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Research Paper

Novel Cyclic Phosphate Prodrug Approach for Cytochrome P450-activated Drugs Containing an Alcohol Functionality

Kristiina M. Huttunen,[1,4] Niina Mähönen,[2] Jukka Leppänen,[1] Jouko Vepsäläinen,[3] Risto O. Juvonen,[2] Hannu Raunio,[2] Hanna Kumpulainen,[1] Tomi Järvinen,[1] and Jarkko Rautio[1]

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Purpose. A cyclic phosphate prodrug of a descriptive molecule containing an alcohol functionality was designed, synthesized and characterized *in vitro* as a cytochrome P450 (CYP) -selective prodrug.
Materials and Methods. To achieve efficient CYP-oxidation and prodrug bioconversion, 1,3-cyclic propyl ester of phosphate was designed to have a C4-aryl substituent and synthesized using phosphorus(III) chemistry. The two-step bioconversion of the cyclic phosphate prodrug was evaluated *in vitro* using human liver microsomes and recombinant CYP enzymes.
Results. This cyclic phosphate prodrug underwent initial CYP-catalyzed oxidation and was mainly catalyzed by the CYP3A4 form. The hydroxylated product was slowly converted to a ring-opened intermediate, which subsequently transformed by β-elimination reaction to a free phosphate. The free phosphate was further dephosphorylated by microsomal phosphatases, releasing the parent molecule with a free hydroxyl group. The cyclic phosphate was reasonably stable in buffer solutions at the pH range 1.0−9.0.
Conclusions. Since CYP enzymes reside predominantly in the liver and secondarily in the small intestine, the results indicate that cyclic phosphate prodrugs represent a very feasible liver- or intestinal-targeted drug delivery strategy for drug molecules containing an alcohol functionality. This may potentially improve the efficacy and the safety profile of the alcoholic parent drugs.

KEY WORDS: bioconversion; cyclic phosphate; cytochrome P450 (CYP); prodrug; targeted drug delivery.

INTRODUCTION

After administration of a drug into the body, several pharmaceutical and pharmacokinetic barriers have to be bypassed before the drug reaches its physiological target, such as a receptor, and can exert its desired effect. Targeted drug delivery is considered as an efficient strategy to produce drugs with increased efficacy and fewer adverse effects by increasing local drug concentrations (1,2). Different drug-carrier delivery systems, such as liposomes (3), glycosylated macromolecules (4), antibodies (5) and nanoparticles (6), have been used to avoid unwanted interactions between the drug and the body, until the drug is released from the carrier at the target site. However, not all of these strategies have been resulted in clinically-proven drugs.

The prodrug approach is a promising strategy to overcome the pharmaceutical and pharmacokinetic limitations of the parent drug, since prodrugs are inactive, bioreversible derivatives of active drug molecules (1). One of the major objectives of the prodrug design is to achieve site-selective drug delivery and tissue or cell-specific enzymes offer an advantageous way of targeting drugs to the desired tissue. Cytochrome P450 (CYP) enzymes are a superfamily of heme containing proteins that catalyze xenobiotic metabolism Phase I reactions, among which the most common is substrate oxidation by insertion of one oxygen atom. Most of the CYP-catalyzed reactions lead to the detoxification of xenobiotics but CYPs can also bioactivate chemicals including drugs (7). Since CYP enzymes have broad substrate specificity and are particularly abundant in the liver and to lesser extent in the small intestine, they are ideal tools for liver- or intestinal-targeted prodrug technology.

Previously, cyclic 1,3-propanyl esters of phosphates and phosphonates have been developed for targeting various nucleoside monophosphates (NMP) to the liver (8–12). These cyclic phosphate prodrugs undergo CYP-catalyzed oxidation predominantly in hepatocytes, although CYPs are also expressed to a lesser extent in the small intestine. Oxidation results in ring opening and β-elimination of an aryl vinyl ketone. The released monophosphates or phosphonates are further converted by nucleotide kinases to active nucleoside

Kristiina M. Huttunen and Niina Mähönen have contributed equally to this work.

[1] Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland.
[2] Department of Pharmacology and Toxicology, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland.
[3] Department of Chemistry, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland.
[4] To whom correspondence should be addressed. (e-mail: Kristiina.Huttunen@uku.fi)

triphosphates (NTP), which act as inhibitors of either viral replication or cell proliferation. In addition to their broad antiviral activities, NTPs may also be used to treat other liver diseases, such as hepatocellular carcinoma.

The purpose of this study was to assess whether the cyclic phosphates could also serve as potential prodrugs targeting drugs containing an alcohol functionality to the liver or intestine. Several drugs used in the treatment of liver or intestinal diseases, such as hypolipidemic and anticancer drugs, as well as glucocorticoids and other immunosuppressive drugs, contain an alcohol functionality and would greatly benefit from liver- or intestinal-targeted drug delivery (13–15). Once liberated from the cyclic phosphates by CYP enzymes in the first step of the bioconversion process (Fig. 1), the released charged free phosphates would have only a poor ability to diffuse passively across the cell membranes and they could be further dephosphorylated by phosphatases within cells (16,17) to their corresponding alcohols (18,19).

In the present study we designed and synthesized a cyclic 1,3-propanyl phosphate ester of a parent drug with a free hydroxyl group that was aimed to undergo a two-step bioconversion process by CYP enzymes and alkaline phosphatases, respectively (Fig. 1). A simple representative alcohol with suitable detection properties and a low amount of other functional groups was used to evaluate the potential of this approach *in vitro*.

MATERIALS AND METHODS

General Synthetic Procedures

All the reactions were performed with reagents of commercial high purity quality without further purification unless otherwise mentioned. Reactions were monitored by thin-layer chromatography using aluminium sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Purifications by flash chromatography were performed on silica gel 60 F_{245} (50 mm). ^{1}H, ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Switzerland) operating at 500.13, 125.75 and 202.46 MHz, respectively. Tetramethylsilane (TMS) was used as an internal standard for ^{1}H and ^{13}C spectra as well 85% H_3PO_4 as an external standard for ^{31}P spectra. ^{1}H NMR spectra of the compounds **1, 2, 5** and **8** were analysed by using PERCH NMR Software (20). Furthermore, the products were characterized by elemental analysis (C, H, N) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy) as well by mass spectroscopy with a Finnigan LCQ quadrupole ion trap mass spectrometer (ESI-MS) equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA, USA).

