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JUHA JUNTUNEN

Water-Soluble Prodrugs of Cannabinoids

Doctoral dissertation

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Department of Pharmaceutical Chemistry
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ABSTRACT

Modern drug development techniques optimize drug-receptor interactions identifying lipophilic compounds that possess good affinity for the receptor *in vitro*. However, these compounds may fail later in the drug development process because of their poor physicochemical properties, such as low aqueous solubility which may prevent the drug molecule from reaching its site of action. Various formulation strategies such as the use of cosolvents, surfactants and cyclodextrins have been used to overcome this problem. Development of more soluble analogs may prove to be difficult, since the receptor binding is also affected when irreversible changes are made to the active molecule structure.

Reversible structural changes are also possible by using the prodrug strategy. Prodrugs are molecules which must undergo chemical or enzymatical transformation within the body before exerting their pharmacological effects. Two different prodrug strategies can be used in order to improve the aqueous solubility: (1) by decreasing the melting point or (2) by introducing polar, ionizable or permanently charged group to the molecule.

Cannabinoids are compounds that interfere with the cannabinoid receptors of the body. Endocannabinoids, such as anandamide (AEA), are endogenous compounds that bind to cannabinoid receptors. There are many potential therapeutical applications for cannabinoids including control of pain, regulation of immune response, antitumor properties, control of appetite, treatment of glaucoma as well as applications in various neurological disorders. However, the pharmaceutical usefulness of cannabinoids is severely limited by their extremely poor aqueous solubilities.

In the present study, several water-soluble prodrugs of endocannabinoids and *R*-methanandamide were designed, synthesized and their properties were evaluated *in vitro*. The usefulness of prodrugs in ophthalmic applications was evaluated in an *in vitro* corneal permeation study and some of the prodrugs were also tested *in vivo* for their ability to reduce intraocular pressure after their topical administration.

Phosphate esters of anandamide, *R*-methanandamide and noladin ether increased the aqueous solubility of parent compounds considerably. The phosphate esters were chemically relatively stable and rapidly released the parent compound after enzymatic hydrolysis.

Tertiary and quaternary methylpiperazine prodrugs of anandamide were more water-soluble than anandamide. Since the pK_a values of tertiary prodrugs were around 7.4, the solubility is strongly pH-dependent near the physiological pH. In contrast, the aqueous solubility of quaternary prodrugs is pH-independent, which is one of their greatest advantages, ensuring good aqueous solubility at all pH values. Some of the tertiary and quaternary prodrugs were not enzymatically cleaved possibly due to steric reasons. However, tertiary prodrug **12** and quaternary prodrug **14** readily released AEA after enzymatic hydrolysis.

The fluxes of phosphate ester prodrugs in the cornea permeation study were smaller than the fluxes of the parent compounds solubilized with cyclodextrin. However, as an example, the flux of noladin ether prodrug was much better than the flux of noladin ether suspension without cyclodextrin. The prodrugs released the parent compound just before or during the absorption, since only the parent compound was detected on the receiver side.

Topically administered phosphate esters of *R*-methanandamide and noladin ether decreased intraocular pressure in normotensive rabbits, this effect was comparable to the parent compounds in a cyclodextrin formulation.

In conclusion, the present results suggest that the prodrug approach is a feasible method for improving water solubility of cannabinoids and other poorly soluble compounds. In addition, *in vitro* and *in vivo* studies show that the phosphate prodrug approach is suitable for ocular applications.

National Library of Medicine Classification: QV 785, QV 744, QV 77.7, WW 166
Medical Subject Headings: prodrugs/chemical synthesis; drug design; cannabinoids; endocannabinoids; solubility; water; esters; phosphates; hydrolysis; permeability; cornea; intraocular pressure; administration, topical; ophthalmic solutions

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I am grateful to the official reviewers of this dissertation, Jeff Krise Ph.D. and Jari Ratilainen Ph.D. for their constructive comments and useful advice. I am also honoured to have Professor Már Másson as my official opponent.

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I want to warmly thank my parents, Ritva and Matti Juntunen, who have always encouraged and supported me. Many thanks also to my dear sister, Anne-Mari, and her husband, Sami, and all their four children Noora, Nelli, Nanna and Nuppu.

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Kuopio, November 2005

Juha Juntunen

ABBREVIATIONS

AEA	arachidonylethanolamide (anandamide)
2-AG	2-arachidonyl glycerol
BCS	biopharmaceutic drug classification system
CB_1	cannabinoid receptor type 1
CB_2	cannabinoid receptor type 2
CD	cyclodextrin
CMC	critical micelle concentration
DMF	dimethyl formamide
DMAP	4-*N,N*-dimethylaminopyridine
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
FAAH	fatty acid amide hydrolase
HPLC	high performance liquid chromatography
IOP	intraocular pressure
J_{ss}	steady-state flux
log D	logarithm of the distribution coefficient
m.p.	melting point
MS	mass spectrometry
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
PG	propylene glycol
pK_a	negative logarithm of the ionization constant
PMSF	phenylmethanesulfonylfluoride
rt	room temperature
S_o	intrinsic solubility
S_{tot}	total solubility
$t_{½}$	half life
THC	tetrahydrocannabinol
TMS	tetramethylsilane
$TRPV_1$	transient receptor potential vanilloid-1

LIST OF ORIGINAL PUBLICATIONS

The present doctoral dissertation is based on the following original papers I-IV:

I Juntunen J, Huuskonen J, Laine K, Niemi R, Taipale H, Nevalainen T, Pate DW, Järvinen T: Anandamide prodrugs 1. Water-soluble phosphate esters of arachidonyl ethanolamide and *R*-methanandamide. Eur J Pharm Sci 19: 37-43, 2003.

II Juntunen J, Niemi R, Vepsäläinen J, Maja T, Järvinen T: Anandamide prodrugs 2. Water-soluble esters containing tertiary and quaternary heterocyclic nitrogen. Submitted.

III Juntunen J, Vepsäläinen J, Niemi R, Laine K, Järvinen T: Synthesis, *in vitro* evaluation and intraocular pressure effects of water-soluble prodrugs of endocannabinoid noladin ether. J Med Chem 46: 5083-5086, 2003.

IV Juntunen J, Järvinen T, Niemi R: *In vitro* corneal permeation of cannabinoids and their water-soluble phosphate ester prodrugs. J Pharm Pharmacol 57: 1153-1157, 2005.

CONTENTS

1 INTRODUCTION

Modern drug development techniques like combinatorial chemistry and high throughput screening produce large numbers of highly potent molecules, many of which have high molecular weights, high lipophilicities and low aqueous solubilities (Lipinski et al. 2001). As the drug molecule has to dissolve before it can be absorbed into the body (Amidon et al. 1995), it is not surprising that poor aqueous solubility is one of the main causes for failure during drug development (Opera 2000). Various formulation strategies have been used to overcome poor aqueous solubility of drugs, including the use of co-solvents, surfactants and soluble complexing agents like cyclodextrins (Fleisher et al. 1996; Loftsson and Brewster 1996). Optimization of physicochemical properties in drug design often involves synthesis of more water-soluble analogs of high affinity receptor ligands, but this may be difficult since their interaction with the receptor will also be affected (Stella 2004).

Instead of making irreversible modifications to a drug molecule, reversible changes are also possible by using the prodrug strategy (Sinkula and Yalkowsky 1975). According to a strict definition, prodrugs are pharmacologically inactive molecules which must undergo chemical or enzymatical transformation within the body before exerting their pharmacological or therapeutic effect (Stella et al. 1985). However, in practice, the prodrug molecule itself may possess some activity although the released parent compound is believed to be the main active species. Depending on the nature of the physicochemical property which causes the poor aqueous solubility, two main prodrug approaches can be used to improve solubility: to decrease the melting point or to introduce ionizable or permanently charged groups into the molecule (Fleisher et al. 1996).

Cannabinoids are compounds that interfere with the cannabinoid receptors of the body (Pate 2002). The active constituent of cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC) was isolated in 1964 (Gaoni and Mechoulam 1964). The discovery of the cannabinoid receptor in 1988 (Devane et al. 1988) triggered the search for endogenous compounds that bind to this receptor. The first endogenous cannabinoid receptor ligand, anandamide, was discovered in 1992 (Devane et al. 1992). Since these breakthrough findings, there has been growing interest to search for potential therapeutic applications for compounds that affect the endocannabinoid system (Piomelli et al. 2000). These drugs have several interesting potential applications e.g. in the control of pain (Pertwee 2001), the regulation of the immune response (Berdyshev 2000) and they have also antitumor properties (De Petrocellis et al. 2000) and effects on the cardiovascular system (Kunos et al. 2000) as well as applications in various neurological disorders (Croxford 2003). Cannabinoids may also have potential in the treatment of glaucoma due to their ability to reduce intraocular pressure and their neuroprotective properties (Järvinen et al. 2002). However, the pharmaceutical usefulness of compounds affecting the cannabinoid system is often hampered by their extremely poor aqueous solubilities.

The objective of the present study was to design, synthesize and evaluate various water-soluble prodrugs of endocannabinoids and their analogs.

2 REVIEW OF LITERATURE

2.1 Aqueous solubility

Solubility can be defined as the concentration of solute in a saturated solution at a given temperature (Martin and Bustamante 1993). In general, the drug has to be in a molecular dispersed form (i.e. in solution) before it can be absorbed (Florence and Attwood 1998). However, there is evidence that very small particles may be transported via endocytosis in some special cases (Desai et al. 1997).

The dielectric constant has been used as a crude indicator of a solvent's polarity, although it actually is a measure of solvent's ability to insulate opposite charges from each other in an electrical condenser (James 1986; Solomons 1992). Water is a polar solvent and it has a high dielectric constant (Table 1). This means that water dissolves polar compounds better than nonpolar compounds. Water and other polar solvents may dissolve substances via at least three different mechanisms. Due to its high dielectric constant, water can overcome the interionic attraction of anions and cations in a crystalline salt. In addition, water can break covalent bonds of strong electrolytes which leads to ionization. The third way in which water can dissolve compounds is by forming hydrogen bonds with solvate molecules (McElvain 1945; Martin and Bustamante 1993). In addition to the dielectric constant, other polarity indexes, such as solubility parameter, interfacial tension, surface tension and partition coefficient, are also used to describe solvent polarity (Rubino and Yalkowsky 1987).

Table 1: Dielectric constants of solvents (modified from Martin and Bustamante 1993)

Dielectric constant		Solvent
Decreasing polarity ↓	80	Water
	50	Glycols
	30	Methyl and ethyl alcohol
	20	Aldehydes, ketones and higher alcohols, ethers, esters and oxides
	5	Hexane, benzene, carbon tetrachloride, ethyl ether, petroleum ether
	0	Mineral oil and fixed vegetable oils

Although the determination of aqueous solubility is a fairly simple procedure, it can be very time consuming when a large number of compounds need to be evaluated. Knowledge of the compound solubility at an early stage during the drug development process is vital for the successful identification of promising compounds with favourable physicochemical properties. However, measurement of thermodynamic solubility with traditional methods at the early

discovery stage may not be feasible due to large sample requirements, low throughput and labour-intensive sample preparation.

Therefore, automated systems for rapid kinetic solubility measurement have been developed having a throughput of thousands of compounds per month (Dehring et al. 2004). Also several computational methods have been developed for the prediction of aqueous solubility enabling compound selection even before synthesis (Huuskonen 2000; Lipinski et al. 2001; McFarland et al. 2001; Ran et al. 2001; Jorgensen and Duffy 2002). Most of the theoretical models used for predicting aqueous solubility produce intrinsic solubility (S_0) values (i.e. solubility of uncharged species) and the Henderson-Hasselbach equation is commonly used to calculate solubility (S_{tot}) at a physiologically relevant pH values (Bergstrom et al. 2004). Although the kinetic solubility measurements and the solubility prediction methods are reliable enough at an early stage of drug development, a more accurate assessment of solubility behaviour is needed later in the development process (Avdeef 1998; Glomme et al. 2005).

2.1.1 The importance of aqueous solubility in drug development

Recent medicinal chemistry reports show a clear tendency of synthesizing more lipophilic compounds (Opera 2000). The reason for this may probably be traced to recent popular combinatorial chemistry approaches, and *in* vitro high throughput screens, which tend to bypass aqueous solubility problems by using solvents like DMSO to dissolve samples. Aqueous solubility and permeability are the two most important factors influencing oral drug absorption and it is not surprising that poor solubility and permeability are among the main causes for failure during drug development (Opera 2000; Lipinski 2000; Bergstrom 2005). In many cases, a drug may show good activity in *in vitro* but may be totally inactive *in vivo* due to its inability to reach the site of action (Glomme et al. 2005).

The required solubility for an orally administered drug depends on the permeability and potency (required dose) of a drug. A drug with a high permeability and potency can have minimum acceptable aqueous solubility as low as 1 μg/ml whereas a second drug with a lower permeability and potency may require 2000 times higher minimum acceptable solubility. (Lipinski 2000). Drugs can be divided into four classes according to a biopharmaceutics drug classification system (BCS) (Table 2) (Amidon et al. 1995). Solubility in BCS is defined in terms of the volume of water (ml) required to dissolve the highest dose at the lowest solubility over the pH range 1-7.5 (Polli et al. 2004). A volume of 250 ml is defined as the limit for high solubility and if larger volumes are required to dissolve the dose, the solubility is defined as being low. Permeability values of above 10^{-4} cm/s are considered high. The absorption of class I drugs is limited by the rate of dissolution while solubility limits the absorption of class II drugs. Permeability is the rate limiting step in the absorption of class III drugs and no *in vitro – in vivo* correlation is expected for class IV drugs (Avdeef 2001).

