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Synthesis and Evaluation of Pharmacological Activities of Cyclodextrin Conjugates of Methotrexate

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In the present investigation methotrexate prodrugs of α-and γ-cyclodextrins were synthesized. The primary hydroxy group of α- and γ-cyclodextrins were used to block the acid group. The synthesis involved a series of protection and deprotection reaction. The esters were evaluated for stability in simulated gastric and intestinal fluid. The hydrolysis of cyclodextrin conjugates in colon is confirmed by the hydrolysis kinetics studies in rat fecal material. The esters were also evaluated for ulcerogenicity. Results of these studies established the primary aim of masking the ulcerogenic potential of free drug, by using 12-fold dose of the normal dose of methotrexate and equivalent doses of the esters.

Cyclodextrins (CDs) belongs to family of cyclicoligosaccharides; the most common being α-, β- and γ-CDs consisting of 6, 7 and 8 glucopyranosyl units, respectively, linked by α (1→4) glucosidic bonds[1]. CDs are obtained by enzymatic degradation of starch, glucosyl transferase a type of amylase of bacterial origin obtained usually from *Bacillus macerans, Bacillus megaterium, Klebsiella pneumoniae* M5, and *Bacillus sterothermophillus*. CDs are moderately soluble in water, methanol and readily soluble in strongly polar aprotic solvents.[2] After oral administration, CDs are not hydrolyzed during their transit through the stomach, hydrolysis occurs only in colon by colonic microflora. The oral administration of CDs does not result in toxicity[3,4]. Thus, CDs were thought to be one of the most suitable promoieties to reduce the ulcerogenic tendencies of methotrexate since they eliminate the exposure of free drug in stomach and small intestine but release the drug in colon.

MTX is N-[4-{[(2,4-diamino-6-pterdinyl)-methyl] methylamine} benzoyl]-L-glutamic acid. MTX is also known as 4-amino-10-methylfolic acid and amethopterin and is identified by the National Cancer Institute code number NSC-740, (www.theaccessproject@aol.com). The primary toxic effects of MTX and other antagonists used in cancer chemotherapy are exerted against rapidly dividing cells of the bone marrow and gastrointestinal epithelium. Gastrointestinal adverse effects include gastric hemorrhage, ulcers and deep perforations. The second toxicity of particular significance during chronic administration in-patients with psoriasis or rheumatic arthritis is hepatic fibrosis and cirrhosis. Increased hepatic portal fibrosis is detected with higher frequency than in control patients, after 6 mo or longer, oral methotrexate treatment of psoriasis[5-7].

It is generally accepted that the GI lesions produced by MTX are the result of two mechanisms, direct contact effect and a generalized systemic effect, which may be manifested after intravenous dosing. The direct contact effect may be attributed to the local irritation produced by the acidic group of MTX. The study of Cioli *et al.*[8], suggests that direct tissue contact due to free –COOH plays an important role in the production of the GIT lesions. MTX alpha–peptides (derivatives in which an amino acid is linked to the alpha–carboxyl group of the glutamate moiety), synthesized, were considered as ideal prodrugs, since they are not transported into the cells and can be converted to the parent drug by

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carboxypeptidases[9].

Based upon the computer model of the human enzyme built from the well known crystal structure of bovine carboxypeptidase A, novel bulky phenylalanine and tyrosine-based prodrugs of MTX were designed and synthesized[10]. The primary toxic effects of MTX are exerted on the bone marrow and the intestinal epithelium; blood cell counts are depressed and there is mucosal ulceration. Thus to circumvent the ulcerogenic potential of the parent drug, prodrug approach was used by conjugating with CDs.

MATERIALS AND METHODS

All melting points were determined by open capillary method and are uncorrected. TLC ascertained the purity of the compounds on precoated silica gel-60 F_{254} plates. Solvent used was butanol:ethanol:water:acetic acid (3:2:3:0.1). Thus different spots for reference and test substance were detected using iodine vapours or by charring the plates using 5% methanolic sulfuric acid. All the final compounds were re-crystallized from water, after extracting the impurities with ethyl acetate. The IR spectra of the synthesized compounds were recorded on a FT-IR spectrophotometer, in potassium bromide (anhydrous IR Grade) pellets. The NMR spectra of the synthesized compounds were determined on a FT-NMR spectrophotometer. The λ_{max} of the synthesized compounds