4-(6-Methoxy-naphthalen-2-yl)-butan-2-ol (1)

$NaBH_4$ (1.2 g, 32.9 mmol) was added in small proportions to the solution of 4-(6-methoxy-2-naphthyl)-2-butanone (5.0 g, 21.9 mmol) in anhydrous EtOH (60 ml) at −10°C under an Ar-atmosphere. The reaction mixture was stirred for 1 h at −10°C and 3 h at RT. The solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane (DCM) (50 ml), washed carefully with cold H_2O (2×30 ml), dried over Na_2SO_4 and concentrated in vacuo to provide the alcohol **1** as white solid (4.8 g, 95%). ^{1}H NMR ($CDCl_3$): δ 1.24 (3H, d, $^{3}J_{HH}$=6.19 Hz), 1.82 (1H, dddd, $^{2}J_{HH}$=−13.63 Hz, $^{3}J_{HH}$=9.58, 6.22, 4.82), 1.85 (1H, dddd, $^{2}J_{HH}$=−13.63 Hz, $^{3}J_{HH}$=9.70, 6.85, 6.51), 2.80 (1H, ddd, $^{2}J_{HH}$=−13.93 Hz, $^{3}J_{HH}$=9.58, 6.85), 2.87 (1H, ddd, $^{2}J_{HH}$=−13.93 Hz, $^{3}J_{HH}$=9.70, 6.22), 3.84 (1H, qdd, $^{3}J_{HH}$=6.19, 6.51, 4.82 Hz), 3.90 (3H, s), 7.10 (1H, d, $^{4}J_{HH}$=2.52 Hz), 7.12 (1H, dd, $^{3}J_{HH}$=8.98 Hz, $^{4}J_{HH}$=2.52 Hz), 7.30 (1H, dd, $^{3}J_{HH}$=8.32 Hz, $^{4}J_{HH}$=1.80 Hz), 7.55 (1H, d, $^{4}J_{HH}$=1.80 Hz), 7.656 (1H, d, $^{3}J_{HH}$=8.98 Hz), 7.659 (1H, d, $^{3}J_{HH}$=8.32 Hz); ^{13}C NMR ($CDCl_3$): δ 23.68 q, 32.07 t, 40.81 t, 55.30 q, 67.52 d, 105.70 d, 118.71 d, 126.24 d, 126.84 d, 127.78 d, 128.89 d, 129.14 s, 133.0 s, 137.21 s, 157.19 s. ESI-MS: m/z = 230.1 $(M+H)^+$. Anal. Calcd for ($C_{15}H_{18}O_2$ *0.1 H_2O): C, 77.62; H, 7.90; Found: C, 77.62; H, 8.11.

Phosphoric Acid mono-[3-(6-methoxy-naphthalen-2-yl)-1-methyl-propyl] Ester (2)

The alcohol **1** (1.2 g, 5.2 mmol) and Et_3N (1.1 ml, 7.8 mmol) in cold anhydrous DCM were added dropwise to a solution of freshly distilled phosphorus oxychloride (0.57 ml, 6.3 mmol) in anhydrous DCM (50 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight, H_2O (30 ml) was added at 0°C to the reaction mixture and stirred for 1 h. The aqueous phase was saturated with NaCl, separated and extracted with DCM (2×15 ml). The combined organic phase was evaporated under reduced pressure. The residue (1.4 g) was dissolved in H_2O, treated with 2 eq. 2M NaOH and evaporated in vacuo. The sodium salt of phosphate **2** was crystallized from acetone as a light yellow solid (1.0 g, 54%). ^{1}H NMR (D_2O): δ 1.33 (3H, d, $^{3}J_{HH}$=6.24 Hz), 1.86 (1H, dddd, $^{2}J_{HH}$=−13.34 Hz, $^{3}J_{HH}$=11.48, 5.19, 6.27 Hz), 1.98 (1H, dddd, $^{2}J_{HH}$=−13.34 Hz, $^{3}J_{HH}$=11.56, 6.32, 5.25 Hz), 2.81 (1H, ddd, $^{2}J_{HH}$=−13.85 Hz, $^{3}J_{HH}$=11.56, 5.19 Hz), 2.91 (1H, ddd, $^{2}J_{HH}$=−13.85 Hz, $^{3}J_{HH}$=11.48, 5.25 Hz), 3.93 (3H, s), 4.29 (1H, qddd, $^{3}J_{HH}$=6.24, 6.32, 6.27 Hz, $^{3}J_{HP}$=8.14), 7.19 (1H, dd, $^{3}J_{HH}$=9.0, $^{4}J_{HH}$=2.61 Hz), 7.31 (1H, d, $^{4}J_{HH}$=2.61 Hz), 7.51 (1H, dd, $^{3}J_{HH}$=8.44, $^{4}J_{HH}$=1.75 Hz), 7.75 (1H, d, $^{4}J_{HH}$=1.75 Hz), 7.80 (1H, d, $^{3}J_{HH}$=8.44 Hz), 7.81 (1H, d, $^{3}J_{HH}$=9.0 Hz, 1H); ^{13}C NMR: δ 21.19 qd ($^{3}J_{CP}$=2.5 Hz), 31.28 t, 39.56 td ($^{3}J_{CP}$=5.1 Hz), 55.29 q, 70.80 dd ($^{2}J_{CP}$=5.3 Hz), 106.16 d, 118.18 d, 125.87 d, 126.86 d, 128.31 d, 128.95 s,

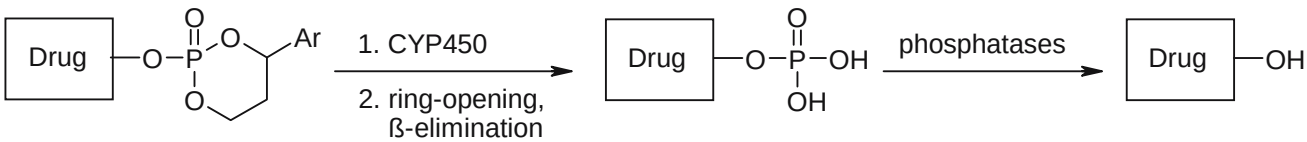

Fig. 1. Proposed bioconversion of cyclic phosphate prodrugs to alcohols in hepatocytes.

129.03 d, 132.49 s, 138.81 s, 156.31 s; ^{31}P NMR: δ 4.55. ESI-MS: anionic m/z = 309.2 $(M+H)^-$. Anal. Calcd for ($C_{15}H_{17}O_5PNa_2$ *3.5 H_2O *1.0 DCM): C, 35.87; H, 5.22; Found: C, 35.91; H, 5.67.