Table 2. Biopharmaceutics drug classification system

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

Low aqueous solubility is a major obstacle in parenteral drug delivery since it can make formulation of parenteral solutions very difficult and sometimes impossible. The drug has to be either solubilized or the particle size has to be below 5 μm in order to avoid the blocking of capillaries (Patravale et al. 2004). Although some conventional formulation strategies have been successfully used to achieve parenteral delivery of poorly soluble drugs, anaphylactic reactions, precipitation and pain at the injection site are commonly encountered problems (Varia et al. 1984; Rajewski and Stella 1996; Kipp 2004).

Optimal topical drug delivery requires both sufficient aqueous and lipid solubilities (Sloan 1992). In fact, independent of the route of delivery, a compromise between reasonable aqueous solubility and hydrophobicity is needed for acceptable membrane permeation (Jorgensen and Duffy 2002). The most difficult compounds are those with both poor aqueous and lipid solubilities. Itraconazole, carbamazepine and amphotericin B are examples of drugs that are poorly soluble both in aqueous and organic media (Washington 1996; Patravale et al. 2004).

2.1.2 Factors affecting aqueous solubility

The aqueous solubility of a crystalline drug is controlled by two kinds of interactions. First, the solubility is affected by how strongly the solute molecules associate with the solvent. The second interaction that controls the solubility is the affinity of the solute for itself, or how tightly bound the molecule is to its own crystal lattice (Delaney 2005). The dissolution process can be divided into three steps (Figure 1): The first step involves the removal of a solute molecule from the crystal. The second step involves a creation of a cavity in the solvent. The last step involves the accommodation of the solute molecule in the cavity of solvent (Valvani and Yalkowsky 1980).

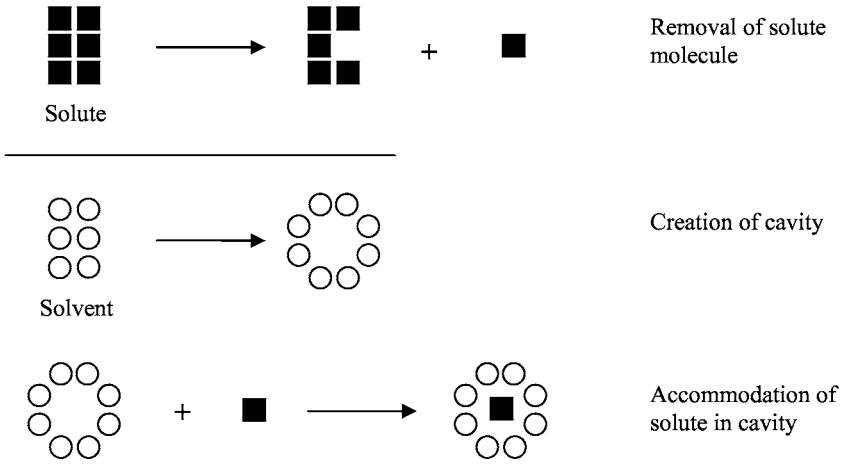
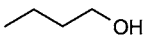
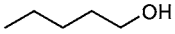
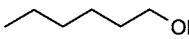
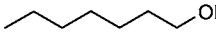
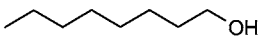
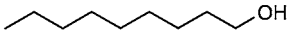
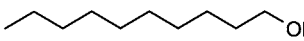


Figure 1. Processes involved in the dissolution of a crystalline solute (modified from Florrence and Attwood 1998)

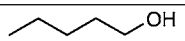
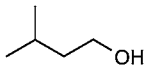
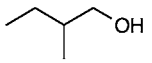
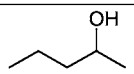
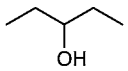
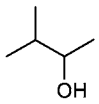
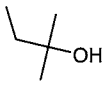
Molecular size has a significant impact on the solubility. Usually when the surface area of the molecule increases then the solubility decreases since a larger cavity has to be formed in the solvent to accommodate the solute molecule (Table 3) (Amidon et al. 1974; Amidon et al. 1975; Valvani et al. 1976).

Table 3. Effect of molecular size on aqueous solubility (modified from Amidon et al. 1974)

Structure	Solubility (molal)	Surface area (nm^2)
	1.0	2.72
	2.6×10^{-1}	3.04
	6.1×10^{-2}	3.36
	1.6×10^{-2}	3.68
	4.5×10^{-3}	3.99
	1.0×10^{-3}	4.31
	2.0×10^{-4}	4.63

Also the shape of the molecule is important. For example chain branching of hydrophobic groups increases the solubility. This is explained by the fact that a straight chain molecule has larger hydrophobic surface area than corresponding branched molecule (Table 4) (Florence and Attwood 1998). However, the correlation between surface area and solubility of branched molecules is not as good as with straight molecules. The boiling point of liquids is an indicator of molecular cohesion and it seems to correlate well with the solubility (Amidon et al. 1974).

Table 4. Effect of molecular shape on aqueous solubility (modified from Amidon et al. 1974)

Structure	Solubility (molal)	Surface area (nm^2)	Boiling point (°C)
	2.6×10^{-1}	3.04	137.8
	3.1×10^{-1}	2.91	131.2
	3.5×10^{-1}	2.89	128.7
	5.3×10^{-1}	2.96	119.0
	6.2×10^{-1}	2.94	115.3
	6.7×10^{-1}	2.84	111.5
	1.4	2.80	102.0

Many of the water's unique properties are a result of hydrogen bonding between water molecules. The influence of different substituents on the solubility of compounds can be due to their effect on the properties of the solid or liquid or to the effect of the substituent with water molecules. The presence of substituents that can form hydrogen bonds with water usually increase a compound's water solubility. Hydrogen bonds are formed between an electronegative atom (for example nitrogen and oxygen) and a hydrogen atom covalently bonded to another electronegative atom (Lehninger et al. 2005). The solubility is also dependent on the ratio of polar to nonpolar groups of the molecule (Martin and Bustamante 1993). The higher the ratio of carbon atoms to polar groups in the molecule, the lower the aqueous solubility of the compound. Substituents can usually be classified as hydrophobic or hydrophilic, depending on their polarity (Florence and Attwood 1998). An increase of hydrogen bonding capability and polarity usually leads to an increase in aqueous solubility although there can be exceptions. An increase of

polarity or hydrogen bonding potential may result in increased intermolecular interactions, stronger packing and poorer aqueous solubility. One indication of stronger packing is the increased melting point (Anderson 1980).

Many drug molecules are weak electrolytes (either acids or bases). The ionization of these molecules depends on the pH. The ionized species are more soluble than the unionized form in aqueous solutions (Ni et al. 2002). Therefore, the solubility of these kinds of molecules is pH-dependent (Figure 2), and pH control or salt formation can be used to improve their aqueous solubility. The observed solubility of an ionizable compound depends on the solubility of the neutral form and the solubility of the ionized form (Albert and Serjeant 1984).

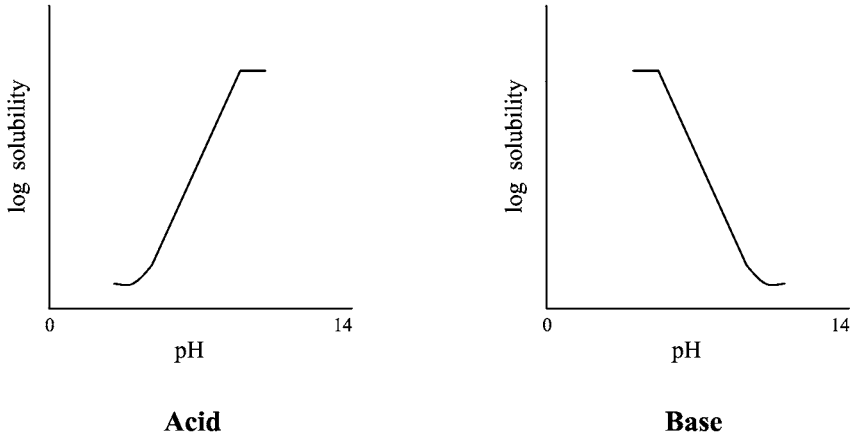


Figure 2. Solubility profiles of monoprotic acid and base

The pH-dependence of the solubility of acids and bases can be expressed by the following equations (Strickley 2004):

For a weak acid: $S_T = S_o(1 + 10^{pH-pKa})$

For a weak base: $S_T = S_o(1 + 10^{pKa-pH})$

Where S_T is the total solubility at a specified pH and S_o is the intrinsic solubility (solubility of the unionized form). However, molecules do not always behave according to these equations since, for example, solubility can also be affected by self association of the solute. If micelles are formed then the ionized solute molecules can solubilize the unionized molecules thereby improving the solubility (Roseman and Yalkowsky 1973).

2.1.3 Methods for improving aqueous solubility

2.1.3.1 Physical methods

The solid state properties of drug candidates can have impact on several important properties including solubility and dissolution rate (Huang and Tong 2004). A solid material can exist in crystalline or amorphous forms. There is a long-range order of molecular packing in crystalline material (Yu 2001). Most compounds can exist in more than one crystalline form. Different crystalline forms of the same chemical composition are called polymorphs (Vippagunta et al. 2001). Molecules are arranged in different ways in each polymorphic form and this leads to differences in the lattice energies (Huang and Tong 2004). The polymorphic form with the highest free energy is the least stable and has the best aqueous solubility (Vippagunta et al. 2001). However, this high-energy form will tend to convert to more stable low-energy forms. This can affect the long term solubility of a metastable form, since the stable crystal form will eventually crystallize out (Figure 3) (Carstensen 1977).

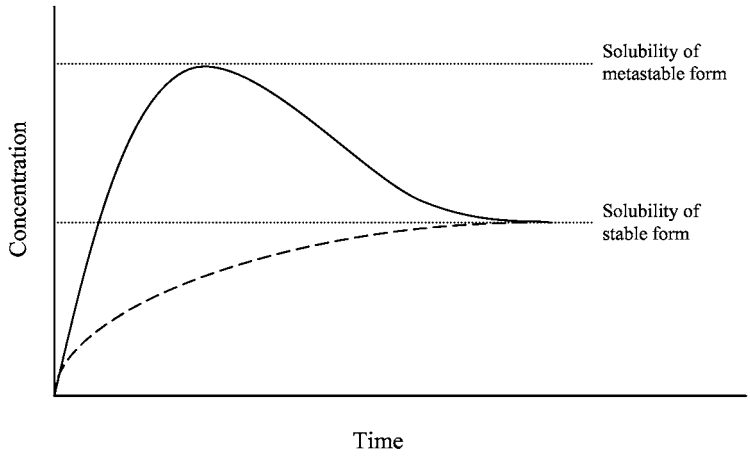


Figure 3. Dissolution of stable (---) and metastable (—) polymorph (modified from Carstensen 1977)

Unlike crystalline material, amorphous solid does not have a long-range molecular order (Yu 2001). Amorphous form has a higher free energy than any of the crystalline forms and thus it has the best solubility (Hancock and Parks 2000). However, due to its high energy state, amorphous material tends to revert back to a more stable crystalline form (Ambike et al. 2004).

The practical impact of using either metastable polymorph or amorphous material to improve solubility depends on the compound. Usually, a more significant enhancement of solubility is gained with the amorphous material than with the metastable crystal form. As an example, amorphous indomethacin was 4.5 times more water-soluble than its stable crystalline form. Use

of metastable polymorphic form gave more modest results since the metastable polymorph was only 1.1 times more water-soluble than the stable form (Hancock and Parks 2000).

2.1.3.2 Cosolvents

One of the most frequently used formulation approach to increase drug solubility is the use of cosolvents (Nuijen et al. 2001). Most cosolvents contain hydrogen bond donor and/or acceptor groups and also small hydrocarbon regions (Millard et al. 2002). Commonly used cosolvents include ethanol, propylene glycol (PG) and polyethylene glycol 400 (PEG 400) (Lee et al. 2003). Properties of different cosolvents are shown in Table 5 (Rubino and Yalkowsky 1987).

Table 5. Properties of water and commonly used cosolvents (modified from Rubino and Yalkowsky 1987)

Solvent	Dielectric constant	Solubility parameter	Interfacial tension	Surface tension
Water	78.5	23.4	45.6	72.7
PG	32.0	12.6	12.4	37.1
Ethanol	24.3	12.7	0.5	21.9
PEG 400	13.6	11.3	11.7	46.0

Solubility usually increases logarithmically with a linear increase in the fraction of organic solvent (Strickley 2004). The solubility enhancement is due to the ability of cosolvents to interrupt the hydrogen bonding structure of water and to lower the dielectric constant of the resulting solvent system (Lee et al. 2003). The polarity of the binary solvent mixture will be intermediate between that of water and the pure cosolvent (Li et al. 1999).