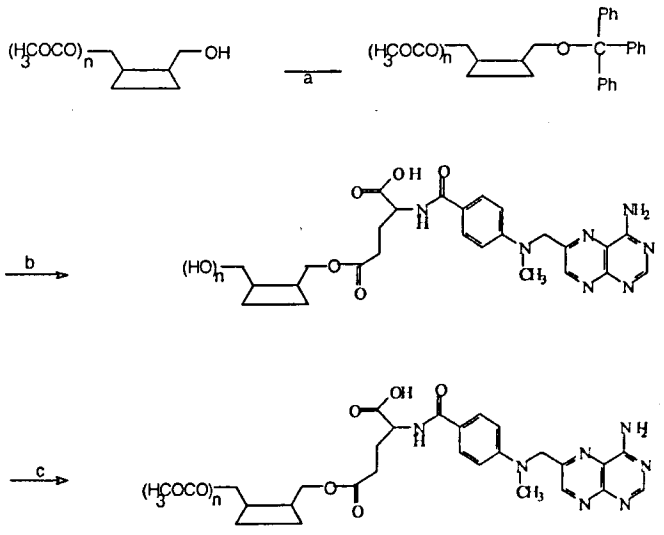

Fig. 1: Synthesis of α cyclodextrin esters of methotrexate SRD-1.

The reagents for each steps are trityl chloride (a), acetyl chloride (b) and triethylamine (c), MPT - 63 resin, MTX free acid, DCC and ethylenediamine.

was determined on UV/Vis double beam spectrophotometer by scanning the compounds between 200-400 nm in various solvents. MTX was obtained as a gift sample from Zyg Pharma Ltd., Indore and Olympia Pharma, Mumbai. CDs were obtained as gift sample from S. A. Chemicals, Mumbai. All the other chemicals used were of synthetic grade.

Synthesis of methotrexate ester of α-CD (SRD1):

Schematic representation for synthesis of SRD1 is shown in Scheme 1. Synthesis of MTX ester of α-CD involved 5 steps. First step involved tritylation of one of the primary hydroxyl groups of α-CD (0.00514 mol, 5 g). This reaction involved reacting the α-CD with trityl chloride (a) (0.00565 mol, 1.57 g) in pyridine (30 ml) by stirring it for 24 h[12]. The residue obtained was refluxed with n-hexane to remove trityl alcohol, the by-product. The solid was dried under vacuum to give tritylated α CD (5.03 g). Next step involved acetylation of tritylated α-CD (0.00411mol, 5 g) using acetyl chloride (b) (0.0698 mol, 4.9 ml) and triethyl amine (c) (0.698 mol, 9.7 ml) in ethylene dichloride (75-80 ml). The temperature was maintained at 0° throughout the reaction. The reaction mixture was filtered and dried under reduced pressure to give acetylated monotritylated α-CD (6.2 g)[13]. Acetylated monotritylated α CD (0.0031 mol, 6 g) was dissolved in N, N-dimethyl formamide (DMF, 200 ml) in round bottom flask. The cation exchange resin T-63 (MP) (d) (3 g) was added slowly into the reaction flask. The reaction mixture was stirred at room temperature and TLC ascertained reaction completion. The reaction mixture was concentrated under vacuum after evaporation of the resin. The residue was refluxed with n-hexane in order to remove trityl alcohol liberated during reaction. The residue obtained after extraction was subjected to esterification reaction.

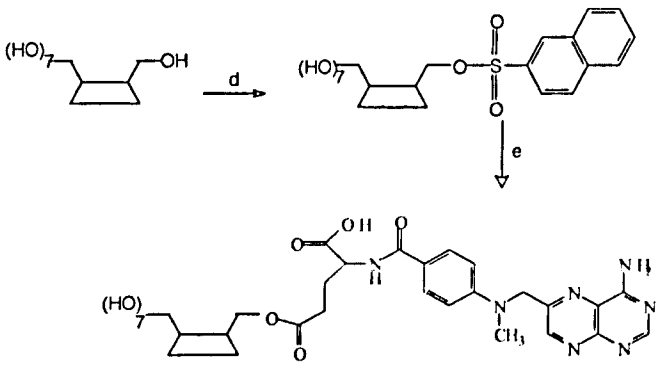

Fig. 2: Synthesis of γ-cyclodextrin ester of methotrexate SRD-2.

The reagents for each step are 2-naphthalenesulfonyl chloride (d), MTX sodium (e).