2-Chloro-1,3,2-dioxaphosphorinane 2-oxide (3)

Freshly distilled phosphorus oxychloride (6.06 ml, 65.0 mmol) in cold anhydrous Et_2O (80 ml) was added dropwise to a solution of 1,3-propanediol (4.7 ml, 65.0 mmol) and anhydrous Et_3N (18.0 ml, 130 mmol) in anhydrous Et_2O (150 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight. The precipitate was filtered out of the mixture and washed thoroughly with Et_2O. The filtrate was concentrated in vacuo to obtain the cyclic phosphoryl chloride **3** as a white solid (10.1 g, 99%). ^{1}H NMR ($CDCl_3$): δ 1.85 (1H, dm, $^2J_{HH}$=15.2 Hz), 2.39–2.49 (1H, m), 4.48–4.61 (4H, m); ^{13}C NMR: δ 25.71 td ($^3J_{CP}$=7.6 Hz), 70.43 td ($^3J_{CP}$=8.2 Hz); ^{31}P NMR: δ −1.68. ESI-MS: m/z = 155.0 $(M-H)^-$. Anal. Calcd for ($C_3H_6O_3ClP$ *0.01 Et_3N): C, 23.33; H, 3.94; Found: C, 23.65; H, 4.22.

2-[3-(6-Methoxy-naphthalen-2-yl)-1-methyl-propoxy]-1,3,2-dioxaphosphorinane 2-oxide (4)

The alcohol **1** (1.2 g, 5.1 mmol) in anhydrous DCM (10 ml) was added dropwise to a solution of cyclic phosphoryl chloride **3** (0.8 g, 5.1 mmol) and 1-methylimidazole (0.41 ml, 5.1 mmol) in anhydrous DCM (20 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight, the solvent was removed in vacuo and the residue was redissolved in DCM (20 ml). The organic phase was washed with 5% $NaHCO_3$ (10 ml) and water (10 ml), dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by flash chromatography eluting with petroleum ether/ethyl acetate (4/1) solution to yield the cyclic phosphate **4** as white solid (1.3 g, 71%). ^{1}H NMR ($CDCl_3$): δ 1.43 (3H, d, $^3J_{HH}$=6.2 Hz), 1.70–1.76 (1H, m), 1.90–1.99 (1H, m), 2.06–2.13 (1H, m), 2.16–2.25 (1H, m), 2.79–2.92 (2H, m), 3.90 (3H, s), 4.30–4.42 (4H, m), 4.64–4.71 (1H, m), 7.09–7.13 (2H, m), 7.29–7.32 (1H, m), 7.56–7.57 (1H, m), 7.65–7.68 (2H, m); ^{13}C NMR: δ 21.63 qd ($^3J_{CP}$=3.0 Hz), 26.03 td ($^3J_{CP}$=7.0 Hz), 31.46 t, 39.14 td ($^3J_{CP}$=6.3 Hz), 55.28 q, 68.27 td ($^2J_{CP}$=7.0 Hz), 68.41 td ($^2J_{CP}$=6.9 Hz), 75.90 dd ($^2J_{CP}$=6.1 Hz), 105.66 d, 118.75 d, 126.27 d, 126.91 d, 127.66 d, 128.90 d, 129.10 s, 133.06 s, 136.50 s, 157.25 s; ^{31}P NMR: δ −7.14. ESI-MS: m/z = 350.9 $(M+H)^+$. Anal. Calcd for ($C_{18}H_{23}O_5P$): C, 61.71; H, 6.62; Found: C, 61.59; H, 6.88.

1-Phenyl-propane-1,3-diol (5)

Ethyl benzoylacetate (5.0 g, 26.0 mmol) in anhydrous Et_2O (100 ml) was dropped to a solution of $LiAlH_4$ (2.0 g, 52.0 mmol) in anhydrous Et_2O (400 ml) at −5°C under an Ar-atmosphere. The reaction mixture was allowed to warm to RT and refluxed overnight. The mixture was cooled in an ice bath and treated with H_2O (2 ml), 1M NaOH (8 ml) and H_2O (10 ml). The precipitate was filtered out of the mixture and washed thoroughly with Et_2O. The filtrate was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography eluting with ethyl acetate to yield the yellow oily propanediol **5** (2.0 g, 51%). ^{1}H NMR ($CDCl_3$): δ 1.92 (1H, dddd, $^2J_{HH}$=−14.58 Hz, $^3J_{HH}$=6.31, 3.73, 3.56 Hz), 1.99 (1H, dddd, $^2J_{HH}$=−14.58 Hz, $^3J_{HH}$=8.99, 8.12, 3.98 Hz), 2.89 (bs, 2H), 3.82 (1H, ddd, $^2J_{HH}$=−10.85 Hz, $^3J_{HH}$=8.12, 3.73 Hz), 3.83 (1H, ddd, $^2J_{HH}$=−10.85 Hz, $^3J_{HH}$=6.31, 3.98 Hz), 4.93 (1H, dd, $^3J_{HH}$=8.99, 3.56 Hz), 7.37–7.24 (1H, m); ^{13}C NMR: δ 40.46 t, 61.35 t, 74.22 d, 125.66 d, 127.55 d, 128.50 d, 144.34 s. ESI-MS: m/z = 151.3 $(M-H)^-$.

2-[3-(6-Methoxy-naphthalen-2-yl)-1-methyl-propoxy]-4-phenyl-1,3,2-dioxaphosphorinane 2-oxide (7)

Phosphorus(V) method. Freshly distilled phosphorus oxychloride (0.60 ml, 6.6 mmol) in cold anhydrous Et_2O (20 ml) was added dropwise to a solution of 1-phenyl-propane-1,3-diol **5** (1.0 g, 6.6 mmol) and Et_3N (1.8 ml, 13.1 mmol) in anhydrous Et_2O (50 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight. The precipitate was filtered out of the mixture and washed thoroughly with Et_2O. The filtrate was concentrated in vacuo to obtain the cyclic phosphoryl chloride **6** as a brownish oily mixture of diastereomers (55:45, 1.1 g, 72%). Due to partial decomposition, the product was not purified further. ^{1}H NMR ($CDCl_3$): Major diastereomer: δ 2.04–2.13 (1H, m), 2.47–2.76 (1H, m), 4.52–4.77 (2H, m), 5.57 (1H, td, $^3J_{HH}$=11.9 Hz, $^3J_{HP}$=2.3 Hz), 7.34–7.50 (5H, m); Minor diastereomer: δ 2.17–2.47 (2H, m), 4.52–4.77 (2H, m), 5.74–5.80 (1H, m), 7.34–7.50 (5H, m); ^{31}P NMR: Major diastereomer: δ −1.85, Minor diastereomer: δ −2.37.