2.1.3.3 Surfactants

Surfactants are amphiphilic compounds containing a polar hydrophilic head and a nonpolar hydrophobic tail (Huang and Lee 2001). There are three different types of surfactants; anionic, cationic and non-ionic. In addition, some surfactants have both negatively and positively charged groups in their structure (Florence and Attwood 1998). Surfactants can self-assemble to form micelles once the surfactant concentration reaches the critical micelle concentration (CMC) (Strickley 2004). Surfactants increase drug solubility by incorporating the lipophilic drug molecules into the micelle interior.

Surfactants also enhance dissolution by lowering the surface tension, which helps to displace the air phase with the advancing liquid phase at the solid drug surface, thereby increasing the

surface area for dissolution (Chen et al. 2003). Surfactants may also protect the drug from hydrolysis (Alkhamis et al. 2003). Surfactants can not retain the drug in solution at concentrations lower than their CMC, which means that the drug may precipitate upon dilution (Lukyanov and Torchilin 2004).

2.1.3.4 Cyclodextrins

Cyclodextrins are a group of cyclic oligosaccharides composed of dextrose units (Figure 4). Natural cyclodextrins can be classified as α-, β- and γ-cyclodextrins according to the number of dextrose units in the molecule. α-Cyclodextrins are composed of 6 dextrose units while β- and γ-cyclodextrins contain 7 and 8 units, respectively (Rajewski and Stella 1996).

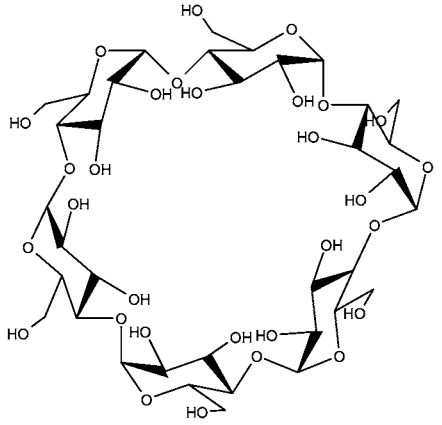


Figure 4. Chemical structure of α-cyclodextrin

Cyclodextrins have mainly been used to increase the aqueous solubility and stability of poorly water-soluble drugs (Loftsson and Järvinen 1999). Cyclodextrins contain a lipophilic cavity and a hydrophilic outer surface. The solubility increasing properties of cyclodextrins are attributable to their ability to form soluble inclusion complexes with appropriately sized hydrophobic molecules (Loftsson and Brewster 1996). The physical, chemical and biological properties of the inclusion complexes can differ dramatically from those of either the parent drug or cyclodextrin. Most drugs form 1:1-complexes with cyclodextrins (Figure 5), but higher order complexes are also possible, depending on the structure and properties of the drug (Rajewski and Stella 1996).

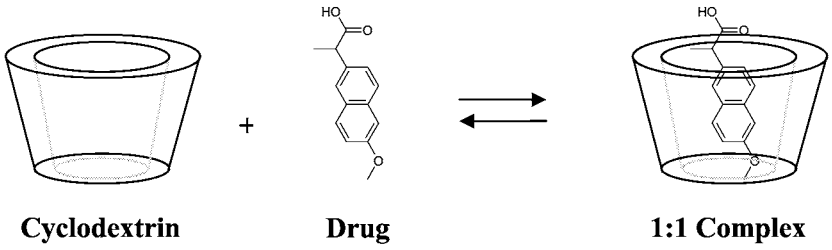


Figure 5. Formation of 1:1-complex between cyclodextrin and drug

The solubilities of natural cyclodextrins are limited but more soluble derivatives have been developed e.g. hydroxypropyl, methylated and sulfobutylether cyclodextrins (Loftsson et al. 2004). The advantage of cyclodextrins as solubilizing agents is that they may also improve the stability of the drug. Cyclodextrins have been successfully used to improve the aqueous solubility and stability of the endocannabinoid, anandamide (Jarho et al. 1996a). In order to achieve the stabilization effect, the labile part of the drug usually has to be located deep inside the cyclodextrin cavity. However, cyclodextrins may also catalyze the breakdown of some compounds (Uekama et al. 1998).

There have been some concerns about the safety of cyclodextrins since kidney toxicity has been associated with iv-administration of natural β-cyclodextrin. However, some of the derivatives, like hydroxypropyl-β-cyclodextrin (HP-β-CD), exhibit improved safety profiles compared to natural β-cyclodextrin (Irie and Uekama 1997).

2.1.3.5 Prodrugs

Prodrugs can be defined as pharmacologically inactive derivatives of parent drugs, which require spontaneous or enzymatic transformation in the body to release the active parent drug (Figure 6) (Friis and Bundgaard 1996). By using the prodrug approach, the physicochemical properties of the parent drug, e.g. its solubility, can be reversibly improved without destroying the optimal receptor binding properties of the parent drug (Sinkula and Yalkowsky 1975). There are two different prodrug strategies to increase the solubility of the parent drug. These techniques are reduction of the intermolecular forces (i.e. decrease melting point) and / or introduction of a polar, ionic or ionizable group to the molecule (Fleisher et al. 1996).

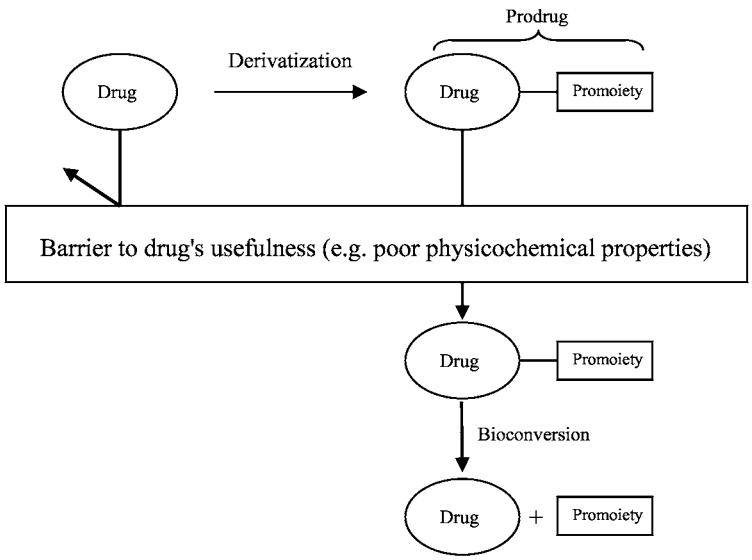


Figure 6. Principle of the prodrug concept

For compounds with high melting points, the solubility limiting factor can be the strong intermolecular forces within the crystal. The intermolecular forces can be reduced by masking the hydrogen bond donor / acceptor groups in the molecule.

Another way to improve solubility is to increase the polarity of the drug molecule. Different types of neutral, ionizable or ionic promoieties can be used depending on the desired properties of the prodrug (Nielsen et al. 1994; Ettmayer et al. 2004). The different types of promoieties are discussed in more detail below. Promoieties can be connected to the drug molecule in various different ways depending on the functional groups present in the drug molecule and the desired way to release the parent compound (Table 6) (Thomas 2000).

Table 6. Examples of functional groups used to link promoieties with drugs (modified from Thomas 2001)

Drug group	Linkage
Alcohol, phenol (D-OH)	Ester: D-COOR
Amine, amide, imide (>NH)	Amide : >NCOR
	Carbamate: >NCOR
	Imine: >N=CHR
Aldehydes and ketones (>C=O)	Acetals: $>C(OR)_2$
	Imine: >C=NR
Carboxylic acids (D-COOH)	Ester: D-COOR

The enzymes hydrolysing the prodrug molecules may be widely distributed (e.g. esterases) or localized in tissue in a site specific manner (e.g. amidases) (Table 7) (Sinkula and Yalkowsky 1975). Since water-soluble prodrugs may be poorly absorbed due to polar or charged groups, it is sometimes preferable that reconversion of the prodrug takes place just prior to absorption. In this way, the soluble prodrug provides a greater concentrational driving force for absorption and the membrane bound enzymes release the more lipophilic and better absorbed parent drug in the vicinity of the absorption site (Fleisher et al. 1996).

Table 7. Examples of prodrug linkages and enzymes hydrolyzing these bonds (modified from Sinkula and Yalkowsky 1975)

Linkage	Hydrolyzing enzymes
Ester: Short-medium chain	Cholinesterase Carboxylesterase Lipases Sterol esterase Acetylcholinesterase Acetylesterase Carboxypeptidases
Ester: Long chain	Lipases Carboxylesterase Carboxypeptidases Cholinesterase Sterol esterase
Ester: Hemiester	Esterases
Ester: Phosphate ester	Acid phosphatase Alkaline phosphatase
Ester: Sulfate ester	Steryl-sulfatase Arylsulfatase
Amide	Amidase
Amino acid	Chymotrypsins Trypsin Carboxypeptidases

2.2 Improvement of aqueous solubility by prodrug technology

2.2.1 Ionizable promoieties

2.2.1.1 Acidic promoieties

Acidic promoieties are commonly used in prodrugs since many of them offer good solubility enhancement at pH 7.4. The most common structures are phosphate esters, sulfate esters and dicarboxylic acid hemiesters (Figure 7) (Bundgaard et al. 1989).

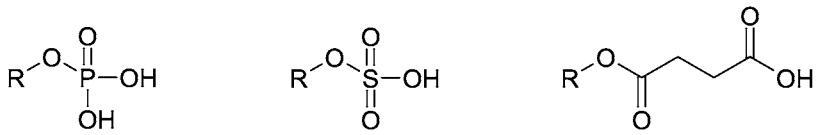
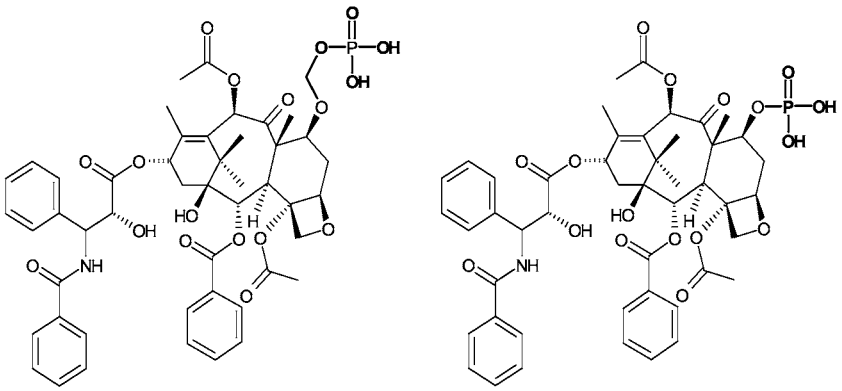


Figure 7. Structures of phosphate, sulfate and hemiester promoieties

Phosphate mono esters contain two ionizable groups and they exist predominantly as dianions at physiological pH 7.4, providing high aqueous solubility (Bundgaard et al. 1984). Phosphate esters generally show good chemical stability and rapid enzymatic bioconversion (Sauer et al. 2000). However, some phosphomonoester prodrugs of sterically hindered secondary and tertiary alcohols suffer from a slow rate of enzymatic conversion (Safadi et al. 1993). This problem can be overcome by using a suitable linker to attach the phosphate promoiety to the parent compound. Phosphonooxymethyl ethers of paclitaxel (Figure 8) readily release the parent compound upon incubation with plasma or alkaline phosphatase. In contrast, the paclitaxel prodrugs where the phosphate moiety was directly attached to the taxane core (Figure 8) were stable *in vitro* in the presence of plasma or alkaline phosphatase (Golik et al. 1996).



Enzymatically cleaved **Not enzymatically cleaved**

Figure 8. Phosphate prodrugs of paclitaxel

Also molecules that do not possess hydroxyl functionality may need a linker group between the parent compound and the phosphate promoiety (Safadi et al. 1993). Fosphenytoin is a water-soluble prodrug of phenytoin where the phosphate group is attached to the phenytoin molecule with a hydroxymethyl linker. The *in vivo* conversion of fosphenytoin to phenytoin occurs in two steps (Figure 9). First, fosphenytoin is enzymatically hydrolysed to hydroxymethylphenytoin which then spontaneously undergoes dehydroxymethylation to phenytoin (Varia et al. 1984).

