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Novel antiglaucoma prodrugs and codrugs of ethacrynic acid

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Abstract—The purpose of this study was to synthesize a novel prodrug of ethacrynic acid (ECA) with short chain polyethylene glycols (PEGs) and codrugs of ECA with the β-adrenergic blocking agent atenolol (ATL) or timolol (TML) to overcome the adverse effects of ECA and to enhance its physicochemical properties.

Elevated intraocular pressure (IOP) in glaucoma, the second most common cause of blindness, is one of the risk factors leading to glaucomatous damage to the eye (optic) nerve. Ethacrynic acid (ECA; [2,3-dichloro-4-(2-methylene-1-oxybuty)phenoxy]acetic acid), a therapeutically useful diuretic and $Na^+/K^+/Cl^-$ co-transport system inhibitor,[1,2] is a potential glaucoma drug that can reduce IOP.[3]

Ethacrynic acid has been reported to lower the IOP in rabbit and monkey eyes.[4,5] Lowering IOP has also been achieved through intracameral injection of ECA into human eyes.[6] A single intracameral injection of ECA in patients with advanced glaucoma reduced IOP for up to 3 days, without corneal and interior chamber side effects.[6] In enucleated calf eyes, living monkey eyes, and enucleated human eyes, ECA increased outflow facility, which was associated with morphologic changes of outflow pathway cells.[7] The ability to lower IOP through cytoskeletal alterations in trabecular meshwork (TM) cells,[4,8] makes ECA a potential glaucoma drug. It is postulated that ECA produces such effects by reacting with sulfhydryl groups in the TM of the eye. Chemical modification of these cellular sulfhydryl groups alters the egress of aqueous humor from the TM.[9,10]

It has been shown in animal studies that long-term topical or intracameral administration of ECA, especially at higher doses, is accompanied by lid, conjunctival, and corneal side effects, such as mild superior eyelid edema, conjunctival hyperemia, and moderate diffuse superficial corneal erosion.[4] Oral administration of ECA to patients with ocular hypertension had little appreciable effect on IOP.[11]

Unfortunately, there are serious limitations on the delivery of ECA because of its physicochemical properties. ECA, a carboxylic acid with a p*K*a around 2.8, exists exclusively (more than 99.9%) in its carboxylate anion form at physiological pH (pH 7.4). Therefore, in spite of the favorable lipophilicity of the neutral species, the predominant anionic form has an extremely unfavorable lipophilicity. Thus, due to the limited ability of ECA or its analogs to effectively penetrate the cornea, a therapeutic concentration of the drug at the postulated site of action (i.e., the trabecular meshwork) will be hard to achieve unless the physicochemical properties of the molecule are altered. Therefore, there is a need for the development of derivatives or prodrugs of ECA with greater ocular safety and corneal penetration, as well as improved therapeutic index.

β-Adrenergic receptor antagonists such as atenolol (ATL) and timolol (TML) provide effective therapy for a vast variety of hypertensive disorders, ischemic heart disease, and certain arrhythmias;[12] also, both drugs exert ocular hypotensive effects in both normal and raised IOP patients.[13] β-Adrenergic receptor antagonists do not usually cause retention of salt and water, and administration of a diuretic is not necessary to avoid edema or development of tolerance to these drugs. However, diuretics do have additive antihypertensive effects

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when combined with β-adrenergic receptor antagonists.[14] In the case of IOP, both ECA and β-adrenergic receptor antagonists can lower elevated IOP by different physiological mechanisms. Although ECA lowers IOP by increasing the outflow facility of aqueous humor from the eye, β-adrenergic receptor antagonists do so by decreasing aqueous humor formation.

To modify the physicochemical properties of ECA to obtain improved delivery to the eye, and in addition, to take advantage of the apparent synergistic mechanism of ECA and β-adrenergic receptor antagonists, we chose to synthesize prodrugs of ECA covalently linked to short chain polyethylene glycols (PEGs), and also to prepare codrugs of ECA covalently linked to either ATL or TML via ester bond linkages having a high degree of chemical or enzymatic lability at physiological pH. These novel codrugs and prodrugs may have less toxicity and afford more efficacious delivery than ECA, ATL, or TML alone.

Ethacrynic acid, atenolol, PEGs, timolol, sodium hydroxide, and triethylamine were purchased from Sigma–Aldrich (St. Louis, MO); acetonitrile, *N*,*N*-dimethylformamide, methylene chloride, oxalyl chloride, toluene, *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-dimethylamine-pyridine (DMAP), 1,1-carbonyldiimidazole (CDI), 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU), and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA).