The alcohol **1** (1.1 g, 4.7 mmol) in anhydrous DCM (20 ml) was added dropwise to a solution of cyclic phosphoryl chloride **6** (1.1 g, 4.7 mmol) and 1-methylimidazole (0.38 ml, 4.7 mmol) in anhydrous DCM (20 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight. The solvent was removed in vacuo and the residue was purified by flash chromatography eluting with petroleum ether/ethyl acetate (2/1) solution to yield the cyclic phosphate **7** as a yellowish oily mixture of four diastereomers (85 mg, 3%). ^{1}H NMR ($CDCl_3$): δ 1.42–1.47 (3H, m), 1.90–2.16 (3H, m), 2.21–2.35 (1H, m), 2.79–2.96 (2H, m), 3.90 (3H, bs), 4.35–4.46 (1H, m), 4.66–4.76 (m, 2H), 5.64–5.70 (1H, m), 7.08–7.13 (2H, m), 7.27–7.41 (5H, m), 7.55 (1H, bs), 7.60–7.67 (2H, m); ^{13}C NMR: δ 21.80 qd ($^3J_{CP}$=2.8 Hz), 31.32 td ($^4J_{CP}$=15.1 Hz), 33.99 td ($^3J_{CP}$=5.0 Hz), 39.01–39.19 tm ($^3J_{CP}$=6.5 Hz), 55.29 q, 66.67–66.80 tm ($^2J_{CP}$=5.4 Hz), 77.40 dd ($^2J_{CP}$=6.7 Hz), 79.72–79.91 dm ($^2J_{CP}$=4.6 Hz), 105.62 d, 118.73 d, 125.55 d (2C), 126.36 d, 126.86 d, 127.72 d, 128.61 d, 128.71 d (2C), 128.92 d, 129.08 s, 133.06 s, 136.52 s, 139.25 sd ($^5J_{CP}$=7.8 Hz), 157.25 s; ^{31}P NMR: δ −3.63, −3.73. According to ^{31}P NMR spectrum, two of four possible diastereomers were observed in a ratio of 56:44). ESI-MS: m/z = 426.8 $(M+H)^+$. Anal. Calcd for ($C_{24}H_{27}O_5P$): C, 65.93; H, 6.50; Found: C, 65.71; H, 6.49.

Phosphorus(III) method. Freshly distilled phosphorus trichloride (0.17 ml, 2.0 mmol) in cold anhydrous Et_2O (10 ml) was dropped to a solution of 1-phenyl-propane-1,3-diol **5** (0.3 g, 2.0 mmol) and pyridine (0.32 ml, 4.0 mmol) in anhydrous Et_2O (10 ml) at −10°C under an Ar-atmosphere. The reaction mixture was stirred at 10°C for 2 h, the precipitate was filtered out of the mixture and washed

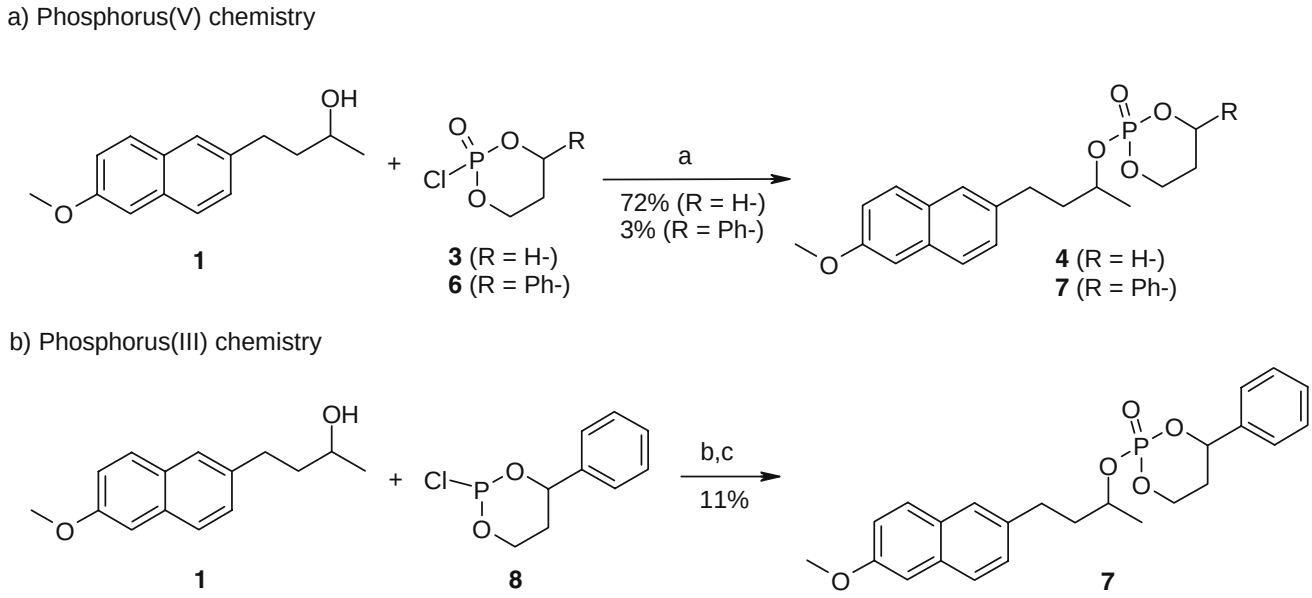

Fig. 2. Synthetic pathways to the cyclic phosphate esters. Reaction conditions: (a) *N*-MeIm, DCM, 0°C, 30 min; 24°C, 20 h; (b) DIPEA, ACN, 0°C, 30 min; 24°C, 20 h; (c) TBHP, 0–24°C, 4 h.

thoroughly with Et_2O. The filtrate was concentrated in vacuo to obtain the yellowish oily cyclic chlorophosphane **8** (0.38 g, 89%). Due to partial decomposition, the product was not purified further. ^{1}H NMR ($CDCl_3$): δ 2.00 (1H, dddd, $^2J_{HH}$=−14.54 Hz, $^3J_{HH}$=2.33, 2.24, 1.70 Hz), 2.51 (1H, dddd, $^2J_{HH}$=−14.54 Hz, $^3J_{HH}$=11.95, 11.03, 5.36 Hz), 4.19 (1H, dddd, $^2J_{HH}$=−11.36 Hz, $^3J_{HH}$=11.03, 1.70 Hz, $^3J_{HP}$=4.49), 4.88 (1H, dddd, $^2J_{HH}$=−11.36 Hz, $^3J_{HH}$=5.36, 2.24 Hz, $^3J_{HP}$=12.76), 5.69 (1H, ddd, $^3J_{HH}$=11.95, 2.33 Hz, $^3J_{HP}$=5.16), 7.33 (m, 5H); ^{13}C NMR: δ 35.75 td ($^3J_{CP}$=2.8 Hz), 62.45 td ($^2J_{CP}$=3.6 Hz), 73.54 dd ($^2J_{CP}$=3.2 Hz), 125.69 d, 128.47 d, 128.71 d, 140.0 sd ($^3J_{CP}$=2.3 Hz).