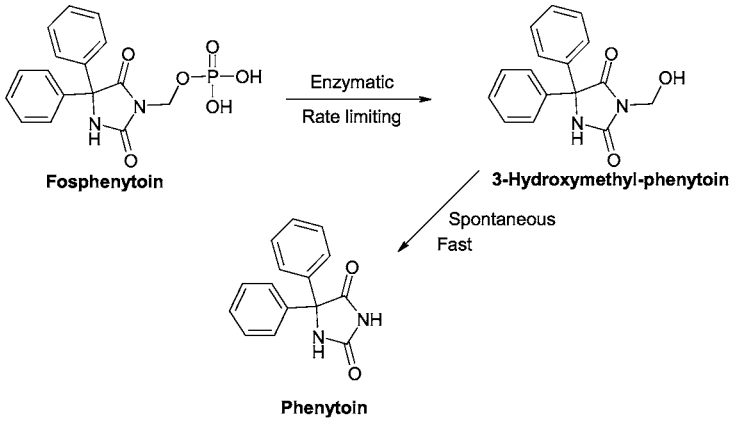


Figure 9. Bioconversion of fosphenytoin (modified from Varia et al. 1984)

Some sulfonamide containing compounds may exhibit poor aqueous solubility at physiological pH 7.4. The sulfonamide group is weakly acidic and the pK_a of the group is usually too high to affect solubility at pH 7.4. Sulfonylphosphoramidic acid derivatives (Figure 10) of the selective COX-2 inhibitor, cimicoxib, show good solubility at neutral pH, enabling formulation as aqueous solutions for parenteral administration (Almansa et al. 2004)

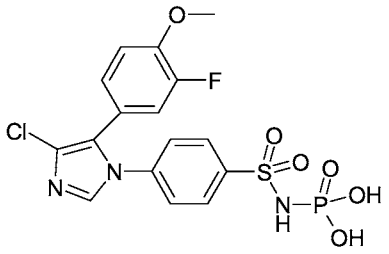


Figure 10. Chemical structure of the phosphoramidic acid derivative of COX-2 inhibitor, cimcoxib

Sulfate esters are known to be chemically stable and they readily increase the aqueous solubility of the parent compound. However, some past reports of sulfate esters indicate that they are not effectively enzymatically cleaved in *in vitro* or *in vivo* tests (Miyabo et al. 1981; Williams et al. 1983). This enzymatic stability of sulfate esters reduces their usefulness as potential water-soluble prodrugs. However, a sulfate group could also be introduced with a enzymatically labile linker so that enzymatic hydrolysis would be carried out by various esterases instead of sulfatases (Anderson et al. 1985b).

Hemiesters of dicarboxylic acids have been used to increase the water-solubility of various lipophilic compounds (Larsen et al. 1988; Möllmann et al. 1988; Pop et al. 1999b). Although succinate esters have been commonly used in the past, solution instability together with a slow and incomplete bioconversion *in vivo* limit their utility (Anderson et al. 1985a). Some improvement of solution stability and enzymatic lability may be gained by using longer hemiesters such as glutarate (Larsen et al. 1988).

2.2.1.2 Basic promoieties

Basic promoieties used for improving aqueous solubility include α-amino acid esters, aliphatic amino acid esters and various heterocycle (e.g. morpholine, piperazine, N-methylpiperazine) containing amino acid derivatives and also small peptides (Figure 11) (Bundgaard et al. 1989; Jensen and Bundgaard 1991; Pop et al. 1996; Rautio et al. 2000). Amino acid prodrugs are suitable for improving the aqueous solubility of compounds containing hydroxyl or amine functionalities. The resulting compounds have the promoiety connected to the parent compound with an ester or an amide bond (Pochopin et al. 1995).

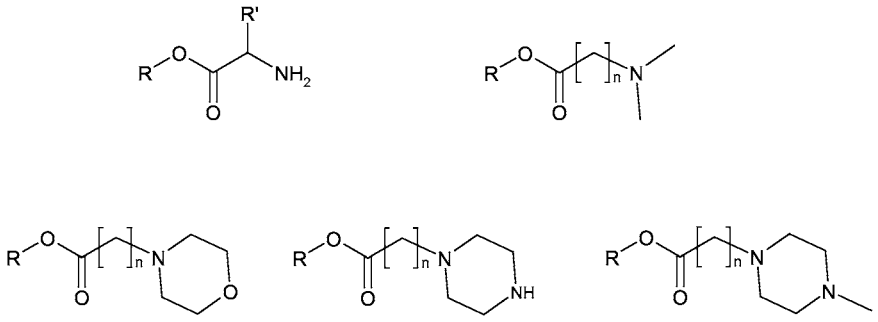


Figure 11. Examples of basic promoieties

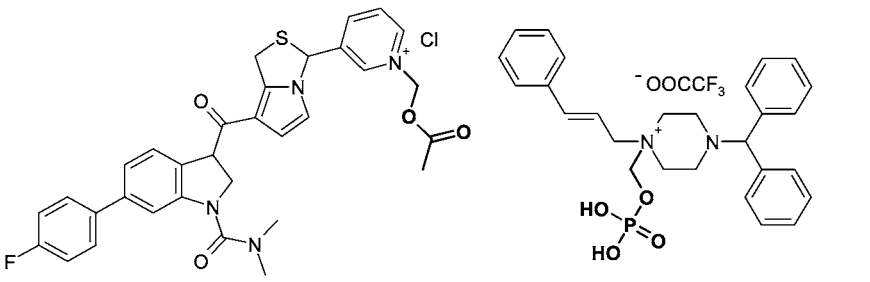
α-Amino acid esters or related short chain aliphatic amino acid esters are readily hydrolyzed by plasma enzymes but their solution stability is poor (Bundgaard et al. 1989). The major reason for the poor solution stability is the electron-withdrawing effect of the protonated amino group which activates the ester bond for hydroxide ion attack (Jensen et al. 1991). Intramolecular catalysis may also be responsible for the high instability of these structures (Jensen and Bundgaard 1991). Increasing the distance between the ionizable amino group and the ester function may improve their chemical stability (Hudkins et al. 1998). Another way to improve stability is to introduce a phenyl group between the amino group and the ester function. The phenyl group prevents intramolecular catalytic reactions and also diminishes the ester activating effect of the protonated amino group (Bundgaard et al. 1989; Jensen et al. 1991). Amino acid prodrugs of amines are usually more chemically stable because they contain an amide bond

instead of an ester bond between the parent drug and the solubilizing promoiety (Pochopin et al. 1995).

Many amino acid prodrugs are prepared as salts (e.g. hydrochlorides) and they readily dissolve in water resulting an acidic solution. However, in neutral, buffered solutions, the solubility increase of amino acid prodrugs may be more modest due to the relatively low pK_a values of the amino moieties.

2.2.2 Permanently charged promoieties

Some of the tertiary amine function containing compounds exhibit poor aqueous solubility even at pH well below the pK_a of the amine (Nielsen et al. 2005). N-alkylation approaches, like acyloxymethylation (Davidsen et al. 1994) and phosphonooxymethylation (Krise et al. 1999) have been shown to produce quaternary ammonium derivatives with improved, pH-independent, solubilities compared to the parent tertiary amines (Figure 12). In fact the promoiety in these prodrugs is not permanently charged but when it is attached to the parent tertiary drug, then a quaternary, permanently charged prodrug is formed. These prodrugs are designed to release the parent amine *in vivo* through a two-step process. The first, rate determining, step involves enzymatic hydrolysis to release a highly unstable intermediate which spontaneously breaks down to release the tertiary parent compound.



N-(acyloxymethyl)pyridinium prodrug of pyrrolothiazole

N-phosphonooxymethyl prodrug of cinnarizine

Figure 12. Quaternary prodrugs of pyrrolothiazole and cinnarizine

Permanently charged promoieties have also successfully been attached to compounds containing hydroxyl group. Trialkylammonium acetoxymethyl esters, N-methylmorpholinium and N-methylpiperazinium iodides and the quaternary pyridinium salt (Figure 13) of dexanabinol were prepared and evaluated as permanently charged water-soluble prodrugs (Pop et al. 1996; Pop et al. 1999a).

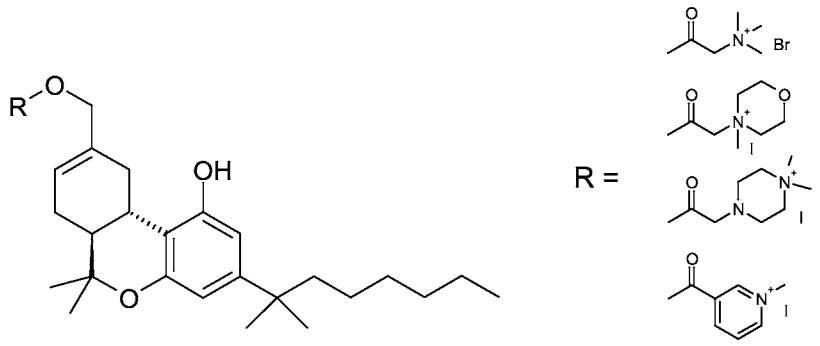


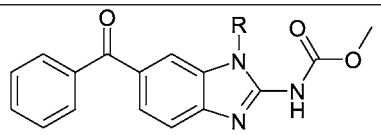
Figure 13. Quaternary prodrugs of dexanabinol

Absorption of permanently charged prodrugs may be reduced due to the highly polar nature of quaternary ammonium compounds. However, results from partition coefficient studies indicate that quaternary ammonium compounds might be able to cross biological membranes by forming hydrophobic ion-pair complexes (Takacs-Novak and Szasz 1999), but the actual significance of ion-pair formation on the absorption of charged species is still not completely understood. Therefore, the adequate absorption of permanently charged prodrugs may require regeneration of the parent compound near to the absorption site (Nielsen et al. 2005).

2.2.3 Other promoieties

Promoieties with no ionizable or charge containing groups can also be used to increase aqueous solubility. One strategy for high-melting compounds is to decrease the melting point by masking groups that are able to form intermolecular hydrogen bonds. Examples of this approach are the prodrugs of mebendazole (Nielsen et al. 1994). The synthesized prodrugs have lower melting points and up to 16-times higher aqueous solubility, while still maintaining the optimal lipophilicity for absorption (Table 8).

Table 8. Melting points, aqueous solubilities (S) and distribution coefficients (D) of mebendazole and the N-alkoxycarbonyl prodrugs (modified from Nielsen et al. 1994)



Mebendazole: R = H

Compound	R	m.p. (°C)	$S_{5.0}$ (M)	log $D_{5.0}$
1	H	289-290	1.7×10^{-6}	2.83
2	$COOCH_3$	156-157	1.9×10^{-5}	2.47
3	$COOCH_2CH_3$	165-166	2.7×10^{-5}	2.93
4	$COO(CH_2)_2CH_3$	113-114	1.3×10^{-5}	3.43

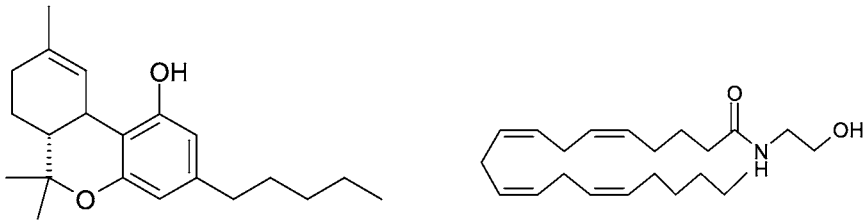
Other methods are usually a combination of decreasing the melting point and the increasing hydrogen bonding capacity. Polyethylene glycol prodrugs of phenytoin are freely soluble in water and exhibit good solution stability. They are rapidly hydrolysed in the plasma to release the parent compound, phenytoin (Dal Pozzo and Acquasaliente 1992). A more modest solubility improvement has been achieved with hydroxybutyrate prodrugs (Scriba and Lambert 1997). Polysaccharide prodrugs intended for colon-specific delivery, including cyclodextrin derivatives, have also been shown to improve aqueous solubility (Zou et al. 2005).

2.3 Cannabinoids

Cannabis has been used in many countries as a medical and narcotic plant for centuries (Frankhauser 2002). The structure of the active ingredient of cannabis, Δ^9-tetrahydrocannabinol (THC) (Figure 14), was established in 1964 (Gaoni and Mechoulam 1964). At first, THC and other cannabinoids were thought to act like general anesthetics by affecting the properties of the biological membranes (Mechoulam et al. 1998). This hypothesis was based on the highly lipophilic nature of cannabinoids. However, in 1988, Devane et al. characterized a cannabinoid receptor in rat brain (Devane et al. 1988).

The discovery of cannabinoid receptor triggered a search for endogenous compounds that bind to these receptors. The first endocannabinoid, anandamide (AEA, arachidonylethanolamide) (Figure 14) was found in 1992 (Devane et al. 1992). Advances in cannabinoid research and a better understanding of the endocannabinoid system have accelerated the search for potential therapeutic applications of cannabinoids (Goutopoulos and Makriyannis 2002). Although the chemical structures of classical cannabinoids like THC and endocannabinoids are very different, they both are highly lipophilic compounds. This high

lipophilicity and the resulting poor aqueous solubility complicate the evaluation of the properties of these cannabinoids. In addition, the poor aqueous solubility hinders the pharmaceutical formulation and drug development of cannabinoids (Jarho et al. 1996b). Cannabinoid receptor agonists can be divided into four main chemical groups. These groups are classical cannabinoids (phytocannabinoids and their analogs), non-classical cannabinoids (bicyclic and tricyclic analogs of THC that lack a pyran ring), aminoalkylindoles and eicosanoids (endocannabinoids and their analogs) (Pertwee 1997). Previously, the term cannabinoids represented only the typical diterpene C_{21} compounds present in Cannabis sativa L., and their analogues and transformation products. However, since the 1990s, the term cannabinoids has been extended to include any molecule that binds to cannabinoid receptors (Lambert and Fowler 2005).



