monkey results. If we extrapolate the data based on this cohort to calculate a PR/TRR ratio, it appears that humans release between 3 and 4% of the attached rotigotine. This value is slightly higher than that for the monkey, which has a ratio of between 1 and 2%. We do not find this to be too surprising, as human plasma was shown to release rotigotine at a slightly faster rate than monkey plasma in vitro (data not shown). After Day 7 there is a fall in both polymer conjugate and plasma rotigotine levels to below the levels of detection.

In summary, a single injection of SER-214 of 20 mg was safe, well-tolerated and demonstrated predictable PK. The clinical study has now entered the multi-dose phase of the trial. To our knowledge, this is the first POZ polymer conjugate to enter human clinical dosing, and the initial results suggest this polymer will be safe and well-tolerated. This information will be invaluable as we and others continue to develop POZ conjugates for additional clinical indications.

11. Summary

In this manuscript we have summarized much of the chemical synthesis and characterization of a 20 kDa polymer of PEOZ with ten pendant alkyne groups that serve as drug attachment sites, and how that approach may be used for development of novel therapeutics. The early preclinical work, and IND-enabling studies that supported the advancement of
SER-214 into patients with Parkinson's disease, has also been summarized. Those studies suggest that POZ may have many of the attributes of an ideal polymer platform, one that addresses multiple classes of molecules for programmed release in either the blood or within cancer cells. We see the major advantages of POZ versus other polymers as (1) programmable size and architecture, with the ability to pre-program the drug load during the synthesis step, (2) high, reproducible yields at the synthesis and drug-conjugate step in a single reaction vessel that can be purified through simple ion exchange chromatography, (3) very low polydispersity index (<1.02) that results in low batch-to-batch variability of polymer size and attached drug, (4) quantitative and facile drug loading through metal-catalyzed "click" chemistry – again, with high yield, single-step purification of API via standard chromatography methods, (5) lack of immunogenicity, (6) low viscosity, such that a therapeutic dose can readily be delivered as a subcutaneous injection in a standard insulin syringe, and (7) a platform that addresses many therapeutic areas of drug development from releasable small molecules in the blood for neurological diseases to targeted therapies for cancer.

The field of POZ polymer therapeutics is at a particularly exciting juncture now after a 50-year history following the initial synthesis by Kagiya and colleagues [1], with the first POZ-therapeutic safely dosed in humans for Parkinson's disease. Novel polymer architectures addressing a multitude of approaches for unmet medical needs are in the works. We anticipate the field will continue to generate new and exciting POZ polymer therapeutics in the decades ahead.

Acknowledgements

The authors wish to acknowledge the outstanding contributions from many in the field of POZ polymers who are too numerous to cite, but we would like to recognize the notable work of Maeda and colleagues, and the Jordan, Luxenhofer and Hoogenboom laboratories. The outstanding administrative assistance of Mrs. Stephanie Green is also noted. In 2011 Mr. John Jurenko, a successful businessman in the Huntsville, AL community, asked if we could use our polymer technology to make a drug for Parkinson's disease - a disorder he eventually succumbed to in 2015. His question set Serina Therapeutics on a course to develop the first drug of its kind.

References

Randall joined Serina Therapeutics in September 2010 as President and Chief Executive Officer. Prior to joining Serina, Dr. Moreadith, 61, was Chief Development Officer at Nektar Therapeutics where he built a clinical and drug development program that successfully moved several of the company’s PEGylated small molecule drugs into the clinic, including the launch of NKTR-102 into four clinical indications (ovarian, breast, cervical and colorectal cancer) and the out-licensing efforts for NKTR-118 (approved product now known as Movantik™, Astra Zeneca and Daiichi Sankyo). He was formerly the Executive Vice President and Chief Medical Officer of Cardium Therapeutics where he led the advancement of novel DNA-based therapeutics into Phase 2b and Phase 3 late-stage development. Before Cardium, Dr. Moreadith served as Chief Medical Officer of Renovis, Inc. where he led the Clinical, Regulatory and Quality Assurance Groups. Prior to that, Dr. Moreadith was co-Founder, President and Chief Operating Officer of ThromboGenics Ltd., a leader in the field of thrombosis drug development. During his tenure at ThromboGenics, the company advanced four biologics into mid-stage development, and one product was later approved (Ocriplasmin™). Dr. Moreadith began his career in the pharmaceutical industry as Principal Medical Officer of Quintiles, Inc., the world’s leading pharmaceutical services organization, where he led the Cardiovascular Therapeutics Group.

Dr. Moreadith has published more than 50 scientific papers and multiple book chapters, is an inventor on multiple patents, and has received numerous awards for his achievements. He received his MD from Duke University and is trained clinically in Internal Medicine and Cardiovascular Diseases. He received his PhD from Johns Hopkins University, and following his Fellowship in Cardiology at Duke University he joined the laboratory of Professor Philip Leder where he was a Howard Hughes Medical Institute Fellow in Genetics at Harvard Medical School. Dr. Moreadith was a member of the faculty of the University of Texas Southwestern Medical Center, where he was an Established Investigator of the American Heart Association.

Tacey joined Serina Therapeutics in November 2006 as Chief Operating Officer. Prior to Serina, Dr. Viegas was an Executive Director of Research and Development at Nektar Therapeutics and at BioCryst Pharmaceuticals (both in Alabama, USA). While at Nektar, Dr. Viegas was a co-inventor of Movantik™ (Astra Zeneca and Daiichi Sankyo) and Onzeald™ (Daiichi Sankyo). At BioCryst, he managed the early development activities of Rapivab™. Dr. Viegas also held positions of increasing seniority at MDV Technologies and at R. P. Scherrer Corporation (both in Michigan, USA), where he developed the Flo-Gel™ polymer technology that was approved and used for wound ulcers and dermal burns. Dr. Viegas has over 25 years of experience in pharmaceutical drug discovery and development in the areas of oncology, neurology, influenza, psoriasis and wound care. He has over 50 peer-reviewed publications and book chapters and has over 50 issued patents. He is a reviewer for several scientific journals and an active participant in various Polymer Therapeutic initiatives. Dr. Viegas received his B.S. in Chemistry (1977) and Pharmacy (1981) from Bangalore University, his M.S. (1984) and Ph.D. (1988) in Pharmaceutical Sciences from the University of Mississippi. Early on in his career, he was the recipient of an American College of Clinical Pharmacology award for his research on the clinical applications of purine nucleosides.