The cyclic chlorophosphane **8** (0.38 g, 1.8 mmol) in anhydrous acetonitrile (ACN) (5 ml) was added dropwise to a solution of alcohol **1** (0.4 g, 1.8 mmol) and diisopropylethylamine (DIPEA) (0.61 ml, 3.5 mmol) in anhydrous ACN (10 ml) at 0°C under an Ar-atmosphere. The reaction mixture was stirred at RT overnight, cooled in an ice bath and treated with *tert*-butyl hydroperoxide (TBHP) (0.59 ml, 5.3 mmol) for 4 h. The solvent was removed in vacuo and the residue was purified by flash chromatography eluting with petroleum ether/ethyl acetate (3/1) solution to yield the cyclic phosphate **7** as a brownish oily mixture of four diastereomers (90 mg, 11%). The chemical shifts of the NMR spectra as well as the ESI-MS spectrum and elemental analysis were identical to the spectra and analysis of compound **7** obtained by the phosphorus(V) method.

Phosphoric Acid 3-hydroxy-3-phenyl-propyl ester 3-(6-methoxy-naphthalen-2-yl)-1-methyl-propyl Ester (9)

The buffer solutions of chemical degradation studies were combined and evaporated under reduced pressure. The residue was extracted with Et_2O (2×15 ml) and the organic phase was washed with H_2O (10 ml), dried over Na_2SO_4 and concentrated in vacuo to provide the hydrolysis product **9** of the cyclic phosphate **7** as a yellowish oily compound (20 mg). ^{1}H NMR (CD_3OD): δ 1.36 and 1.37 * (3H, d, $^3J_{HH}$=6.1, 5.9 Hz), 1.87–1.96 (1H, m), 1.98–2.10 (3H, m), 2.74–2.93 (2H, m), 3.89 (3H, s), 3.97–4.05 (1H, m), 4.12–4.20 (1H, m), 4.42–4.51 (1H, m), 4.78–4.82 (1H, m), 7.08 (1H, dd, $^3J_{HH}$=9.0, $^4J_{HH}$=2.6 Hz), 7.15 (1H, bs), 7.20–7.24 (1H, m), 7.27–7.35 (5H, m), 7.57 (1H, bs), 7.63–7.69 (2H, m); ^{13}C NMR ($CDCl_3$): δ 21.58 qd ($^3J_{CP}$=3.2 Hz), 29.71 t, 31.38 td ($^3J_{CP}$=6.1 Hz), 39.26 td ($^3J_{CP}$=7.1 Hz), 55.24 q, 63.88 t, 70.35 dd ($^2J_{CP}$=6.9 Hz), 75.45 dd and 75.53 dd * ($^2J_{CP}$=6.9, 5.6 Hz), 105.59 d, 118.59 d and 118.67 d *, 125.38 d, 126.31 d, 126.77 d and 126.79 d *, 127.69 d and 127.74 d *, 127.80 d and 127.82 d *, 128.05 d, 128.39 d, 128.66 s, 128.96 d, 129.08 d and 129.11 d *, 133.96 s and 132.99 s *, 136.84 s and 136.88 s *, 144.46 s, 157.13 s and 157.17 s *;

Table I. Half-Lives of the Cyclic Phosphate **7** in 50 mM Buffer Solutions and in Alkaline Phosphatase (ALP) Solution at 37°C (Mean±SD, n=3 Unless Otherwise Mentioned)

	$T_{1/2}$ (h)						
Compound	pH 1.0	PH 3.0	pH 5.0	pH 7.4[a]	pH 7.4[b]	pH 9.0	ALP
7	68±5.6	73±4.3	73±4.0 $^{n=2}$	78±0.02 $^{n=2}$	74±2.2	78±0.4	–[c]

[a] Phosphate buffer. [b] Borate buffer. [c] No degradation was observed during the 24 h incubation.

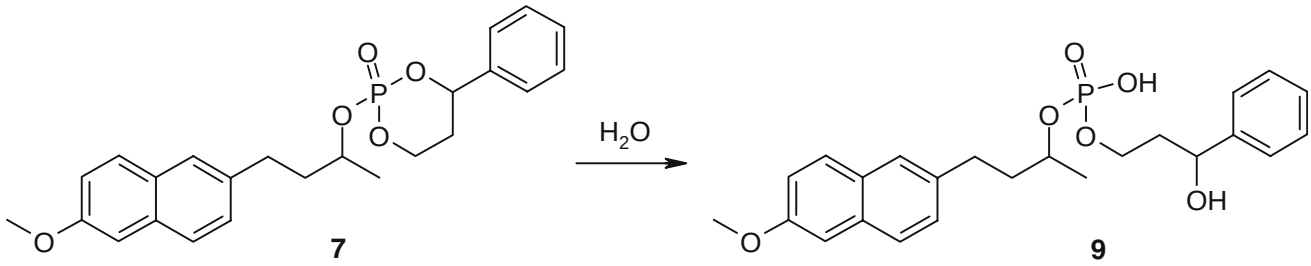

Fig. 3. Hydrolysis of the cyclic phosphate **7** in buffer solutions.

^{31}P NMR ($CDCl_3$): δ −1.52, −1.39. ESI-MS: m/z = 443.1 $(M-H)^-$. * Two chemical shifts were detected due to the chiral centres of the molecule **9**.

HPLC Analysis

The analysis was performed on the high performance liquid chromatography (HPLC) system, which consisted of an Agilent 1100 binary pump (Agilent Technologies Inc., Wilmington, DE, USA), a 1100 micro vacuum degasser, a HP 1050 Autosampler, a HP 1050 variable wavelength detector (operated at 225 nm) and an Agilent Zorbax SB-C18 analytical column (4.6 mm×150 mm, 5 μm). The chromatographic separations were achieved by using isocratic elution of acetonitrile and 50 mM phosphate buffer (pH 2.0) with a ratio of 55:45 (v/v) at the flow rate 1.0 ml/min at 25°C. The retention times of analytes were 11.9 min for the cyclic phosphate prodrug **7**, 5.1 min for the parent alcohol molecule **1** and 2.8 min for the free phosphate **2**.

