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Research paper

Thiomers: Inhibition of cytochrome P450 activity

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ABSTRACT

The aim of the present study was to investigate the potential of different thiolated polymers (thiomers) on the catalytic activity of CYP450s on one hand and to explore new inhibitors for CYP activity on the other hand. Several thiolated polymers including poly(acrylic acid)–cysteine (PAA–cysteine), chitosan–thioglycolic acid (chitosan–TGA), and thiolated PEG-*g*-PEI copolymer along with brij® 35, myrj® 52 and the well-established CYPP450 inhibitor verapamil were screened for their CYP3A4 and CYP2A6 inhibitory activity, and their IC_{50} values were determined. Both enzyme inhibition assays were performed in 96-well microtiter plates. 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC) and 7-hydroxycoumarin (7-HC) were used as fluorescent substrates in order to determine CYP3A4 and CYP2A6 catalytic activity, respectively. All investigated compounds inhibited CYP3A4 as well as CYP2A6 activity. All tested (thiolated) polymers were found to be more potent inhibitors of CYP3A4 than of CYP2A6 catalytic activity. Apart from verapamil that is a known CYP3A4 inhibitor, brij® 35 and myrj® 52 were explored as potent inhibitors of CYP3A4 and CYP2A6 catalytic activity. Among the tested polymers, the rank order for CYP3A4 inhibition was PAA–cysteine (100 kDa) > brij® 35 > thiolated PEG-*g*-PEI copolymer (16 kDa) > myrj® 52 > PAA (100 kDa) > PAA–cysteine (450 kDa) > verapamil > PAA (450 kDa) > chitosan–TGA (150 kDa) > chitosan (150 kDa). On the other hand, the rank order of CYP2A6 inhibition was brij® 35 > PAA–cysteine (100 kDa) > chitosan–TGA (150 kDa) > PAA (100 kDa) > thiolated PEG-*g*-PEI copolymer (16 kDa) > PAA–cysteine (450 kDa) > chitosan (150 kDa) > verapamil > PAA (450 kDa) > myrj® 52. Thus, this study suggests that (thiolated) polymers display a promising potential to inhibit cytochrome P450s activity and might turn out to be potentially valuable tools for improving the oral bioavailability of actively secreted compounds by avoiding intestinal metabolism.

1. Introduction

Oral administration of drugs is strongly recommended because of its convenience, the relative low production costs, and the high level of patient compliance and safety. However, a noticeable percentage of drugs do not exhibit the characteristics required for oral administration. A prerequisite for a drug to be efficient after oral administration is that it mainly bypasses a series of physical and biochemical barriers in the gastrointestinal tract. Out of which an important element of barrier function is a large super family of heme-thiolate proteins, cytochrome P450s (CYP450s).

CYP450s isoenzymes play an important role in the phase I oxidative metabolism of structurally diverse xenobiotics, such as drugs, toxic chemicals, carcinogens as well as endobiotic chemicals including steroids, fatty acids, fat-soluble vitamins, and prostaglandins [1]. The ability of CYP450 to effectively limit the penetration of compounds into select tissues can profoundly influence the efficacy of a drug by restricting its interaction with the target site. Conversely, this same process may effectively prevent agents from reaching toxic levels in these protected tissues [2].

CYP3A4 is the most important drug-metabolizing enzyme belonging to CYP 3A family that metabolizes a wide variety of xenobiotics, endogenous substrates, and more than 50% of administrated drugs. Thus, it represents a major contributor in the reduced bioavailability of numerous drugs. On the other hand, human CYP2A6 is expressed predominantly in the liver, representing between 1% and 10% of total hepatic P450s and responsible for the metabolism of various clinically relevant compounds.

Over the past few years, thiolated polymers so-called thiomers that are obtained by the immobilization of thiol-bearing ligands onto the polymeric backbones have been introduced in the pharmaceutical literature. These thiolated polymers demonstrated not only improved mucoadhesive, controlled release and permeation-enhancing properties but also enzyme inhibitory properties [3]. Recently, Werle and Hoffer reported a significantly improved

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transmucosal transport of P-gp substrate rhodamine-123 (Rho-123) in the presence of thiolated chitosan as a result of P-gp efflux pump inhibition [4].

CYPs and P-gp are both expressed in the intestinal mucosa and function as a barrier to oral drug delivery either by transmembranal efflux or by metabolizing drugs. There is a noticeable overlap in the drugs that interact with these two proteins, and both proteins share many inhibitors, substrates, and inducers. For instance, previous studies provide evidence that CYPs are inhibited by amiodarone, ketoconazole, quinidine, and verapamil that are known to inhibit P-gp as well. [5,6].