Δ^9-THC AEA

Figure 14. Chemical structures of Δ^9-THC and anandamide (AEA)

2.3.1 Cannabinoid receptors

According to current knowledge, the endocannabinoid system includes two cannabinoid receptors, CB_1 (Matsuda et al. 1990) and CB_2 (Munro et al. 1993). CB_1 receptors are expressed in various regions of the brain, particularly in basal ganglia, substantia nigra, globus pallidus, hippocampus and cerebellum (Herkenham et al. 1990). These regions of the brain are associated with memory and movement (Porter and Felder 2001). CB_1 receptors are also present in many peripheral tissues like lung, vascular tissue and testis (Gerard et al. 1991; Rice et al. 1997; Liu et al. 2000). CB_2 receptors are predominantly expressed in spleen and other parts of the immune system (Munro et al. 1993; Lynn and Herkenham 1994)

Cannabinoid receptors belong to the superfamily of G protein-coupled membrane receptors (Giuffrida and Piomelli 2000). The affinity and properties of cannabinoid receptor ligands for CB_1 and CB_2 receptors tends to be rather similar, although the amino acid sequence homology at the binding site is only 68% and the overall sequence homology is 45% (Giuffrida and Piomelli 2000; Porter and Felder 2001). Recent findings indicate that there may be additional cannabinoid receptors since some of the cannabinoid effects are not mediated by either CB_1 or CB_2 receptors (Begg et al. 2005). Some of the cannabinoid effects may be mediated by transient receptor potential vanilloid 1 ($TRPV_1$) receptors (Ralevic et al. 2000; Ralevic et al. 2002;

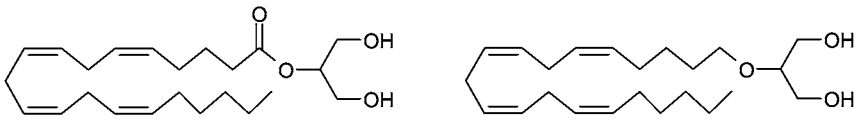
Zygmunt et al. 2002). However, some of the non CB_1 and CB_2 actions cannot be explained by the involvement of $TRPV_1$ receptors alone. There seems to be an endothelial cannabinoid receptor, which is activated by anandamide and *R*-methanandamide but not by THC. In addition to the endothelial cannabinoid receptor, there is evidence for the existence of an additional central nervous system cannabinoid receptor, located in the hippocampal area (Begg et al. 2005).

2.3.2 Endocannabinoids

The search for endogenous compounds that bind to cannabinoid receptors began shortly after the discovery of CB_1 receptor. Anandamide was the first endocannabinoid to be discovered (Figure 14) (Devane et al. 1992). Anandamide is a fatty acid derivative which is rapidly inactivated in the body by cellular uptake, followed by hydrolysis by fatty acid amide hydrolase (FAAH) to produce arachidonic acid and ethanolamine (Deutsch and Chin 1993; Cravatt et al. 1996; Deutsch et al. 2002).

The second endogenous cannabinoid receptor agonist, 2-arachidonyl glycerol (2-AG) (Figure 15) was discovered in 1995 (Mechoulam et al. 1995). Instead of an amide structure, it contains an ester bond. Although 2-AG was originally identified in rat peripheral tissues, it is also present in the brain. In fact, levels of 2-AG in the brain have been reported to be up to 1000-fold higher than levels of anandamide (Sugiura et al. 1995; Goparaju et al. 1999). The inactivation pathway of 2-AG in the body is not yet fully understood, but it has been suggested to occur via catalysis by monoacylglycerol lipase (MGL) or FAAH to produce arachidonic acid and glycerol (Di Marzo et al. 1999; Lambert and Fowler 2005).

A third endogenous cannabinoid receptor ligand, 2-arachinoyl glyceryl ether (noladin ether) (Figure 15), resembles 2-AG, but instead of an ester bond it possesses an ether moiety (Hanus et al. 2001). The concentration of noladin ether in the brain is lower than the concentration of 2-AG. There are also differences in the regional distribution of noladin ether in the brain compared to 2-AG and anandamide (Fezza et al. 2002). Noladin ether differs structurally from the other ethers in mammalian tissues since ether bonds are usually located at the 1-position of the glycerol backbone. Therefore, it has been suggested that noladin ether might not be an endogenous cannabinoid receptor ligand (Oka et al. 2003).



2-AG Noladin ether

Figure 15. Chemical structures of 2-AG and noladin ether

Anandamide and 2-AG are rapidly inactivated in the body, whereas noladin ether is much more metabolically stable due to its ether linkage (Di Marzo et al. 1999; Suhara et al. 2000). More stable analogs of anandamide, like *R*-methanandamide have been synthesized (Abadji et al. 1994). The precence of a methyl group adjacent to the amide nitrogen in *R*-methanandamide hinders the breakdown of this molecule by FAAH. *R*-methanandamide also exhibits 4-fold higher affinity to the CB_1 receptor compared to AEA in the presence of phenylmethanesulfonylfluoride (PMSF), a FAAH inhibitor. In the absence of PMSF, the binding affinity of *R*-methanandamide is unchanged, whereas anandamide affinity decreases by 30-fold due to its enzymatic hydrolysis by FAAH (Khanolkar et al. 1996).

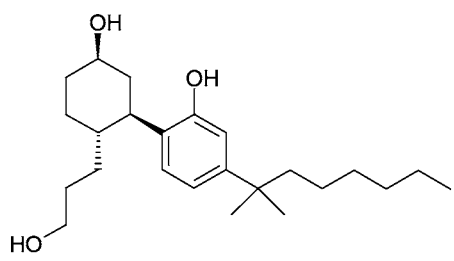
The selectivity of endocannabinoids for CB_1 and CB_2 receptors differs to some extent. 2-AG has similar affinities for both CB_1 and CB_2 receptors, while anandamide activates predominantly the CB_1 receptor and to a lesser extent the CB_2 receptor (Fezza et al. 2002). Noladin ether binds selectively to the CB_1 receptor (Hanus et al. 2001).

2.3.3. Other cannabinoids

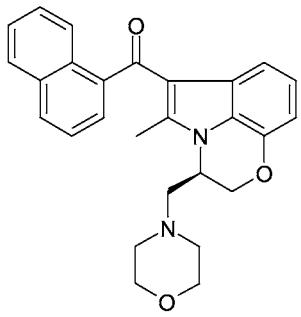
Plant derived cannabinoids, like Δ^9-THC (Figure 14), and their analogues belong to the group of classical cannabinoids. Other examples of classical cannabinoids are plant derived cannabidiol and cannabinol and the synthetic analogs nabilone and synhexyl. The phenolic hydroxyl group appears to be a key requirement for maintaining cannabimimetic activity. Also the hydrophobic side chain on the phenolic ring and an unsaturated Δ^8 or Δ^9 ring with a exocyclic methyl or hydroxymethyl can be associated with cannabinoid activity (Tius et al. 1995; Nadipuram et al. 2003).

Non-classical cannabinoids differ from the classical cannabinoids by the absence of tetrahydropyran ring (Xie et al. 1996). One of the most important non-classical cannabinoids is CP-55,940 (Figure 16), which was crucial in the process of cannabinoid receptor characterization and localization. CP-55,940 is 10-100 times more potent agonist *in vivo* than THC (Devane et al. 1988; Herkenham et al. 1990).

A structurally more different group of cannabinoids is the aminoalkylindoles, like WIN-55212-2 (Figure 16). Aminoalkylindoles were originally designed as nonulcerogenic non-steroidal anti-inflammatory drugs, but they were later found to bind to cannabinoid receptors. Actually, WIN-55212-2 was used, together with CP-55,940, in the development of radioligand binding assays and in the localization of cannabinoid binding sites in the brain (D'Ambra et al. 1992; Eissenstat et al. 1995).



CP-55,940



WIN-55212-2

Figure 16. Chemical structures of CP-55,940 and WIN-55212-2

2.3.4. Therapeutical effects of cannabinoids

A better understanding of the endogenous cannabinoid system, due to discovery of CB_1 and CB_2 receptors and endogenous cannabinoid receptor ligands, has triggered the search for potential therapeutic applications (Piomelli et al. 2000). There are several attractive features in the endocannabinoid system which could be targeted by therapeutical agents though the most obvious targets remain the CB_1 and CB_2 receptors. The molecules interfering with the cannabinoid receptors can be agonists, antagonists or inverse agonists (Pertwee 2000). In addition, endocannabinoid inactivation mechanisms can be viewed as targets (Goutopoulos and Makriyannis 2002). The endocannabinoid inactivation is a two-step process starting with cellular uptake, followed by intracellular hydrolysis by FAAH and MGL. Currently there are different proposed mechanisms for the uptake of endocannabinoids including carrier mediated transport, simple diffusion and endocytosis (McFarland and Barker 2004).

There are already cannabinoids, like THC and nabilone, which have been approved for the treatment of the nausea and vomiting evoked by cytostatic drug therapy (Pertwee 2000). Cannabinoids also hold potential in the treatment of pain (Pertwee 2001), anxiety (Marsicano et al. 2002), cancer (De Petrocellis et al. 2000), glaucoma (Järvinen et al. 2002), cardiovascular diseases (Kunos et al. 2000) and various neurological disorders like Alzheimer's disease (Milton 2002; Pazos et al. 2004), multiple sclerosis (Baker et al. 2000) and Parkinson's disease (Sanudo-Pena et al. 1998).

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3 AIMS OF THE STUDY

The general objective of the present study was to investigate usefulness of the prodrug approach to improve the physicochemical properties, especially the aqueous solubility, of endocannabinoids and their analogues. The specific aims were:

1. To design, synthesize and characterize acidic, basic and permanently charged derivatives as possible water-soluble prodrugs of cannabinoids.

2. To evaluate the physicochemical properties of the synthesized prodrugs.

3. To evaluate *in vitro* enzymatic lability of the prodrugs in various media including pure enzyme solutions, serum, and liver and cornea homogenates.

4. To determine *in vitro* corneal permeation of the phosphate prodrugs compared to the cyclodextrin formulation of the parent drug.

5. To evaluate the usefulness of the synthesized prodrugs in ocular applications *in vivo*.

4 WATER-SOLUBLE PHOSPHATE ESTERS OF ARACHIDONYLETHANOLAMIDE AND *R*-METHANANDAMIDE*

Abstract: Phosphate esters of arachidonylethanolamide (AEA) and *R*-methanandamide were synthesized and evaluated as water-soluble prodrugs. Various physicochemical properties (pK_a, partition coefficient, aqueous solubility) were determined for the synthesized phosphate esters. The chemical stability of phosphate esters was determined at pH 7.4. *In vitro* enzymatic hydrolysis rates were determined in 10% liver homogenate, and in a pure enzyme-containing (alkaline phosphatase) solution at pH 7.4. The intraocular pressure (IOP) lowering properties of *R*-methanandamide phosphate ester were tested on normotensive rabbits. The phosphate promoiety increased the aqueous solubility of the parent compounds by more than 16500-fold at pH 7.4. Phosphate esters were stable in buffer solutions, but rapidly hydrolyzed to their parent compounds in alkaline phosphatase solution ($t_{½} << 15$ s) and liver homogenate ($t_{½}$ = 8-9 min). The phosphate ester of *R*-methanandamide (or the released parent compound) reduced IOP in rabbits after topical administration. These results indicate that the phosphate esters of AEA and *R*-methanandamide are useful water-soluble prodrugs.

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4.1 Introduction

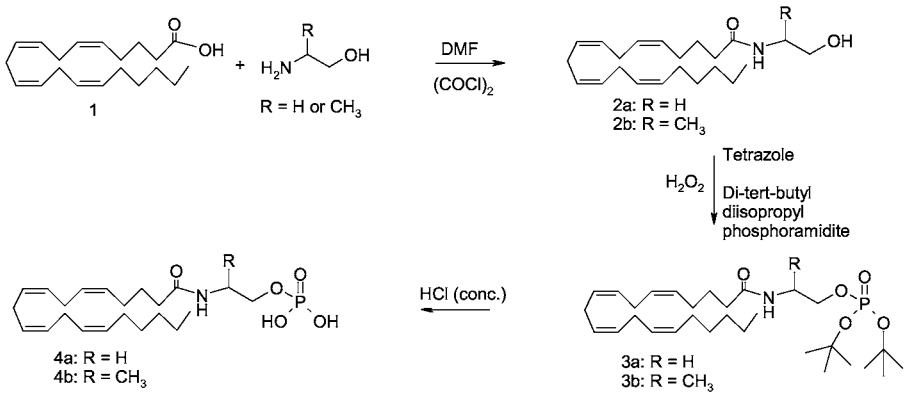
As with the classical cannabinoids, anandamides are very lipophilic and poorly water-soluble molecules. The poor aqueous solubility of anandamides causes problems in pharmacological experiments and decreases their pharmaceutical usefulness. Various approaches such as non-aqueous solvents (Wenger et al. 1995; Cabral et al. 1995) and emulsifiers (Adams et al. 1995) have been used to overcome this problem. Jarho et al. (1996) used cyclodextrins to significantly improve both the aqueous solubility and chemical stability of AEA.

Introduction of a polar or permanently charged moiety to the anandamide structure opens up new possibilities for use of these molecules as water-soluble prodrugs or analogs. Phosphate esters have been synthesized in the past to increase the water solubility of sparingly soluble molecules (Varia et al. 1984; Cho et al. 1982; Jadhav et al. 1996). Sheskin et al. (1997) synthesized AEA O-phosphate and they speculated that it might be a precursor of AEA in the body. They also measured binding affinities of various arachidonyl amides, and AEA O-phosphate was 5 times less active than AEA in their binding assay. Hopper et al. (1996) introduced new phosphorylation methods for mono- and poly-unsaturated fatty acid chains, including AEA. In the present study, we have synthesized phosphate esters of AEA and *R*-methanandamide to evaluate their use as water-soluble prodrugs for various dosage forms and administration routes.