Hydrolysis in Aqueous Solutions

The rates of chemical hydrolysis of the cyclic phosphate **7** were determined at 37°C in 50 mM (ionic strength 0.15) HCl buffer at pH 1.0, citric acid buffer at pH 3.0, acetate buffer at pH 5.0, phosphate buffer at pH 7.4, borate buffer at pH 7.4 and at pH 9.0. The incubation mixtures were prepared by dissolving 12 mM of prodrug **7** in EtOH in preheated buffer solutions. The EtOH concentration in the incubation mixtures was 1.5% and the prodrug concentration about 200 μM. The mixtures were incubated at 37°C and the samples were withdrawn at appropriate intervals. Acetonitrile (ACN) was added to the samples (1:1, v/v) to hinder further hydrolysis during the HPLC analyses. The pseudo-first-order half-lives ($t_{1/2}$) for the hydrolysis of the prodrug were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

Biological Material

Human liver samples used in this study were obtained from the University Hospital of Oulu (Oulu, Finland) as surplus from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Oulu, Finland. The livers were transferred to ice immediately after surgical excision, cut into pieces, snap-frozen in liquid nitrogen and stored at −80°C until the microsomes were prepared by standard differential ultracentrifugation. The metabolic characteristics of these microsomes have been published earlier (21). A weight-balanced microsomal pool of seven liver microsomal preparations which have been extensively characterized for primary metabolic screening was employed.

Alkaline phosphatase EC 3.1.3.1 (Type VII-S: from bovine intestinal mucosa, 2.430 units/mg protein) was purchased from Sigma (St. Louis, MO, USA). Baculovirus-insect cells expressing human recombinant CYP1A2, CYP3A4, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 were purchased from Gentest Corp. (Woburn, MA, USA).

Hydrolysis in Alkaline Phosphatase Solution

Hydrolysis in alkaline phosphatase solution was determined by incubating mixture (final volume of 1,600 μl) of 50 μM cyclic phosphate **7** in ethanol or 50 μM free phosphate **2** in water, 50 mM Tris-HCl buffer (pH 7.4) and 4 μl (24.3 units) alkaline phosphatase at 37°C. In blank solutions, alkaline phosphatase was replaced with the same volume of water. The ethanol concentration in the incubation mixture was 1% or less. The incubation time was 10 min for the free phosphate **2** and 24 h for the cyclic phosphate **7** and the samples were withdrawn at appropriate intervals. The enzymatic reaction was terminated by the addition of the same amount of ice-cold stopping-solution (93% acetonitrile, 7% orthophosphoric acid) as the sample. The samples were kept on ice, centrifuged for 15 min at 11000 rpm, and the supernatant was analyzed by the HPLC. Half-lives ($t_{1/2}$) for the hydrolysis of the free phosphate in alkaline phosphatase solution were calculated from the slope of the linear portion of the plotted logarithm of remaining free phosphate against time.

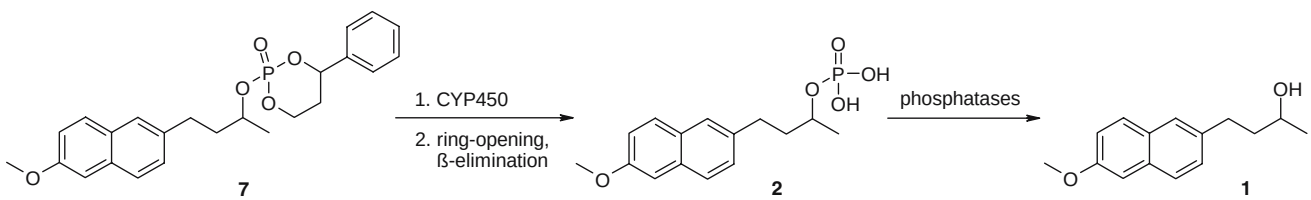

Fig. 4. The two-step bioconversion of the cyclic phosphate **7** to the free phosphate **2** and further to the alcohol **1** *in vitro*.

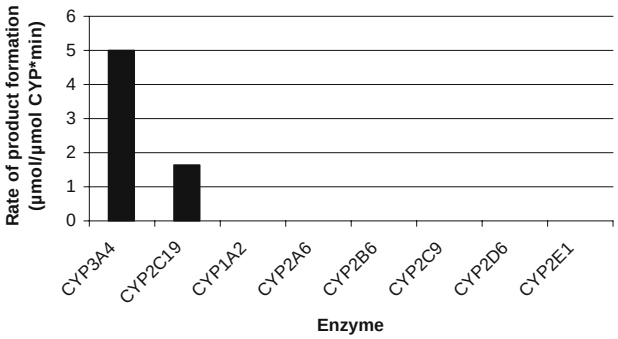

Fig. 5. The formation of the free phosphate **2** with eight recombinant human CYP forms.

In vitro Metabolism and Enzyme Kinetic Analyses

The oxidation of the cyclic phosphate **7** was determined in isolated human liver microsomes and with eight recombinant human CYP forms (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4). A typical incubation mixture, in a final volume of 150–1,600 µl, contained 50 µM of cyclic phosphate **7** in ethanol, 50 mM of Tris-HCl buffer (pH 7.4) and 100 µg microsomal protein or 5 pmol of recombinant CYP enzyme. Ethanol concentrations in the incubation mixtures were 1% or less. The reaction was started by the addition of a NADPH-regenerating system (1.15 mM NADP, 12.5 mM isocitric acid, 56.25 mM KCl, 187.5 mM Tris-HCl, 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$ and 0.77 µl/ml isocitric acid dehydrogenase, pH 7.4) following 2 min of pre-incubation at 37°C. In blank solutions, liver microsomes or CYPs were replaced with the same volume of water. Incubation time ranged from 15 min to 24 h depending on the experiment. The samples were withdrawn at appropriate intervals and the reactions were terminated by the addition of the same amount of ice-cold stopping-solution (93% acetonitrile, 7% orthophosphoric acid) as the sample. The samples were kept on ice, centrifuged for 15 min at 11000 rpm, and the supernatant was analyzed by the HPLC.