It was therefore the aim of this study to evaluate the influence of different thiolated polymers on the catalytic activity of CYP450s on one hand and to explore new inhibitors for CYP activity on the other hand. For this purpose, several thiolated polymers along with brij® 35 and myrj® 52 in comparison with well-established cytochrome P450 inhibitor verapamil were screened for CYPs inhibitory activity, and their IC_{50} values were determined. CYP3A4 and CYP2A6 were chosen for CYP inhibition studies as these enzymes are jointly responsible for more than 60% of overall drug metabolism.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI) 600 Da was purchased from Polysciences. Homo-functional polyethylene glycol (OH-PEG-OH) 6000 Da was from Rapp Polymere GmbH, Germany. Hexamethylene diisocyanate (HMDI) and chitosan (from crab shell; degree of deacetylation >85%) were purchased from Fluka. (±)-Verapamil hydrochloride, γ-thiobutyrolactone, and thioglycolic acid (TGA) were purchased from Sigma. Coumarin, β-nicotinamide adenine dinucleotide phosphate reduced tetra(cyclohexylammonium) salt (NADPH), L-cysteine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), and poly(acrylic acid) (PAA) having molecular mass of 100 kDa and 450 kDa, respectively, were purchased from Sigma. 7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC), baculovirus-expressed human CYP3A4, and CYP2A6 supersomes were purchased from BD Biosciences Discovery Labware (Bedford, MA). All other reagents used were of analytical grade.

2.2. Methods

2.2.1. Polymers synthesis

2.2.1.1. Poly(acrylic acid)–cysteine (PAA–cysteine; 100 kDa and 450 kDa). PAA–cysteine conjugates (100 kDa and 450 kDa) were synthesized by the covalent attachment of cysteine to poly(acrylic acid) according to a method described previously [7]. Briefly, 1 g each of PAA 100 kDa and PAA 450 kDa were hydrated separately in demineralized water, and the pH value of the PAA solutions was adjusted to 6 by the addition of 5 M NaOH. Then, EDAC in the final concentration of 200 mM was added in order to activate the carboxylic acid moieties of each of the hydrated polymers. After 20 min of incubation under stirring at room temperature, 1 g of L-cysteine was added to each of the hydrated PAA solutions and the pH was readjusted to 6. Reaction mixtures were incubated for 3 h at room temperature under stirring.

2.2.1.2. Chitosan–thioglycolic acid conjugate (chitosan–TGA; 150 kDa). Chitosan–TGA (150 kDa) was synthesized by covalent attachment of TGA to chitosan as described previously [8]. Briefly, 1% (m/v) solution of chitosan hydrochloride was obtained by the initial hydration of 500 mg of chitosan in 4 ml of 1 M HCl followed by addition of the according volume of demineralized water. Thereafter, 500 mg of TGA was added. After TGA was completely dissolved in the chitosan hydrochloride solution, EDAC was added in a final concentration of 125 mM in order to activate the carboxylic acid moieties of TGA. The reaction mixture was incubated at pH 5 for 3 h at room temperature under stirring.

2.2.1.3. Thiolated PEG-g-PEI copolymer (PEG-g-PEI-SH; 16 kDa). Thiolated PEG-*g*-PEI copolymer was synthesized according to a method described previously [9]. In brief, 4 g of PEG were dissolved in 40 ml of chloroform followed by the addition of 36 ml of HMDI and refluxed for 48 h. The activated polymer formed was precipitated by the addition of 600 ml of petroleum ether, and the resulting solid product was re-dissolved in 15 ml of chloroform. Precipitation was repeated four times. The activated polymer was vacuum dried and weighed. A solution of 4 g of PEI 600 Da in 50 ml $CHCl_3$ was added dropwise (with continuous stirring) to a solution of 3.8 g of activated PEG in 50 ml $CHCl_3$. The mixture was refluxed for 48 h. The copolymer was precipitated by the addition of 500 ml of petroleum ether and 2.6 g of PEG-*g*-PEI copolymer were dissolved in 50 ml of distilled water. pH value was adjusted to 5.75 with 1 M HCl followed by the addition of 1000 μl of γ-thiobutyrolactone under continuous stirring for 2 h. The pH value after thiolation was 5.30.