4.2 Results and discussion

4.2.1 Synthesis

Parent compounds and prodrugs were synthesized according to the route illustrated in Scheme 1. Anandamide and *R*-methanandamide were synthesized from arachidonic acid and the corresponding aminoalcohol as described by Abadji et al. (1994). Phosphorylation of cannabinoids was carried out using di-*tert*-butyl-*N,N*-diisopropylphosphoramidite in the presence of tetrazole (Hopper et al. 1996). The protecting *tert*-butyl groups were removed with concentrated HCl.



Scheme 1. Synthesis of phosphate esters of AEA and *R*-methanandamide

4.2.2 Aqueous solubility

Jarho et al. (1996) measured the aqueous solubility of AEA to be 0.4 μg/ml (1.15×10^{-6} M) at pH 7.4. Phosphate esters **4a** and **4b** were completely dissolved during the solubility experiment. Because there was no undissolved compound left after the experiment, exact solubilities cannot be given. The maximum solubility of **4a** and **4b** could not be measured due to the small amount of each phosphate ester available and their high respective aqueous solubilities. The aqueous solubilities of **4a** and **4b** in a pH 7.4 solution at room temperature were determined to be at least 8.15 mg/ml (19.06×10^{-3} M) and 3.8 mg/ml (8.60×10^{-3} M), respectively (Table 1). However, it is clear that introduction of a phosphate moiety to the anandamide structure significantly increased the aqueous solubility of the parent drug.

4.2.3 pK_a values

The two pK_a values of phosphate ester **4a** and **4b** are shown in Table 2. Goodness-of-fit values (Avdeef 1993) for the multiset of two determinations were 1.48 and 1.31 for **4a** and **4b**, respectively. The good solubility of the phosphate esters at pH 7.4 can be readily explained by the pK_a values. At pH 7.4 the hydroxyl group with the lower pK_a (2.65) is completely ionized and more than 80% of the other hydroxyl group is in the ionized form.

4.2.4 Distribution coefficient

4b showed a higher distribution coefficient (log D) at pH 5.0 and 7.4 compared to **4a** (Table 1) which is probably due to the α-methyl substituent on the former structure. The high partition coefficients suggest ion-pair partitioning. Partition of **4a** or **4b** between octanol and the aqueous phase is strongly dependent on the pH of the aqueous phase due to their respective ionizable

phosphate moieties. The lipophilic character of both **4a** and **4b** decreases when pH is increased. These phosphate esters **4a** and **4b** have high aqueous solubilities (>8.15 mg/ml and >3.8mg/ml, respectively) and adequate distribution coefficients (log $D_{7.4}$ = 1.15 and 1.53, respectively) which is a very good combination for further studies and possible drug development.

Table 1. Aqueous solubility (one determination), distribution coefficient at pH 5.0 and 7.4 (log D , mean ± SD; n = 3) and pK_a of AEA, **4a** and **4b**.

Compound	Aqueous Solubility (mM) (pH 7.4)	Solubility compared to AEA	Log $D_{5.0}$ (pH 5.0)	Log $D_{7.4}$ (pH 7.4)	pK_{a1}	pK_{a2}
AEA	$1.15*10^{-3}$ [a]	-	-	-	-	-
4a	> 19.06	> 16500	1.91 ± 0.02	1.15 ± 0.09	2.68	6.88
4b	> 8.6	> 7500	2.60 ± 0.30	1.53 ± 0.02	2.65	6.73

[a] Jarho et al., 1996

4.2.5 Hydrolysis in aqueous solution

The effect of a buffer system on the stability of phosphate esters **4a** and **4b** was considerable. Phosphate esters **4a** and **4b** hydrolyzed more slowly in 185 mM borate buffer ($t_{½}$ = 11.9 days and 13.9 days, respectively) compared to 50 mM phosphate buffer ($t_{½}$ = 2.4 days and 3.1 days, respectively), which is most probably due to a catalytic effect of phosphate-ions on the hydrolysis of the phosphate ester group (Table 2).

Table 2. Half-lives (days) of 4a and 4b in phosphate buffer (50 mM; pH 7.4) and in borate buffer (185mM; pH 7.4) at 37 °C.

Compound	$t_{½}$ (days) Phosphate buffer (50 mM; pH 7.4)	$t_{½}$ (days) Borate buffer (185 mM; pH 7.4)
4a	2.4	11.9
4b	3.1	13.9

4.2.6 Hydrolysis in enzyme solution

Enzymatic hydrolysis of phosphate esters **4a** and **4b** in borate buffer was very rapid (completely hydrolyzed before the first sample was taken) in the presence of alkaline

phosphatase (Table 3), releasing AEA and *R*-methanandamide, respectively. The buffer system is important in these enzymatic hydrolyses, as the reaction was much slower in phosphate buffer than in borate buffer. Both phosphate and borate are known to be inhibitors of alkaline phosphatase (Fernandez et al. 1981). The phosphate ion inhibits alkaline phosphate much more effectively than the borate ion, which is logical as the inorganic phosphate binds to alkaline phosphatase, thereby inhibiting the enzyme. Inorganic phosphate is also produced when alkaline phosphatase hydrolyses phosphate esters. The slow dissociation of inorganic phosphate from the enzyme could be avoided by using a buffer like Tris, which is a good phosphoryl acceptor (Simopoulos and Jencks 1994).

4.2.6 Hydrolysis in liver homogenate

The breakdown of **4a** and **4b** in liver homogenates released AEA and *R*-methanandamide, respectively. Both **4a** and **4b** were hydrolyzed with half-lives of 8 and 9 minutes respectively (Table 3) and AEA was further hydrolyzed to arachidonic acid. The breakdown of *R*-methanandamide to arachidonic acid was probably prevented by the *R*-α-methyl group.

Table 3. Half-lives (min) (mean ± SD, n =3) of **4a** and **4b** in alkaline phosphatase solution (pH 7.4) and liver homogenate (pH 7.4) at 37 °C

Compound	$t_{½}$ (min) Alkaline phosphatase		$t_{½}$ (min) 10% Liver homogenate
	Borate Buffer	Phosphate buffer	
4a	Rapid hydrolysis[a]	209 ± 9	8 ± 1
4b	Rapid hydrolysis[a]	473 ± 24	9 ± 0

[a]Completely hydrolyzed before first sample (15 s)

4.2.7 Intraocular pressure effects

R-methanandamide was chosen for the *in vivo* IOP study because it is enzymatically more stable than AEA in ocular tissues (Pate et al. 1997). Both *R*-methanandamide (dissolved in HP-β-CD solution) and phosphate ester **4b** caused a statistically significant ($p < 0.05$) fall of IOP in the treated eye when compared to a buffer solution (Figure 2). The maximal observed decrease of IOP was 3.1 ± 0.7 mmHg and 2.4 ± 0.4 mmHg at 2 hours after administration of 173 μmol of *R*-methanandamide or **4b**, respectively. No statistically significant difference between *R*-methanandamide and **4b** could be observed.

Administration of 173 μmol of *R*-methanandamide or **4b** did not cause a statistically significant decrease of IOP in the corresponding untreated eyes when compared to buffer solution (data not shown) which suggests that the reduction of IOP was a local effect.

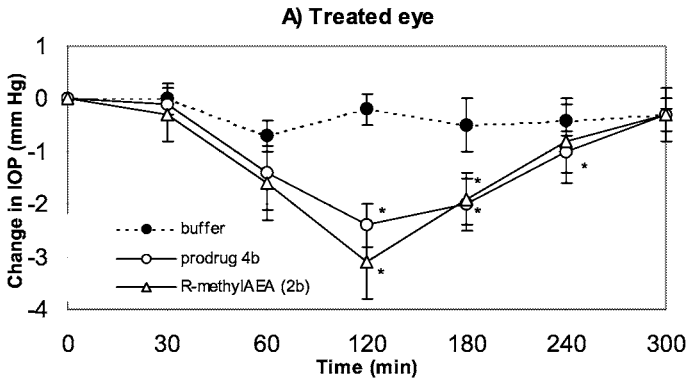


Figure 2. IOP changes (mean ± S.E.M., n=5) in treated eyes of normotensive rabbits after unilateral ocular administration of isotonic phosphate buffer (•), 173 nmol *R*-methanandamide in HP-β-CD solution (△) or 173 nmol phosphate ester **4b** (○). Symbols (*) indicate data significantly different from values for the phosphate buffer.

4.3 Conclusions

Phosphate esters of AEA and *R*-methanandamide significantly increased the aqueous solubility of their respective parent compounds, were adequately stable in buffer solutions, and released the parent drugs after enzymatic hydrolysis in alkaline phosphatase-containing solution and in liver homogenate. The phosphate ester of *R*-methanandamide (or the released parent compund) reduced IOP in rabbits, and the effect was comparable to *R*-methanandamide in hydroxypropyl-*β*-cyclodextrin solution. Thus, phosphate esters of arachidonylethanolamide and *R*-methanandamide can be considered as potential water-soluble prodrugs of anandamides.

4.4 Experimental

General procedures. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance (Bruker, Rheinstetter, Germany) operating at 500.1 MHz and 125.6 MHz, respectively. Chemical shifts are reported as parts per million (δ) using TMS as an internal standard. The following abbreviations are used for describing ^{1}H NMR data: s = singlet, d = doublet, t = triplet, q = quartet, qui = quintet, m = multiplet and br = broad. Mass spectra were recorded using a LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). The spray needle was set at 4.5 kV in the positive ion mode and to –4.5 kV in the negative ion mode. The spray

was stabilized by a nitrogen sheath flow, the value set to 100. The inlet capillary temperature was 225 °C. The samples were dissolved in 50% methanol/water or in 100% methanol (100 μg/ml) and 5 µl samples were injected. The mobile phase consisted of acetonitrile/water-mixture (50:50) and the flow was set to 200 µl/min. Elemental analyses were carried out with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer. pH-metric pK_a determinations were carried out using a Sirius PCA200 automatic titrator (Sirius, Forest Row, UK) and data was analyzed using Refine200 software (Sirius, Forest Row, UK).

Preparation of (5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoic acid (2-hydroxy-1-methyl-ethyl) -amide (2b). Arachidonic acid (**1**) (300 mg, 0.985 mmol) was dissolved in dry methylene chloride (10 ml) and DMF (72 mg, 0.985 mmol) was added. The solution was cooled to 0°C and oxalyl chloride (250 mg, 1.970 mmol) was added dropwise. The solution was stirred at room temperature for 2 hours. Solvent was evaporated to yield arachidonic acid chloride. Arachidonic acid chloride was dissolved in dry methylene chloride and the solution was cooled to 0°C. *R*(-)-2-amino-1-propanol (740 mg, 9.85 mmol) in 5 ml of methylene chloride was added dropwise and the reaction mixture was stirred for 1h under a nitrogen atmosphere. The mixture was washed twice with water (15 ml) and dried with magnesium sulfate. The product was purified with flash chromatography on 40 μm silica gel (J.T. Baker, Deventer, Holland) using 4% methanol / 96% methylene chloride as eluent to give 279 mg (78.3%) of *R*-methanandamide (**2b**) as a slightly yellow oil. ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, ^{3}J = 6.8 Hz, 3H), 1.16 (d, ^{3}J = 6.8 Hz, 3H), 1.23-1.39 (m, 6H), 1.63-1.76 (m, 2H), 2.05 (q, ^{3}J = 6.9 Hz, 2H), 2.12 (q, ^{3}J = 6.8, 2H), 2.19 (t, ^{3}J = 7.4 Hz, 2H), 2.76-2.88 (m, 6H), 3.48-3.56 (m, 1H), 3.60-3.69 (m, 1H), 4.00-4.11 (m, 1H), 5.28-5.43 (m, 8H), 5.62 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.04, 17.04, 22.55, 25.45, 25.62, 25.63, 26.61, 27.20, 29.30, 31.50, 36.07, 47.80, 67.38, 127.51, 127.83, 128.14, 128.26, 128.61, 128.83, 129.03, 130.52, 173.64. ESI-MS: 362.3 (M+1)

Preparation of phosphoric acid di-tert-butyl ester 2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoylamino)-ethyl ester (3a). AEA (**2a**) (222 mg, 0.638 mmol) and tetrazole (134 mg, 1.92 mmol) were dissolved in a 50 ml mixture of dry tetrahydrofuran and methylene chloride (1:1). Di-*tert*-butyl diisopropyl-phosphoramidite (531 mg, 1.92 mmol) was added and the reaction mixture was stirred overnight under a nitrogen atmosphere. After addition of 0.2 ml 30% hydrogen peroxide, the reaction mixture was stirred for 2 hours. The mixture was cooled to 0°C and saturated sodium metabisulfite solution was added to hydrolyze excess hydrogen peroxide. The mixture was diluted with 50 ml of ethyl acetate and washed twice with saturated sodium metabisulfite solution (2 x 50 ml). The organic phase was dried with sodium sulfate and the solvent was evaporated *in vacuo*. The product was purified by flash chromatography on silica gel with ethyl acetate to give 190 mg (55%) of **3a** as a clear oil. The purification was done as fast as possible to avoid breakdown of the protecting *tert*-butyl groups. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, ^{3}J = 7.3 Hz, 3H), 1.20-1.40 (m, 6H), 1.49 (s, 18H), 1.72 (qui, ^{3}J = 7.6 Hz, 2H), 2.01-2.14