Enzyme kinetic studies of oxidation of the cyclic phosphate prodrug **7** by recombinant CYP3A4 and CYP2C19 were conducted in the same conditions as described above using 100 µM–10 mM of cyclic phosphate **7**, 1 pmol of CYP3A4, 5 pmol of CYP2C19 and incubating for 20 min (CYP3A4) or 240 min (CYP2C19).

The bioconversion of the alcohol **1** was determined in human liver microsomes. The incubation mixture contained 10 µM of alcohol **1** in ethanol, 50 mM of Tris-HCl buffer (pH 7.4) and 100 µg of microsomal protein. The experiment was carried out as described earlier for the cyclic phosphate **7**. Incubation time was 360 min.

Half-lives ($t_{1/2}$) for the enzymatic degradations of the prodrug **7**, the free phosphate **2** and the alcohol **1** at liver microsomes and recombinant CYP enzymes were calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time.

RESULTS

Synthesis of Cyclic Phosphate Prodrugs

Two different 1,3-cyclic propyl esters of phosphate, with and without an aryl ring substituent, were synthesized (Fig. 2). The unsubstituted cyclic phosphate **4** was obtained at good yields using the phosphorus(V) approach, but the yields of the cyclic phosphate **7** with the C4-aryl ring substituent were considerably lower (Fig. 2a). The phosphorus(III) approach produced slightly better yields of the cyclic phosphate **7** (Fig. 2b). The phosphorus(V) method involved the treatment of the alcohol **1** with phosphoryl chlorides **3** or **6** in the presence of 1-methylimidazole (22). In the phosphorus(III) method, the alcohol **1** was first reacted with chlorophosphane **8** in the presence of diisopropylethylamine (DIPEA) and subsequently oxidized with *tert*-butyl hydroperoxide (TBHP) to the cyclic phosphate **7** (8). The cyclic phosphoryl chlorides **3** and **6**, as well the chlorophosphane **8**, were prepared by treating phosphorus oxychloride or phosphorus trichloride, respectively, with propane-1,3-diols in the presence of triethylamine.

Stability of the Cyclic Phosphate Prodrug in Buffer Solutions and in Alkaline Phosphatase Solution

The chemical stability of the cyclic prodrug **7** in aqueous solutions was evaluated at pH 1.0−9.0, with half-lives ranging from 68 to 72 h (Table I). The quantitative hydrolysis followed first-order kinetics and led to the formation of a stable ring-opened product **9** (Fig. 3). The enzymatic stability of cyclic phosphate **7** was also studied in alkaline phosphatase (ALP) solution, and no degradation was observed during 24 h incubation (Table I).

Bioconversion of the Cyclic Phosphate *in Vitro*

The first step in the bioconversion process of the cyclic phosphate **7** to the corresponding alcohol **1** (Fig. 4), is a CYP-catalysed oxidation reaction producing a free phosphate **2** as an intermediate. This reaction was initially studied in isolated human liver microsomes and liver microsomes of rats treated with CYP-inducing agents, dexamethasone (CYP3A) and phenobarbital (CYP2B) or of mice treated with pyrazole (CYP2A) (23). The most efficient reactions were detected in

Table II. Half-Lives of the Cyclic Phosphate **7**, the Free Phosphate **2** and the Alcohol **1** in Human Liver Microsomes (HLM), Human Recombinant CYP Enzymes (3A4, 2C19) and Alkaline Phosphatase (ALP) Solution (Mean ± SD, n=3)

Compound 7		Compound 2		Compound 1	
Enzyme	$T_{1/2}$ (min)	Enzyme	$T_{1/2}$ (min)	Enzyme	$T_{1/2}$ (min)
HLM	14±0.57	ALP	2.2±0.0058	HLM	13±0.51
CYP3A4	34±1.7	HLM (+NADPH)	37±1.5		
CYP2C19	290±16	HLM (−NADPH)	43±0.36		

Table III. Enzyme Kinetic Parameters for the Cyclic Phosphate **7**

Recombinant enzyme	K_m [μM] of **7**	95% confidence intervals	$V_{max(a)}$	95% confidence intervals	$V_{max}/K_{m(b)}$
3A4	37	17–70	83	64–170	2.2
2C19	20	5–50	0.65	0.51–0.92	0.033

[a] μmol free phosphate **2** (pmol CYP)$^{-1}$ (min)$^{-1}$. [b] l (pmol CYP)$^{-1}$ (min)$^{-1}$

isolated human liver microsomes and dexamehasone-pretreated rat liver microsomes (data not shown).

CYP enzyme specificity was further evaluated with eight recombinant human CYP forms (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4). The human liver microsomes and recombinant CYP3A4 and CYP2C19 produced the free phosphate **2** from the cyclic phosphate **7**, while CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6 and CYP2E1 did not catalyze this reaction (Fig. 5).

The oxidation of the cyclic phosphate **7** occurred in human liver microsomes and recombinant CYP3A4 and CYP2C19 with half-lives of 14, 34 and 290 min, respectively (Table II). The formation rates of free phosphate **2** were 5.0 μmol (μmol enzyme)$^{-1}$ (min)$^{-1}$ with CYP3A4 and 1.6 μmol (μmol enzyme)$^{-1}$ (min)$^{-1}$ with CYP2C19. Enzyme kinetic parameters, K_m and V_{max}, were determined for the cyclic phosphate **7** using recombinant CYP3A4 and CYP2C19. Four separate experiments were analyzed by non-linear regression using GraphPad Prism software (Table III).

The second step of the bioconversion process of the cyclic phosphate **7** (Fig. 4) is the hydrolysis of the free phosphate **2** to the alcohol **1** catalyzed by phosphatases. The reaction was initially studied in alkaline phosphatase solution with a half-life of 2.2 min (Table II), releasing the alcohol **1** quantitatively. The hydrolysis of the free phosphate **2** was also evaluated in human liver microsomes with and without NADPH, an electron source for the CYP-catalyzed reactions, with half-lives of 37 and 43 min, respectively (Table II).

In the human liver microsomes, the bioconversion of cyclic phosphate prodrug **7** proceeded through two phases catalyzed by CYP and phosphatase enzymes, respectively, and the formation of the free phosphate **2** preceded the release of the alcohol **1** (Fig. 4). However, to detect also the parent molecule **1**, a long incubation time was needed. The parent molecule, the alcohol **1** was furthermore degraded in the human liver microsomes with half-life of 13 min (Table II).