2.2.2. Purification

In order to eliminate unbound reacting species from the polymers, each of the above four reaction mixtures was dialyzed five times using Spectra/Por® 3 membrane (MWCO: 1200) at (low acidic) pH ~ 3 according to a method described previously [10] for 3 days in total at 10 °C in the dark. In detail, the thiolated polymers were dialyzed one time against 5 mM HCl and then two times against the same medium but containing 1% NaCl, finally two times against 1 mM HCl. After dialysis, the pH of PAA–cysteine conjugates was readjusted to 6. Thereafter, the dialyzed products were freeze-dried for 3 days at −80 °C under reduced pressure and stored at 4 °C until use [11].

2.2.3. Determination of the thiol group content

The amount of thiol groups immobilized on the polymer conjugates was determined spectrophotometrically using Ellman's reagent as described previously [12]. TGA standards were used to calculate the amount of thiol groups immobilized on the chitosan–TGA conjugate while L-cysteine. HCl was employed to establish calibration curve for all other polymer conjugates.

2.2.4. CYP3A4 and CYP2A6 enzyme inhibition assays

All incubations were performed in 96-well plates, and the experimental conditions are summarized in Table 1. For CYP3A4 in each well, 150 μl of incubation medium containing 100 mM Tris–HCl buffer (pH 7.4), 4.2 mM $MgCl_2$, 50 μM 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), 0.001–1000 μM or 0.001–1.00% test substance/polymer, and 0.75 pmol cDNA expressing CYP were transferred. The reaction was initiated by the addition of 0.3 mM NADPH (50 μl) after 10 min preincubation at 37 °C. The reaction mixture was incubated at 37 °C for 30 min and terminated by the addition of 110 μl of 80% acetonitrile/20% 0.5 M Tris base. The formed fluorescence was measured using microplate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). For CYP2A6 in each well, 100 μl of incubation medium containing 75 μl of 50 mM Tris–HCl buffer (pH 7.4), 5.0 mM $MgCl_2$, 10 μM coumarin, 0.001–1000 μM or 0.001–1.00% test substance/polymer, and 0.75 pmol cDNA expressing CYP were transferred. The reaction was initiated by the addition of 0.3 mM NADPH (25 μl) and terminated by the addition of 60 μl of 10% trichloroacetic acid (TCA) after 15 min of incubation at 37 °C. Immediately before the measurement, 140 μl of 1.6 mM glycine–NaOH buffer pH 10.4 was

Table 1
Experimental conditions for CYP3A4 and CYP2A6 enzyme inhibition assays.

CYP Isoform	Substrate and concentration (μM)	Fluorescent metabolite	Incubation time (min)	λem (nm)	λex (nm)	Enzyme concentration (pmol)
3A4	BFC (50)	HFC	30	538	410	0.75
2A6	Coumarin (3)	7-HC	15	460	355	0.75

BFC: 7-benzyloxy-4-(trifluoromethyl)coumarin; HFC: 7-hydroxy-4-(trifluoromethyl)-coumarin; 7-HC: 7-hydroxycoumarin.

added. The formed fluorescence was measured using microplate reader (Tecan infinite M200 spectrophotometer, Grödig, Austria). All IC_{50} values were determined in triplicate. The enzyme activities in the presence of test compounds/polymers were compared with the control incubations without inhibitor. Supersomes or substrates were omitted from the blank wells [13,14].

2.2.5. Statistical data analysis

Concentration response data for CYP3A4 and CYP2A6 enzymes were analyzed using nonlinear curves fit based on the equation: $y = a \times \exp(b \times x)$. The IC_{50} values at 95% confidence intervals (95% Cls) for each compound were determined by nonlinear regression analysis using GraphPad Prism™ v 5.0 (GraphPad Software, Inc., San Diego, USA) and expressed as the mean of three individual experiments with lower and upper 95% confidence intervals.

Statistical data analyses were performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Characterization of thiolated polymers

The chemical substructures of the chitosan–TGA (150 kDa), PAA–cysteine conjugates (100 kDa and 450 kDa), and thiolated PEG-*g*-PEI copolymer (16 kDa) used in this study are illustrated in Fig. 1. All of the lyophilized thiolated polymers appeared as white, odorless powder of fibrous structure. PAA–cysteine conjugates (100 kDa and 450 kDa) and thiolated PEG-*g*-PEI copolymer showed good solubility in water. On the other hand, chitosan–TGA conjugate was easily solublized in 1% (v/v) acetic acid. Comparison of different experimental parameters used for thiolation is presented in Table 2. Determination of thiol groups attached to the polymers by Ellman's test demonstrated that on average 171 ± 20 μmol and 656 ± 79 μmol thiol groups were immobilized