(m, 4H), 2.22 (t, ^{3}J = 7.6 Hz, 2H), 2.74-2.87 (m, 6H), 3.53 (q, ^{3}J = 5.1 Hz, 2H), 4.05 (qui, ^{3}J = 4.3 Hz, 2H), 5.29-5.43 (m, 8H), 6.52 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.06, 22.58, 25.54, 25.66, 25 .68, 26.76, 27.25, 29.34, 29.87, 29.90, 31.54, 36.07, 40.00, 40.04, 65.97, 66.02, 127.59, 127.92, 128.25, 128.63, 128.76, 129.14, 130.52, 173.23

Preparation of phosphoric acid di-tert-butyl ester 2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoylamino)-propyl ester (3b). Compound **3b** was synthesized from *R*-methanandamide (**2b**) (210 mg, 0.580 mmol), tetrazole (483 mg, 1.74 mmol) and di-*tert*-butyl diisopropyl phosphoramidite (122 mg, 1.74 mmol) as described for **3a**. The product was purified by flash chromatography on silica gel with ethyl acetate to give 150 mg (47%) of **3b** as a clear oil. The purification was done as fast as possible to avoid breakdown of the protecting *tert*-butyl groups. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, ^{3}J = 6.8 Hz, 3H), 1.21 (d, ^{3}J = 6.8 Hz, 3H), 1.23-1.40 (m, 6H), 1.48 (s, 9H), 1.49 (s, 9H), 1.62-1.77 (m, 2H), 2.00-2.14 (m, 4H), 2.19 (t, ^{3}J = 7.6 Hz, 2H), 2.73-2.87 (m, 6H), 3.84-3.93 (m, 1H), 3.95-4.04 (m, 1H), 4.15-4.26 (m, 1H), 5.26-5.45 (m, 8H), 6.43 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.08, 17.15, 22.58, 25.56, 25.61, 25.62, 26.64, 26.70, 27.22, 29.33, 29.81, 29.84, 29.88, 31.52, 36.13, 45.15, 45.20, 69.66, 69.71, 127.56, 127.89, 128.21, 128.22, 128.59, 128.69, 129.13, 130.51, 172.79

Preparation of phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoyl amino)-ethyl] ester (4a). To a solution of **3a** (95 mg, 0.175 mmol) in ethyl acetate (2 ml) 3 drops of concentrated hydrochloric acid were added. The mixture was stirred at room temperature for 6 hours and evaporated *in vacuo* to give 75 mg (100%) of **4a**. ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, ^{3}J = 6.8 Hz, 3H), 1.23-1.42 (m, 6H), 1.64-1.76 (m, 2H), 2.00-2.12 (m, 4H), 2.33 (t, ^{3}J = 7.5 Hz, 2H), 2.70-2.86 (m, 6H), 3.51 (br s, 2H), 4.07 (br s, 2H), 5.25-5.43 (m, 8H), 8.43 (br s, 2H). ^{13}C NMR ($CDCl_3$) δ: 14.07, 22.58, 25.64, 25.65, 25.66, 26.62, 27.24, 29.34, 31.53, 35.49, 127.56, 127.86, 128.13, 128.40, 128.70, 128.83, 128.98, 130.55, 176.32. ESI-MS: 426.5 (M-1). Anal. ($C_{22}H_{38}NO_5P \cdot 0.33H_2O$) C, H, N

Preparation of phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenoyl amino)-propyl] ester (4b). Compound **4b** was synthesized from **3b** (110mg, 0.199 mmol) as described for **4a** to give 85 mg (97%) of **4b**. ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, ^{3}J = 6.7 Hz, 3H), 1.20 (d, ^{3}J = 6.0 Hz, 3H), 1.23-1.42 (m, 6H), 1.62-1.76 (m, 2H), 1.99-2.14 (m, 4H), 2.22-2.38 (m, 2H), 2.73-2.88 (m, 6H), 3.91 (br s, 1H), 4.04 (br s, 1H), 4.21 (br s, 1H), 5.26-5.45 (m, 8H), 8.25 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.09, 16.46, 22.58, 25.61, 25.64, 25.70, 26.55, 27.22, 29.33, 31.51, 35.56, 127.53, 127.85, 128.14, 128.33, 128.64, 128.84, 128.92, 130.52, 175.44. ESI-MS: 440.5 (M-1). Anal. ($C_{23}H_{40}NO_5P$) C, H, N.

HPLC analysis. HPLC determinations were performed with a Merck LaChrom HPLC system consisting of Model L-7250 programmable autosampler, Model L-7100 intelligent pump, Model D-7000 interface, Model L-7455 diode array detector and a Model D-7000 HPLC system manager software (Hitachi, Tokyo, Japan). A Purospher RP-18e (125 mm x 4.0 mm,

5μm) (Merck, Darmstadt, Germany) reversed phase column was used for all HPLC separations. Gradient elution with a 20 mM phosphate buffer (pH 7.4) / 90% acetonitrile solvent system achieved a good chromatographic separation of arachidonic acid, anandamides and their phosphate esters. A flow rate of 0.9 ml/min was used and compounds were detected at 211 nm.

Aqueous solubility. The aqueous solubilities of phosphate esters were determined at room temperature in 185 mM borate buffer (pH 7.4). Compounds **4a** (8.43 mg) and **4b** (3.81 mg) were added to 1 ml of buffer solution and the solution was vigorously shaken for 4 hours, filtered (0.45 μm Millipore), diluted and analyzed for phosphate ester by HPLC. The pH of the mixtures was held constant throughout the study.

pK_a values. pK_a was determined by the pH-metric technique (Avdeef, 1993) using Sirius PCA200 computerized titration system. Measurements were performed at 25 ± 1 °C under an argon stream to minimize CO_2 absorption. Sample concentrations ranged from 0.40 mM to 0.65 mM. Two independent measurements were made for each compound and the results were combined into a multiset. HCl and KOH solutions used in titrations were made using standardized ampoules (Merck, Darmstadt, Germany). Average ionic strength during the titrations was 0.151 M (adjusted with 0.15 M KCl). Titrations were performed from high to low pH (pH 9.88 - 1.66).

Distribution coefficient. The distribution coefficients (log D) of phosphate esters **4a** and **4b** were determined at 25 °C in a 1-octanol-borate buffer (pH 7.4 and pH 5.0) system. Before use, the 1-octanol was saturated with borate buffer by vigorously shaking for 24 h. A known amount of phosphate ester was dissolved in the borate buffer and the pH was checked and adjusted (if necessary). The solution was filtered using a 0.45 μm membrane filter and 2 ml of the filtered solution was shaken with 0.4 ml 1-octanol for 4 hours. The phases were separated by centrifuging the tubes 10 minutes at 1700 rpm. The concentration of phosphate esters in the buffer, before and after the shaking, was determined by HPLC.

Hydrolysis in aqueous solution. The rates of chemical hydrolysis for phosphate esters at 37°C were determined in 50 mM phosphate buffer (pH 7.4, ionic strength 0.5) and in 185 mM borate buffer (pH 7.4). The solutions were prepared by dissolving an appropriate amount of phosphate esters in buffer solution (initial concentration 198-246 μM). The filtered solutions (Millipore 0.45 μm) were placed in a water bath at 37 °C and samples were withdrawn at appropriate intervals. Samples were analyzed for remaining phosphate ester by HPLC.

Hydrolysis in enzyme solution. The rate of hydrolysis in alkaline phosphatase solution was determined at 37 °C. Alkaline phosphatase (Type VII-S: from bovine intestinal mucosa, 2.745 u/mg protein) was purchased from Sigma (St. Louis, MO, USA). **4a** (0.5 μmol) or **4b** (0.6 μmol) were each dissolved in an 185 mM borate buffer or 50 mM phosphate buffer (pH 7.4) and each solution was filtered through 0.45 μm membrane filter. A tube containing each filtered solution (2 ml) was placed in a 37 °C water bath and 4 μl (20.9 units) of alkaline phosphatase

was added. Samples (120 μl) were removed from each tube and 120 µl of acetonitrile was added to stop enzymatic hydrolysis. After centrifugation (14000 rpm, 10 min), samples were analyzed for remaining phosphate ester and for released anandamide by HPLC.

Hydrolysis in liver homogenate. The hydrolysis of **4a** and **4b** was studied in 10% rabbit liver homogenate at 37 °C. The 20% liver homogenate was prepared by homogenizing rabbit liver with four equivalents of isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000 x g at 4°C and the supernatant was stored at –80 °C until used. One volume of 1.15 mM phosphate ester solution in borate buffer (pH 7.4) was added to one volume of stirred, pre-incubated (37°C) 20% liver homogenate to give 10% liver homogenate. The solution was kept at 37 °C and 200 μl samples were withdrawn and added to 300 μl of cold acetonitrile. After mixing and centrifugation (14000 rpm, 10 min), the samples were analyzed for remaining phosphate ester, and for released anandamide, by HPLC.

Formulation of eyedrops. *R*-methanandamide was dissolved in aqueous 15% HP-β-CD solution. Earlier studies (Pate et al. 1996, 1998) have demonstrated that topical administration of HP-β-CD (up to 30% solution) does not affect the IOP of rabbits. The pH of *R*-methanandamide solution was adjusted to 7.4 with sodium hydroxide and the solution was made isotonic with sodium chloride. Isotonicity of the solution was confirmed by using Osmostat OM-6020 Auto-Osmometer (Kyoto Daiichi Kagaku, Kyoto). The phosphate ester of *R*-methanandamide (**4b**) was dissolved to an isotonic 50 mM phosphate buffer at pH 7.4. Final drug concentration of *R*-methanandamide and of the phosphate ester of *R*-methanandamide in the eyedrop solutions was 6.91 mmol/ml. The isotonic 50 mM phosphate buffer was used as a negative control treatment. Drug concentrations were analyzed by HPLC.

Intraocular pressure measurements. The method for IOP measurements has been described earlier (Pate et al. 1998; Laine et al. 2002). The experimental animals used were normotensive Dutch Belted rabbits of either gender (2.4 - 3.5 kg, n = 5). The rabbits were housed singly in cages under standard laboratory conditions: 12 h dark / 12 h light cycle, 20.0 ± 0.5 °C and 55 - 75% relative humidity. Rabbits were given water and food *ad libitum* except during the tests. Animals were treated in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research.

During the tests, rabbits were placed in plastic restraining boxes located in a quiet room. A drop (25 μl) of the test solution was instilled unilaterally into each left eye of the rabbits. During installation, the upper eyelid was pulled away slightly from the globe. IOP was measured using a BioRad (Cambridge, MA) Digilab Modular One Pneumatonometer. Before each measurement, one or two drops of 0.06 % oxybuprocaine were applied to the cornea to eliminate discomfort. For each determination, at least two readings were taken from the treated and untreated eye, and the mean of these readings was used. IOP of the rabbits were measured 1 and 0 h before, and at 0.5, 1, 2, 3, 4 and 5 h after the administration of the eyedrop. IOP at the

time of eyedrop administration (0 h) was used as the baseline value. Baseline IOPs ranged from 25.5 to 16.8 mmHg. All studies were arranged using a randomized crossover design. At least 72 h of wash-out time was allowed for the rabbits between dosings.

Statistical analysis of IOP data. Results are given as a change in IOP (mmHg) mean ± S.E. (standard error). A one-factor analysis of the variance (ANOVA) for repeated measurements was used to test the statistical differences between the *R*-methanandamide, the phosphate ester **4b** and control treated groups. Significance in differences of the means was tested using Fisher's Protected Least Significant Difference (PLSD) method at the 95 % confidence level.

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5 WATER-SOLUBLE ESTERS CONTAINING TERTIARY AND QUATERNARY HETEROCYCLIC NITROGEN*

Abstract. Various tertiary (**11** and **12**) and quaternary (**13-16**) nitrogen containing esters of arachidonylethanolamide (AEA) were synthesized and evaluated as water-soluble bioreversible prodrug structures. All the synthesized prodrugs were significantly more water-soluble than the parent AEA at pH 7.4 and 5.0. Aqueous solubilities of the tertiary prodrugs were significantly affected by the pH since their pK_a values were close to 7.4. In contrast, the aqueous solubilities of the quaternary prodrugs were pH-insensitive, which may offer advantages for certain pharmaceutical applications. Enzymatic hydrolysis studies revealed the importance of the distance of the ester bond from the piperazine ring to the enzymatic lability of the prodrugs. Prodrugs **11**, **13**, **15** and **16** were enzymatically reasonably stable ($t_{½}$ = 46-870 min in human serum and liver homogenate) but prodrugs **12** and **14** rapidly ($t_{½}$ = 3-16 min) hydrolyzed to the parent compound AEA. Prodrugs **12** and **14** appear to be the most promising compounds since they significantly enhance aqueous solubility, are chemically stable and enzymatically release the parent compound AEA. Both tertiary and quaternary methylpiperazine derivatives seem to be potential pro-moieties for increasing the aqueous solubility of cannabinoids.