DISCUSSION

The cyclic phosphate prodrug approach offers a very feasible liver-targeted drug delivery strategy for drug molecules containing an alcohol functionality. The syntheses of cyclic phosphates are straightforward, although the variable yields of synthetic conversion to the cyclic prodrug may offer challenges depending on the disposition of the parent drug and the employed phosphorus approach. In this study, the phosphorus(III) approach produced better yields of the cyclic phosphate **7** rather than the phosphorus(V) method, although due to steric and inductive effects the overall yields were quite poor (Fig. 2).

At the beginning, an unsubstituted cyclic phosphate **4** (Fig. 2) was also evaluated as a CYP-selective prodrug. However, the bioconversion of this cyclic phosphate to the free phosphate **2** did not occur in the liver microsomes, probably due to incorrect CYP-catalyzed ring oxidation (10,13,14) resulting in a rearrangement of phosphate in the aliphatic chain. This was confirmed by isolating the compound produced by the CYP-catalyzed reaction and characterizing the product by both electrospray ionization quadrupole ion trap mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectrometry. According to MS and MS^2 fragmentation spectra, a PO_3-group was present in this compound. Also a naphthalene ring and the O-CH(R)-CH_2-O moiety were detected in ^{1}H and ^{1}H-^{1}H correlated NMR spectra. To achieve the CYP-oxidation at the desired carbon followed by efficient prodrug cleavage, we came to the same conclusion as Erion et al. (2004), i.e. the 1,3-cyclic propyl ester of phosphate has to possess a C4-aryl ring substituent (cyclic phosphate **7**, Fig. 2) (10). In that case, the ring of the CYP-oxidized product will open to the form which subsequently undergoes a β-elimination reaction and produces the free negatively charged phosphate (Fig. 6).

Due to proper chemical stability of cyclic phosphates in aqueous solutions and extreme enzymatic stability toward alkaline phosphatase, cyclic phosphates are potential site-selective drug carriers and suitable for oral drug delivery. The hydrolytic rate of the cyclic phosphate **7** in aqueous buffer solutions at pH 1.0−9.0 was not dependent to any extent on pH (Table I). Furthermore, the catalytic effect of phosphate ions on the hydrolysis of the phosphate ester group at pH 7.4 was not significant.

According to the bioconversion results (Table II, Fig. 4), the first step of the bioconversion process was catalyzed by CYP enzymes and the main hepatic CYP form oxidizing the cyclic phosphate **7** to the free phosphate **2** was CYP3A4 (Table III, Fig. 5). CYP3A4 was also a more efficient enzyme than CYP2C19 based on V_{max}/K_m values that were 2.2 and 0.033 l (pmol CYP)$^{-1}$(min)$^{-1}$, respectively. These findings are consistent with the earlier studies on cyclic phosphates of nucleosides (10) and also with our results obtained with microsomes from dexamethasone (CYP3A)–pretreated rats. The formation of the free phosphate **2** was considerably affected by the dexamethasone enzyme induction (data not shown).

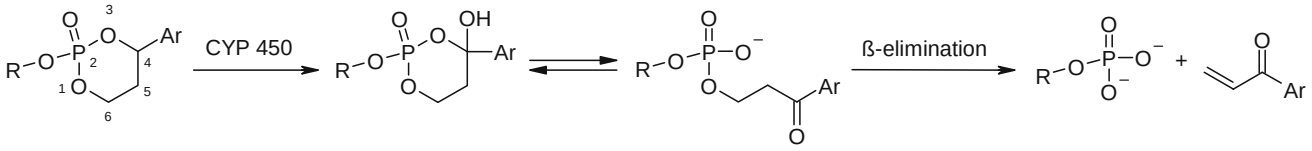

Fig. 6. Proposed CYP-catalyzed bioconversion mechanism of cyclic phosphate prodrugs.

Based on the half-lives of CYP-catalyzed oxidation of the cyclic phosphate **7** and dephosphorylation of the free phosphate **2** (Table II, Fig. 4), the formation of the free phosphate **2** appeared to be the slowest step of the bioconversion process. Since the hydrolysis of the free phosphate **2** occurred also without NADPH in human liver microsomes, the dephosphorylation was apparently catalyzed by microsomal dephosphorylative enzymes rather than via CYP enzymes. The slow formation rate of the free phosphate **2** is probably a consequence of a reversible reaction of ring opening after oxidation reaction of the cyclic phosphate **7** (Fig. 6) (10). The slow ring opening reaction, and consequently, the long prodrug conversion time can most likely be overcome by using an electron withdrawing group, such as chlorine, in the C4-aryl ring substituent (10–12). Furthermore, the prodrug byproduct of the CYP-catalyzed and followed spontaneous reactions, aryl vinyl ketone (Fig. 6) associated with toxicity, is rapidly and quantitatively detoxified with intracellular glutathione in the liver (11,12).

In the experiments with human liver microsomes, the parent alcohol **1** was found to be unstable in the presence of active CYP enzymes and the formation of the alcohol **1** from the cyclic phosphate prodrug **7** was not entirely quantitative. The methoxy group of the alcohol **1** was most likely oxidized by CYP enzymes (Table II) (24). Therefore, each cyclic phosphate prodrug of distinct parent drug molecule should undergo full metabolic and toxicological evaluation during the phase of prodrug development. Optical purity of cyclic phosphate prodrugs should also be taken into account when evaluating the pharmacological properties of the real parent drugs. In this study unseparated diastereomeric cyclic phosphates were used to evaluate the prodrug bioconversion and it showed to be insensitive to the absolute stereochemistry at the C4-position.

Our results clearly indicate that the quite fast and CYP-catalyzed cleavage combined with high stability outside of the liver mean that these kinds of cyclic phosphate prodrugs could be considered as potential candidates to achieve increased drug levels in the liver.

CONCLUSIONS

The 1,3-cyclic propanyl phosphate ester with a C4-aryl ring substituent was evaluated *in vitro* as a potential means for CYP-mediated drug delivery for a representative molecule containing an alcohol functionality. The results confirmed that the cyclic phosphate prodrug is oxidized by liver microsomal CYP enzymes, primarily by CYP3A4 and CYP2C19, to the free phosphate and dephosphorylated further by microsomal phosphatases to the parent alcohol. Consequently, this cyclic phosphate prodrug approach is a novel extension to the cyclic phosphate prodrugs of nucleosides and represents an efficient drug delivery strategy for targeting drug molecules containing the alcohol functionality to the liver or intestine. Since many drugs possess an alcohol functionality and exert their action specifically in the liver or intestine, this site-specific prodrug approach could serve as a potential method to increase the therapeutic efficacy and/or to decrease adverse effects of these drugs.

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