An Agilent 1100 series HPLC analytical system was used for the hydrolysis studies and consisted of an L-4000 UV detector, an L-6000 intelligent pump, an AS-2000 autosampler, and a D-2500 chromato-integrator, Hitachi (Tokyo, Japan).

Analysis of the ECA–PEG prodrugs and the ECA–ATL codrugs in the hydrolysis studies was performed utilizing a reversed phase Axxion Ultrasphere ODS C-18 column (250 × 4.6 mm; 5 μm). The mobile phase was either (A) a mixture of acetonitrile and 0.02% acetic acid solution, adjusted to pH 4.0 with NaOH, or (B) a mixture of acetonitrile and 1% triethylamine solution, adjusted to pH 6.8 (for the analysis of ECA), or adjusted to pH 3.0 (for the analysis of ATL) with phosphoric acid. The mobile phase flow-rate for the assay of ATL was 0.5 mL/min, and 1 mL/min for the other studied compounds.

The prodrugs of ECA were prepared using short chain PEGs containing 2, 4, or 6 oxyethylene units, which were linked to ECA via an ester bond, as shown in Figure 1.[15] The acid chloride of ECA was first prepared by gently refluxing ECA (0.012 mol) with oxalyl chloride (1.6 mL, 0.018 mol) in toluene for a short period of time until a clear yellow solution was formed. The solution mixture was then flushed very gently with a stream of nitrogen to remove excess oxalyl chloride leaving behind a solid residue. The solid residue was cooled in an ice-bath and was dissolved by adding 10 mL of *N*,*N*-dimethylformamide slowly to the reaction vessel. (*Note:* the reaction is exothermic!) The appropriate PEG (0.01 mol) was dissolved in methylene chloride (280 mL) in the presence of pyridine (10 mL), and the solution was cooled in an ice-bath for 15 min. The DMF solution containing the ethacrynic acid chloride was added drop-wise to the cooled solution of the appropriate PEG with stirring for a further 15 min, to yield a mixture of two major products, that is, the desired mono-esters and the corresponding di-ester side products (prodrugs **1** and **2**, respectively). Two side products were also formed in this reaction, which were identified as the mono- and di-PEG esters of a chlorinated ECA molecule, formed by the addition of HCl across the double bond of ECA (compounds **3** and **4**, respectively). These products were successfully separated from the desired prodrugs by silica gel column chromatography.

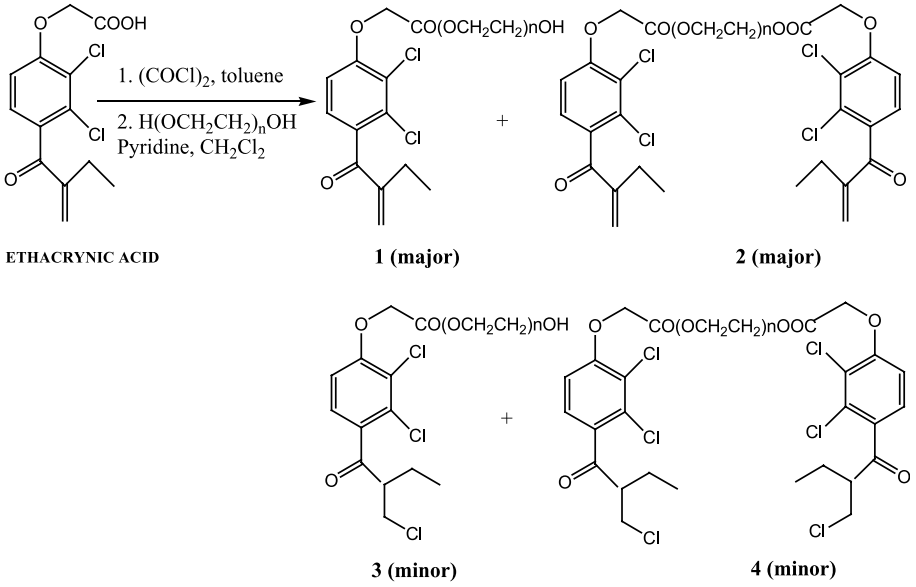

Figure 1. Synthesis of ECA–PEG prodrugs (n = 2, 4, or 6).