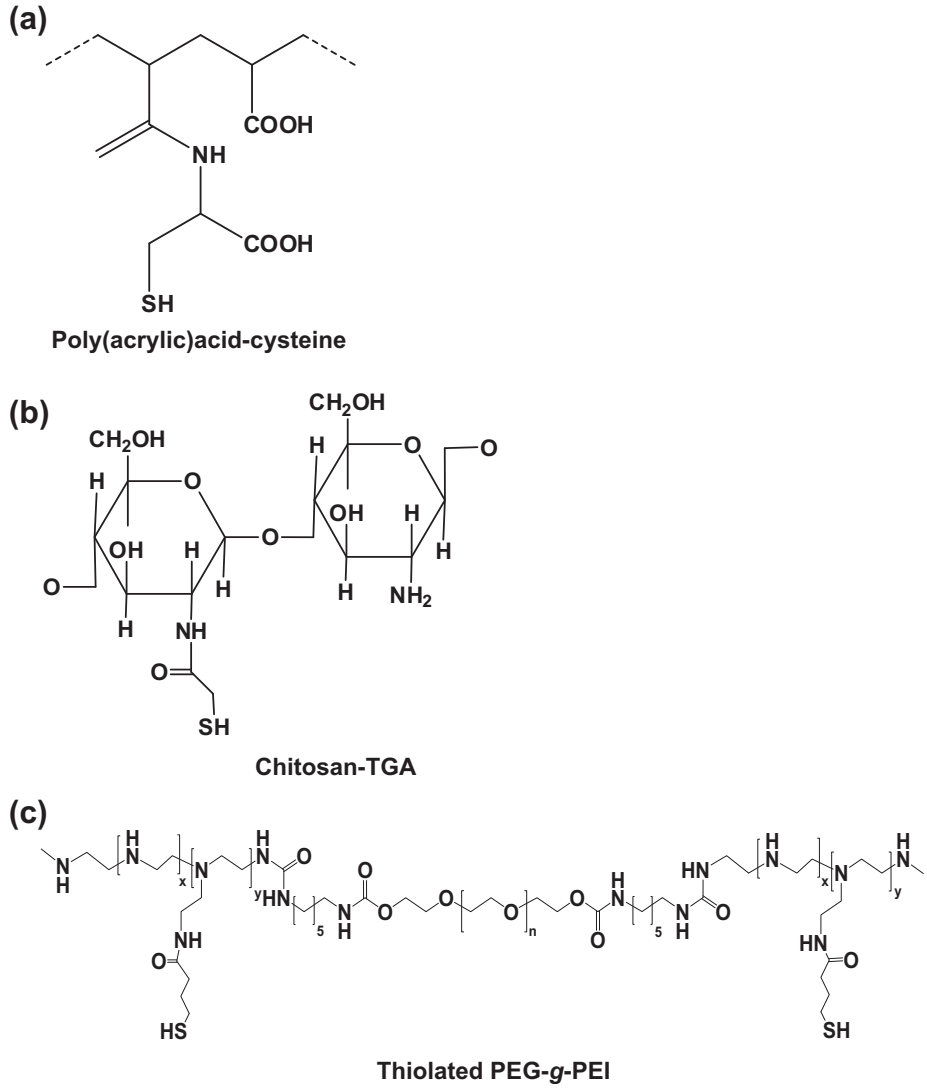

Fig. 1. Presumptive chemical structures of thiolated polymers: (a) chitosan–TGA (b) poly(acrylic acid)–cysteine (c) thiolated PEG-*g*-PEI copolymer.

Table 2
Comparison of experimental conditions for the thiolation of different thiolated polymers and the resulting amount of immobilized thiol groups per gram of each thiomer.

Thiolated polymer[a]	Thiolating reagent[b]	Ratio [a:b] (g)	pH during the reaction	Immobilized thiol groups (μmol/g)	Standard deviation (μmol/g)
Thiolated PEG-*g*-PEI copolymer (16 kDa)	γ-Thiobutyrolactone	2.2:1	5.75	84	±5
Chitosan–TGA (150 kDa)	Thioglycolic acid	1:1	5.0	624	±46
PAA–cysteine (100 kDa)	L-Cysteine	1:1	6.0	171	±20
PAA–cysteine (450 kDa)	L-Cysteine	1:1	6.0	656	±79

[a] Thiolated polymer.
[b] Thiolating reagent.

per gram of 100 kDa and 450 kDa poly(acrylic acid)–cysteine conjugates, respectively. Chitosan–TGA (150 kDa) and thiolated PEG-*g*-PEI copolymer displayed 624 ± 46 μmol and 84 ± 5 μmol thiol groups per gram of polymer, respectively.