Selectively detritylated α-CD (0.0024 mol, 4 g) was dissolved in DMF (50 ml) in a round flask. 1,3-dicyclohexyl carbodiimide (DCC) (d) (0.0024 mol, 0.49 g) was dissolved in DMF. Both the solutions were ice cooled to 0°. The DCC solution and MTX free acid (e) (0.0023 mol, 0.57 g) were added to CD solution[10] at 0°. The reaction mixture was stirred at 0° for 2 h and then at room temperature for 12 h. The reaction mixture was then filtered to separate the precipitate of N,N-dicyclohexyl urea, the by product. The filtrate was concentrated under reduced pressure, to give acetylated MTX ester of α-CD (3.7 g). This ester was deacetylated by dissolving the ester (0.0015 mol, 3 g) in methanol at room temperature and reacting with ethylenediamine (0.15 mol, 10.12 ml) and triethyl amine (c) (0.15 mol, 20.2 ml)[14]. The reaction was stirred for 5 h and large amount of acetone (400 ml) was added to precipitate the deacetylated ester. Molecular formula of α-CD ester of MTX (SRD1) is $C_{56}H_{79}N_7O_{34}$. Melting point: 232-234°,TLC; R_f: 0.74; butanol:ethanol:water:acetic acid (3:2:3:0.1) and λmax in distilled water: 259.2 nm, in 0.05 M HCl buffer (pH 1.2): 207.6 nm and in 0.05 M phosphate buffer (pH 7.4): 260.6 nm. PMR spectra was recorded on YH 300 NMR spectrophotometer with the sample as a solution in DMSO-d6.The chemical shift was in the ppm relative to TMS designated as 0.00. The δ in the range of 7-8 confirmed peculiar aromatic peaks e.g. of H- 2',6',3',5', benzylic protons and H2N-2,4 protons nearby δ=7.4 nearby δ=8.6 represented the protons on pteridine ring. Benzylic –CH2 protons (H-9) and H-α d nearby 4-5, -CH3-11 protons represented (H-9) and H-α represented (H-9) and H-α represented (H-9) and H-α represented δ nearby 3-3.4 and peak due to H- (COOH; HOH) was represented at δ=6.1-6.4.

TABLE 1: HYDROLYSIS KINETICS OF SRD 1 AND SRD 2-IN RAT FAECAL MATTER

Time (min)	% Drug released	
	SRD 1	SRD 2
15	-	-
30	-	-
45	5.00	3.20
60	7.65	6.90
75	25.1	15.2
90	76.0	37.3
105	81.9	51.0
120	83.0	64.8
240	85. 2	82.0
24 h	85. 3	85.1

The hydrolysis of SRD 1 and SRD 2 in rat fecal contents was studied with time.

Synthesis of methotrexate ester of γ-CD (SRD 2):

Schematic representation for synthesis of SRD2 is shown in Scheme 2. γ-CD (0.0008 mol, 4 g) was dissolved in dry pyridine (250 ml). To it 2-naphthalene sulfonyl chloride (h) (0.00924 mol, 2.09 g) was added. The reaction mixture was allowed to stir at room temperature for 8 h. the reaction mixture was concentrated under reduced pressure to give 2-naphthalene sulfonyl γ-CD (4 g). 2-Naphthalene sulfonyl γ-CD (0.002 mol, 3 g) was dissolved in DMF (100 ml) and MTX sodium (i) (0.002 mol, 0.537 g) was added to the reaction solution and the mixture was stirred at 0° for 30 h[15]. Reaction mixture was concentrated under reduced pressure and the final ester was precipitated using 500 ml amount of acetone. Molecular formula of MTX ester of γ-CD (SRD2) is $C_{68}H_{99}N_7$. Melting point: 240-242°, TLC; R_f: 0.68; butanol:ethanol:water:acetic acid (3:2:3:0.1) and λmax in distilled water: 283.2 nm, in 0.05 M HCl buffer (pH 1.2): 306.2 nm and in 0.05 M phosphate buffer (pH 7.4): 261.8 nm. A PMR spectrum was recorded on YH 300 NMR spectrophotometer with the sample as a solution in DMSO-d6. The chemical shift was in the ppm relative to TMS designated as 0.00. The δ in the range of 7-8 confirmed peculiar aromatic peaks e.g. of H-2',6',3',5', benzylic protons and H2N-2,4 protons nearby δ=7.4. nearby δ=8.6 represented the protons on pteridine ring. Benzylic –CH2 protons (H-9) and H-α δ nearby 4-5, -CH3-11 protons represented (H-9) and H-α represented (H-9) and H-α represented (H-9) and H-α represented δ nearby 3-3.4 and

TABLE 2: SCORING OF GASTRIC ULCERS

Ulcerogenic Response	Score
Ulcers in between 0-1 mm	1
Ulcers in between 1-2 mm	2
Ulcers in between 2-3 mm	3
Ulcers in between 3-4 mm	4
Ulcers in between 4-5 mm	5
Ulcers greater than 5 mm	10
Perforated lesions	25

peak due to H- (COOH; HOH) was represented at δ=6.1-6.4.