An alternative but more problematic method of synthesizing prodrugs **1** and **2** via activation of the carboxylic group with *N,N*-dicyclohexylcarbodiimide (DCC) in 4-dimethylamine pyridine (DMAP) was also carried out and afforded the desired products. However, it was found difficult to separate the mono-ECA–PEG esters from the byproduct, *N,N′*-dicyclohexylurea, which is formed in the DCC coupling reaction.

Codrugs of ECA with the β-adrenergic receptor antagonists ATL and TML were synthesized as shown in Figure 2.[15] During the activation of the carboxylic acid group of ECA with CDI, and coupling with ATL, addition of CDI to the double bond of ECA also occurred. Fortunately, treatment with DBU removed the CDI moiety and afforded the desired ECA–ATL codrug, **5**, which could be easily purified by silica gel column chromatography. Using the above synthetic procedure, the ECA–TML ester, **6**, was also prepared (the structure of the crude compound could be confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy), but unfortunately, this ester was not stable enough to be fractionated and isolated by column chromatography in a pure form, since it hydrolyzed very rapidly to the parent drugs. Attempts to crystallize the ECA–TML ester were also unsuccessful for the same reason.

Table 1 lists the concentrations of acetonitrile in the mobile phase, the detection wavelength, and the retention times (RT) for the selected drug conjugates, and also for the parent drugs.

Buffer hydrolysis of the ECA–PEG prodrugs and the ECA–ATL codrug was examined by dissolving 10 mg of the codrug or prodrug in 10 mL of acetonitrile. This stock solution was added to phosphate buffer (0.1 M, pH 7.4 at 37 °C) to give a final concentration of 100 μg/mL. Samples (100 μL) were periodically removed and assayed utilizing the previously described HPLC system.

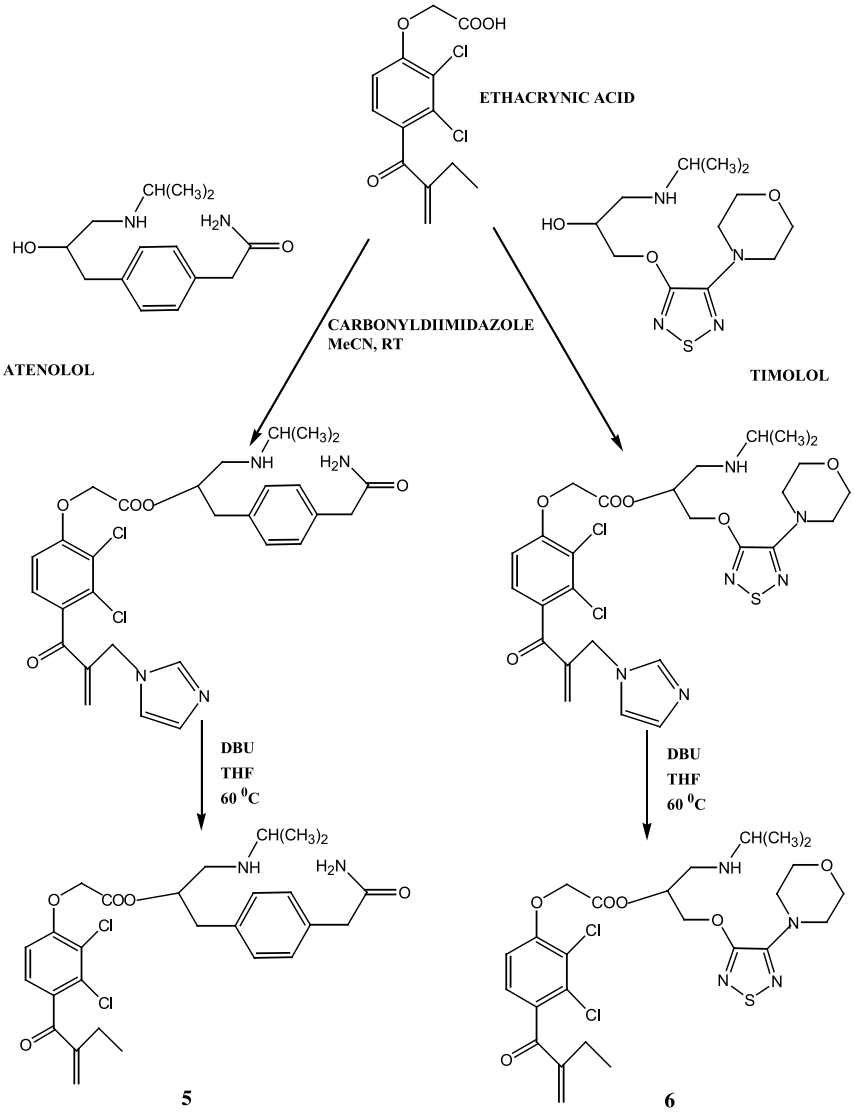

Figure 2. Synthesis of ECA–ATL (**5**) and ECA–TML (**6**) codrugs.