3.2. CYP3A4 and CYP2A6 enzyme inhibition assays

Several methodologies and different experimental systems are currently in practice to monitor the inhibition of drug-metabolizing CYP activity. Some are rather time-consuming or quite complicated associated with several problems [15]. Fluorescent probe assays were chosen in this study for screening of in vitro inhibitory potential of thiolated polymers, as they offer the advantage of high throughput along with selectivity and ease of implantation. Enzyme inhibition studies were performed using human liver supersomes containing either CYP3A4 or CYP2A6 protein expressed on baculovirus/insect cell system. This system was found to be suitable for the expression of phase I drug-metabolizing enzymes, such as cytochromes P450, as it ensures that these proteins are expressed in appropriate environment, the endoplasmic reticulum (ER) of a higher eukaryote and allows higher levels of expression with the capability of correct post-translational modification and subcellular targeting of the expressed protein. BFC was used as fluorescent substrate for the determination of catalytic activity of CYP3A4 [16] while fluorescence emitted by coumarin 7-hydroxide as a result of coumarin 7-hydroxylation reaction is a specific and well-characterized marker for CYP2A6 enzyme activity [17]. It has been reported previously [18,19] that the presence of more than 1% of organic solvents greatly affects the in vitro metabolism of a variety of CYP enzymes. Therefore, methanol in the final concentration of 0.2% (v/v) was used to dissolve coumarin and BFC to avoid enzyme inhibition, as solvent concentrations $\leqslant$0.5% were reported safe for use owing no significant effects on CYP-mediated metabolism. On the other hand, several control experiments were also carried out during both fluorescent assays to monitor and nullify potential risk of fluorescence modification by chemical interaction with test compounds/inhibitors and other interfering factors. Preliminary results clearly indicated that all of the tested (thiolated) polymers display a great potential to inhibit CYP3A4 as well as CYP2A6 catalytic activity. Results for CYP3A4 inhibition are shown in Table 3. It is clearly indicated that thiolation process greatly improves inhibitory properties of all of the polymers. Thus, all of the tested thiomers possess higher potential to inhibit cytochrome P450 catalytic activity and displayed lower IC_{50} values than those of corresponding unmodified polymers. PAA–cysteine (100 kDa) was the strongest inhibitor among the investigated compounds/polymers with an IC_{50} value of 0.007%, and chitosan (150 kDa) was the weakest with an IC_{50} value of 0.063%.

All polymers displayed also inhibition of CYP2A6 catalytic activity. Results are shown in Table 4. Brij® 35 was identified as most potent inhibitor for CYP2A6 activity with an IC_{50} value of 2.47×10^{-7}% followed by PAA–cysteine (100 kDa) with an IC_{50} value of 0.001% and myrj® 52 being the weakest with an IC_{50} value of 0.061%. Thiolated polymers were found to be potent inhibitors of CYP2A6 catalytic activity as well, and each of them displayed a lower IC_{50} value for CYP2A6 inhibition in comparison with corresponding unmodified polymers. Apart from verapamil that is a known CYP3A4 inhibitor, brij® 35 and myrj® 52 were explored as potent inhibitors of CYP3A4 and CYP2A6 catalytic activity. Verapamil and myrj® 52 displayed strong inhibition towards CYP3A4 in comparison with CYP2A6. On the other hand, brij® 35 was found more potent against CYP2A6 in comparison with CYP3A4.

Previous studies reported that the active site of CYP2A6 is smaller than that of CYP3A4, suggesting that smaller molecules are probably more potent inhibitors of CYP2A6 activity [20]. On the other hand, thiolated polymers are generally high molecular weight compounds. Therefore, it is more likely that the inhibitory potential of thiolated polymers is due to the interaction of immobilized thiol groups within the structure of thiolated polymers with the active site of CYP2A6. This hypothesis correlates with Fujita and Kamataki [21] who suggest that the sulfur atom may play an important role in the inhibition of CYP enzymes. Moreover, organosulfur compounds such as *S*-allyl cysteine, 2-allylthiopyrazine, allylsulfide, allylmercaptan, and allylmethylsulfide can be potent

Table 3
IC_{50} values of test compounds against CYP3A4 inhibition.