Hydrolysis kinetics:

Hydrolysis of α-CD ester and γ-CD ester was studied in simulated gastric fluid (0.05 M HCl buffer, pH 1.2) and simulated intestinal fluid (0.05 M phosphate buffer, pH 7.4).

Release studies in 0.05 M HCl buffer, pH 1.2 and 0.05 M phosphate buffer, pH 7.4:

Same procedure was followed for both the esters. α-CD ester and γ-CD were dissolved in HCl buffer and in phosphate buffer so that the final concentration of the two esters is equivalent to 1 μg/μl of MTX. To each of the flasks of dissolution apparatus (Veego Scientific DA 6D model) 800 ml of buffer was added when the temperature reached 37° the ester solution was added to the flask and stirred at 75 rpm. Two ml of aliquot portions were withdrawn at various time intervals, dilution factor was corrected immediately by adding 2 ml buffer solution and the aliquots were diluted with 6 ml of acetone and solution was spotted on precoated silica gel F254 silica gel HPTLC plates using Camag Linomat IV instrument. After running the plate in mobile phase, peak area for corresponding concentration was determined.

Release study in rat faecal matter, pH 7.4:

All the esters were dissolved in phosphate buffer (pH 7.4) so that final concentration of solution was 250 μg/ml. Fresh fecal material of rats was weighed (about 1 g) and placed in different sets of test tubes. To each test tube, 1 ml of the ester solution was added and diluted to 5 ml with phosphate buffer (50 μg/ml). The sets of test tubes were incubated at 37 ° for different intervals of time. For analysis the free drug was extracted in 5 ml $CHCl_3$ and directly estimated on double beam UV-spectrophotometer (Jasco, V-530 model, Japan). The concentration of free drug was determined using K and b values obtained from calibration curve. The esters showed negligible release in both the hydrolysis media. The hydrolysis of the esters was studied in rat faecal material (pH 7.4) to confirm the colonic hydrolysis of the esters[16]. The release of drug in rat faecal material was almost complete, maximally being for γ-CD ester. The results of hydrolysis in rat faecal content are quoted in Table 1.

TABLE 3: ULCEROGENIC ACTIVITY OF SRD 1 AND SRD 2

Compound	Dose (mg/kg)	Ulcer index ± S.D.*
Control	-	Nil
MTX	12.5	63 ± 0.94
SRD 1	38.7	Nil
SRD 2	49.6	14.6 ± 0.82

*Average of six readings, p<0.05.

Ulcerogenicity:

Ulcerogenicity was determined by the method reported by Rainsford *et al.*[17] The animal study protocols have met with IAEC's approval. According to this method sensitivity of gastric mucosa to ulcerogenic –COOH group is increased by exposing the animals to a short period of physical stress (i.e. cold or restraint)[18-20]. Sprague Dawley rats of either sex (150-200 g) were selected. They were kept for fasting for 24 h. Following oral administration of drug that is 12 times the normal dose in case of test and vehicle dose in case of control, the animals were stressed by exposure to cold (-15° for 1.5 h). The animals were sacrificed 3 h after drug administration and the number and severity of gastric mucosal lesions were determined.

RESULTS AND DISCUSSION

Ulcerogenicity studies showed that esters are not ulcerogenic (Table 2 and 3). *In vitro* hydrolysis studies have shown that the esters were quite stable in simulated gastric and intestinal fluid where as they hydrolyzed almost completely in rat fecal contents representing the colon. Although, this is the method of confirming the hydrolysis of esters in colon, it gives an idea about the behavior of ester in gastrointestinal tract (GIT). This can be further confirmed by improved analysis, one approach to it is to isolate the specific strain of microorganism responsible for hydrolysis of CD and study the hydrolysis of conjugates in presence of them. The present study clearly indicates that conjugation of CDs with MTX is a good method of masking the -COOH group and thus reducing the ulcerogenicity, a major drawback. The field is open for developing an animal model for anticancer evaluation of the synthesized compounds, determination of LD_{50} and ED_{50} and clinical trials to establish them as prodrugs. The synthesized compound need to be further studied before being considered as potentially useful prodrugs.

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