Table 1. The concentrations of acetonitrile in the mobile phase, the detection wavelength, and the retention times (RT) for the selected drug-conjugates and for the parent drugs[a]

Compound	Mobile phase	RT (min)
ECA–ATL, **5**	A/45% MeCN	14.3
ECA–PEG, **1a**	A/45% MeCN	12.9
ECA–PEG4, **1b**	A/45% MeCN	12.3
ECA–PEG6, **1c**	A/45% MeCN	11.8
ECA	B/30% MeCN	10.5
ATL	B/10% MeCN	8.5

[a] Wavelength was 254 nm for all except for ATL, it was 225 nm.

Enzymatic hydrolysis was determined in a similar way using human serum, and the samples were deproteinized with acetonitrile before HPLC analysis.

A number of prodrugs containing ECA linked to a short chain PEG moieties consisting of 2, 4, or 6 oxyethylene units, and two codrugs of ECA covalently linked to the β-adrenergic receptor antagonists ATL or TML, have been synthesized. The parent drugs were linked via ester moieties to permit facile non-enzymatic hydrolytic cleavage at physiological pH or via enzyme-mediated catalysis in plasma. The main goal of this approach was to improve drug delivery of the parent drugs by overcoming solubility problems and to enhance permeation through topical membranes, as well as to afford a reduction in toxicity.

The reaction of the acid chloride of ECA with a variety of short chain PEGs yielded both the desired mono-ester prodrug and di-ester side products, which were easily separated. All the ECA–PEG monoesters studied underwent facile hydrolysis in both phosphate buffer at pH 7.4 (half-lives 110–130 min) and in human serum (half-life <2 min for all ECA–PEG monoesters studied), to generate ECA and the corresponding PEG. Esterfication of ECA with ATL afforded the mono-substituted codrug (half-life in serum = 30 min, and in buffer, pH 7.4 = 14 h). The hydrolysis of the ECA–ATL codrug and the ECA–PEG mono-esters prodrugs exhibited first order kinetics under the conditions used to generate the corresponding drugs, that is, ECA and ATL, or ECA and PEG, respectively. The half-lives of the ECA prodrugs and the ECA–ATL codrug in pH 7.4 buffer and in serum are listed in Table 2.

The ECA–ATL codrug exhibited a longer half-life, both in buffer and in serum, compared to the ECA mono-esters prodrugs; this might be due to the steric hindrance around the ester linkage in the codrug that could compromise efficient binding at the active site of the plasma esterase enzyme; also, this same steric hindrance may shield the ester moiety from general base hydrolysis in phosphate buffer.

Table 2. Hydrolysis of the ECA–ATL codrug and the ECA–PEG prodrugs

Compound	Half-life (time)	
	In buffer, pH 7.4	In serum (min)
ECA–ATL, **5**	14 h	30
ECA–PEG2, **1a** ($n = 2$)	110 min	<2
ECA–PEG4, **1b** ($n = 4$)	130 min	<2
ECA–PEG6, **1c** ($n = 6$)	120 min	<2

For the treatment of glaucoma, both the ECA–ATL codrug and the ECA–PEG mono-ester prodrugs represent less toxic and more efficacious forms for the delivery of both ECA and/or ATL. Furthermore, these novel drug conjugates could provide a longer duration of action for the pharmacological effect of these compounds (i.e., reducing the IOP) compared to the parent drug(s) alone. Incorporation of a diuretic drug with a β-adrenergic receptor antagonist into a codrug structure may also provide better efficacy in the treatment of a number of other therapeutic conditions, such as ischemic heart disease, certain arrhythmias, and airway diseases such as bronchitis.

The ECA–ATL codrug and several of the ECA–PEG mono-ester prodrugs described above are currently undergoing further evaluation in in vivo studies.

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