Inhibitor	CYP3A4 inhibition	
	IC_{50} (μM)	IC_{50} (%) (m/v)
PAA–cysteine (100 kDa)	–	0.007^{**}
Brij® 35	212.41	0.008
Thiolated PEG-*g*-PEI copolymer (16 kDa)	–	0.010
Myrj® 52	–	0.014
PAA (100 kDa)	–	0.022^{*}
PAA–cysteine (450 kDa)	–	0.027^{++}
Verapamil	631.24	0.031
PAA (450 kDa)	–	0.045^{+}
Chitosan–TGA (150 kDa)	–	$0.054^{!!}$
Chitosan (150 kDa)	–	$0.063^{!}$

* Differs from ** $p < 0.0001$. $^{!}$ Differs from $^{!!}$ $p < 0.0001$. $^{+}$ Differs from $^{++}$ $p < 0.0001$.

Table 4
IC_{50} values of test compounds against CYP2A6 inhibition.

Inhibitor	CYP2A6 inhibition	
	IC_{50} (μM)	IC_{50} (%) (m/v)
Brij® 35	0.0068	2.47×10^{-7}
PAA–cysteine (100 kDa)	–	0.001^{**}
Chitosan–TGA (150 kDa)	–	$0.017^{!!}$
PAA (100 kDa)	–	0.023^{*}
Thiolated PEG-*g*-PEI copolymer (16 kDa)	–	0.026
PAA–cysteine (450 kDa)	–	0.027^{++}
Chitosan (150 kDa)	–	$0.028^{!}$
Verapamil	875.4	0.043
PAA (450 kDa)	–	0.047^{+}
Myrj® 52	–	0.061

* Differs from ** $p < 0.0001$. $^{!}$ Differs from $^{!!}$ $p < 0.0001$. $^{+}$ Differs from $^{++}$ $p < 0.0001$.

inhibitors of CYPs activity [22–24]. However, the exact mechanism for the role of the sulfur atom in these organosulfur compounds on the inhibition of CYPs activity remains to be elucidated at present. In case of CYP3A4, no common relationship was found among the active site of CYP3A4 and molecular structure of CYP3A4 inhibitors. As the thiolated polymers displayed a lower IC_{50} value in comparison with unmodified polymers, it is suggested that the low IC_{50} value is due to the structural and conformational changes in the membrane structure of CYP proteins as a result of interaction of CYP3A4 and CYP2A6 with the immobilized thiol groups within the structure of thiolated polymers.

CYP450 enzymes are a family of heme-thiolate proteins with the identity of the fifth ligand to the heme formed by a cysteine residue. In mammals, these enzymes are membrane-bound (usually in the endoplasmic reticulum) [25]. However, only rudimentary information is available on the exact localization and distribution of CYP450 proteins in the GI tract [26]. CYP450 enzymes require reducing equivalents, i.e., electrons, from the cofactor NADPH for their catalytic activity. Those electrons are usually provided indirectly via an accessory enzyme, NADPH-P450 reductase that is also a membrane-bound enzyme [27]. The proposed mechanism responsible for the inhibition of CYP450 activity across the cell membrane by non-absorbable sulfhydryl compounds like thiomers seems to be based on: (1) the attachment of mucoadhesive thiomers with mucus layer via formation of disulfide bonds with cysteine-rich subdomains of the mucus layer [28] and (2) formation of disulfide bonds between thiomers and CYP450 enzymes across the semipermeable cell membrane utilizing electrons coming from flavoprotein NADPH-P450 reductase. Thus, unavailability of reducing environment, i.e., electrons on one hand and the formation of disulfide bond on the other hand makes CYP450 enzymes inactive for metabolism. Whether thiomers can be uptaken by enterocytes in significant quantities and can subsequently also interact with CYP450 enzymes in the endoplasmic reticulum will strongly depend on their molecular mass and hydrophilic/lipophilic character. Detailed investigations on that topic are subject of ongoing studies.

4. Conclusion

Several (thiolated) polymers were screened for CYP3A4 and CYP2A6 inhibitory activity, and their IC_{50} values were determined. All of the tested (thiolated) polymers demonstrated a great potential to inhibit CYP3A4 as well as CYP2A6 catalytic activity. In addition, brij® 35 and myrj® 52 were explored as potent inhibitors of CYP3A4 and CYP2A6 catalytic activity. Because of high potential to inhibit CYP450s activity, the thiolated polymer conjugates described herein represent a useful polymeric carrier matrix to avoid intestinal metabolism and could be potentially valuable tools for improving the oral bioavailability of various pharmaceutical active ingredients.

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