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5.1 Introduction

The discovery of the central cannabinoid receptor (CB_1) in 1988 (Devane et al. 1988) and the characterization of the first endogenous cannabinoid receptor ligand, arachidonylethanolamide (AEA) in 1992 (Devane et al. 1992), initiated a new phase in cannabinoid research. Since then, numerous studies have described the physiological significance of the endocannabinoid system and there is a growing interest in the search for novel therapeutic applications of cannabinoids (Piomelli et al. 2000). Some of the most interesting potential applications include the control of pain (Pertwee 2001), regulation of the immune response (Berdyshev 2000), antitumor properties (De Petrocellis et al. 2000), cardiovascular properties (Kunos et al. 2000), applications in various neurological disorders (Croxford 2003) and treatment of glaucoma (Järvinen et al. 2002). However, the pharmaceutical usefulness of compounds affecting the cannabinoid system is often severely limited due to their extremely poor aqueous solubility. The low aqueous solubility has led to the use of non-aqueous solvents (Wenger et al. 1995), emulsifiers (Cabral et al. 1995) and cyclodextrins (Jarho et al. 1996). However, the use of non-aqueous solvents or emulsifiers is not always possible due to their toxicity and irritation.

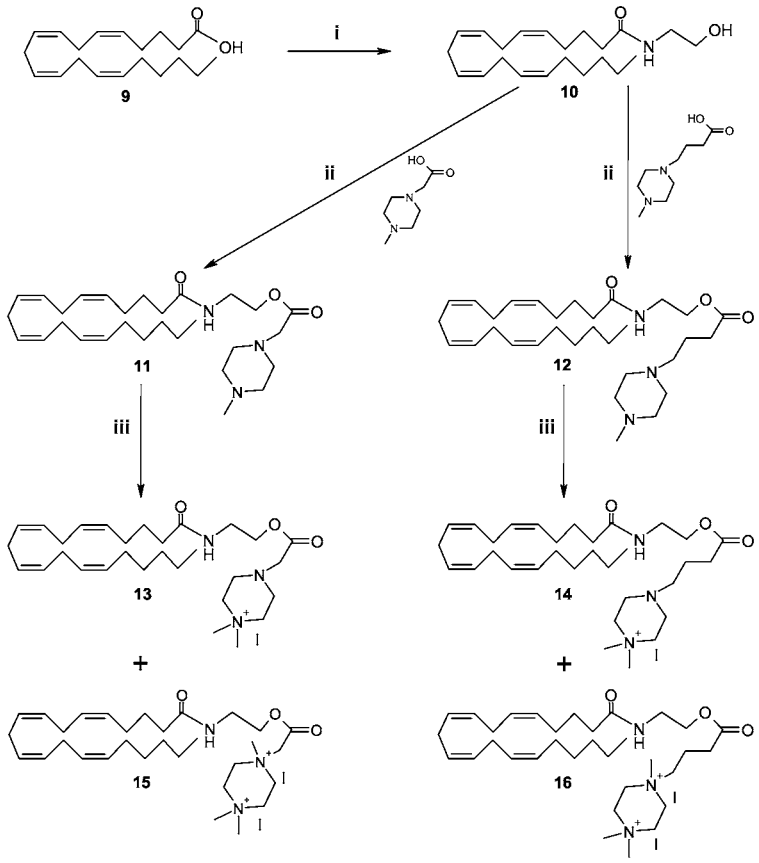
One potential solution to overcome the poor aqueous solubility of cannabinoids is the use of the prodrug approach. Aqueous solubility can be increased by introducing an ionizable or permanently charged promoiety into the lipophilic parent compound. The resulting prodrugs are amphiphilic since there is a lipophilic chain and a hydrophilic head-group in the molecule. These amphiphilic molecules tend to form aggregates like micelles. The ability to undergo micelle formation has an effect on the solubility behaviour of the prodrugs because micelles formed by the ionized prodrug molecules can solubilize the lipophilic unionized form of the prodrug. Additionally, the micelles can also solubilize poorly soluble degradation products (for example, the parent compound) which would otherwise precipitate at very low concentrations. Micelle formation can also have an effect on chemical stability since the prodrug molecules may be better protected against hydrolysis in the micelles.

In the present study, we describe the synthesis, and *in vitro* evaluation of water soluble tertiary and quaternary esters of AEA. Basic tertiary prodrugs often have different solubility properties compared to, for example, phosphate esters; phosphate esters have good solubility from mildly acidic to basic conditions whereas tertiary amine prodrugs have good solubility in conditions ranging from neutral to acidic pH. The most significant advantage of quaternary prodrugs is their pH-independent solubility, ensuring good solubility at any pH and also in unbuffered solutions.

5.2 Results and discussion

5.2.1 Synthesis

Tertiary promoieties were synthesized from bromoacetic or bromobutyric acid and methylpiperazine. Arachidonic acid was used as the starting material for the synthesis of arachidonylethanolamide as described earlier (Juntunen et al. 2003a). Tertiary prodrugs were prepared by coupling the arachidonylethanolamide with the (4-methyl-piperazin-1-yl)-acetic acid or (4-methyl-piperazin-1-yl)-butyric acid in the presence of dicyclohexylcarbodiimide and 4-(dimethylamino)-pyridine (Scheme 1). Quaternary prodrugs were synthesized from tertiary prodrugs by adding excess amounts of methyl iodide. Methyl iodide reacts with nucleophilic amine groups in the piperazine ring producing mono- and diquaternary compounds which were separated by using preparative HPLC.



Scheme 1. Synthesis of tertiary and quaternary prodrugs. (i) DMF (1 equiv), oxalyl chloride (2 equiv) dropwise 0 °C, RT 2h, ethanolamine (10 equiv) dropwise 0 °C, RT 1h; (ii) DCC (2 equiv), DMAP (2 equiv), RT 48h; (iii) MeI (10 equiv) RT 48h

5.2.2 Aqueous solubility

All the synthesized prodrug molecules were more water-soluble both at pH 7.4 and 5.0 than the parent AEA (Table 1). Due to the small amounts of compounds available, saturated solutions could not be achieved for compounds other than **11**.

Tertiary prodrugs **11** and **12** have pK_a values 7.3 and 7.5, respectively, which means that the aqueous solubilities of these prodrugs are highly pH-dependent near the physiological pH 7.4. Tertiary prodrug **12** is more soluble (> 5mg/ml) at pH 7.4 than compound **11** due to its higher pK_{a2}. The proximity of the electron withdrawing carbonyl group in **11** reduces the basicity of both amine groups in the piperazine ring. The large difference between the solubilities of **11** and **12** near pH 7.4 is not in agreement with the theoretical solubility profile. This phenomenon is most probably due to the formation of micelles, which increases the solubility by incorporating the unionized lipophilic form of the prodrug molecule into the micelle interior. Similar solubility behaviour has been observed with prostaglandin $F_{2\alpha}$ (Roseman and Yalkowsky 1973). All the quaternary prodrugs had a solubility of more than 5mg/ml at pH 5.0 and pH 7.4. Compound **15** was, however, chemically unstable and was partly converted to the parent AEA during the solubility determination.

5.2.3 pK_a and distribution coefficient

Ionization constants (pK_a) were determined for the tertiary prodrugs **11** and **12** using Sirius PCA 200 (Table 1). The higher pK_a of **11** was determined to be 7.3 ± 0.1 but the lower pK_a of **11** could not be determined. The calculated value for lower pK_a of **11** is 1.7. The pK_a values of prodrug **12** are higher (3.6 ± 0.1 and 7.5 ± 0.0) than the corresponding values of **11** because there are three carbon atoms between the methyl piperazine ring and carbonyl group of ester bond in **12** instead of one in **11**. Thus, the electron withdrawing effect (base weakening) of the ester carbonyl group is more evident in compound **11**. The determination of pK_a for amphiphilic molecules is difficult since the molecules tend to associate and this can influence the pK_a values. We performed the pK_a determinations using methanol as a co-solvent to minimize the effect of association and also to avoid precipitation near to the pK_a value. In our earlier study (Juntunen et al. 2003a) we measured pK_a values of phosphate esters of AEA without co-solvent and the relatively high lower pK_a (2.7) of phosphate group suggests that an aggregation of phosphate ester molecules might have occurred. Ionization constants for monoquaternary prodrugs **13** and **14** were calculated with ACD labs log *D* suite because pH-metric pK_a measurements were not successful.

The distribution coefficients (log *D*) for tertiary prodrugs **11** and **12** were determined by Sirius PCA 200 (Table 1) but for the other prodrugs, the shake flask method was used. Lipophilicity profiles of **11** and **12** are shown in Figure 1. Tertiary prodrug **12** has higher pK_a values than **11,** and is therefore more ionized. This explains the lower log *D* of **12** from pH 8 to

3. The distribution coefficients of quaternary prodrugs are pH dependent and the compounds are more lipophilic at pH 5.0 than at pH 7.4. This behaviour can be explained by ion-pair formation between the quaternary prodrug and the phosphate anion of the buffer used. For example, the higher log $D_{5.0}$ of **13** compared to log $D_{7.4}$ is due to ion-pair formation with the phosphate anion resulting in formation of a neutral ion-pair (Figure 2) which can distribute to the octanol phase. At pH 7.4, the situation is different since phosphate exists predominantly in a dianionic form and if an ion-pair is formed with **13** and the phosphate dianion, it will have a negative net charge (Figure 2) not favoring its distribution into the octanol phase. The impact of ion-pair formation was confirmed by performing the same experiment in pure water without any buffer or ionic strength adjustment; the distribution coefficient of **13** in pure water was 1.2. Diquaternary prodrug **16** probably forms an ion-pair with two phosphate molecules resulting in the formation of a negatively charged ion-pair at pH 7.4 and a neutral ion-pair at pH 5.0. The significance of ion-pair formation for *in vivo* absorption of charged molecules is not yet completely understood but some promising results to improve drug absorption by ion-pair formation have been reported (Hatanaka et al. 2000; Higashiyama et al. 2004).

Table 1. pK_a values, distribution coefficient (log D; pH 7.4 and 5.0) and aqueous solubility of AEA and prodrugs (mean ± SD; n = 3).

Compound	pK_{a1}	pK_{a2}	log $D_{7.4}$	log $D_{5.0}$	Solubility (mg/ml) pH 7.4	Solubility (mg/ml) pH 5.0
AEA	-[b]	-[b]	6.0[a]	6.0[a]	0.0004[d]	-
11	1.7[a]	7.3 ± 0.1	3.9	2.4	0.3 ± 0.0	> 5.0
12	3.6 ± 0.1	7.5 ± 0.0	3.8	2.2	> 5.0	> 5.0
13	1.8[a]	-[b]	2.1 ± 0.0	3.1 ± 0.2	> 5.0	> 5.0
14	4.0[a]	-[b]	2.3 ± 0.1	2.8 ± 0.1	> 5.0	> 5.0
15	-[b]	-[b]	-[c]	-[c]	> 5.0	> 5.0
16	-[b]	-[b]	1.0 ± 0.0	1.7 ± 0.0	> 5.0	> 5.0

[a] Calculated using ACD labs log D suite 4.5

[b] No ionizable groups present

[c] Not determined due to instability of prodrug

[d] From Jarho et al. 1996

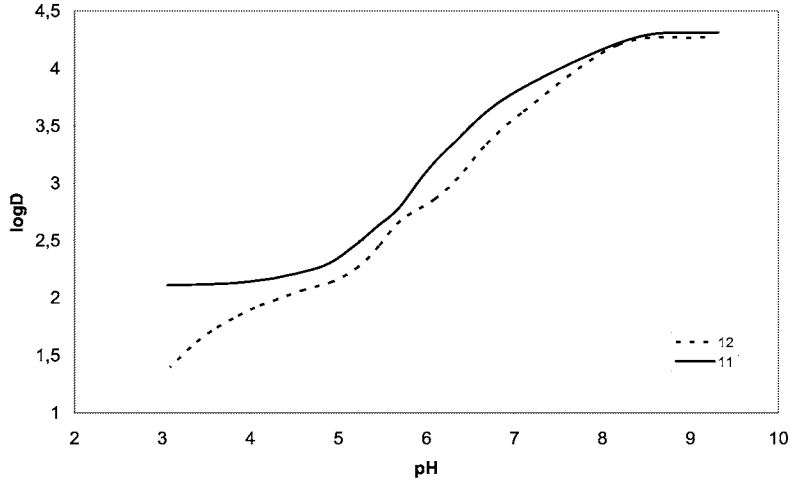


Figure 1. Lipophilicity profile (log *D*) of prodrugs **11** and **12** as a function of pH

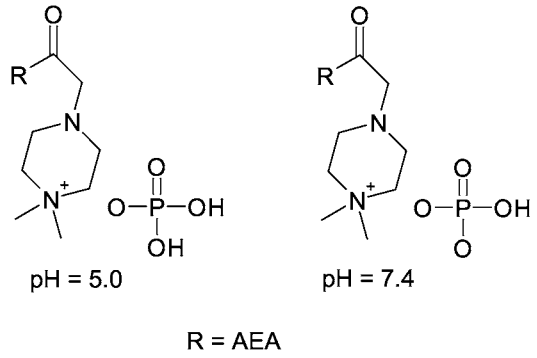


Figure 2. Proposed ion-pair formation between quaternary prodrug **13** and phosphate anion.

5.2.4 Hydrolysis in aqueous solution

Prodrug **11** was slightly more stable than **12** at pH 7.4 ($t_{½}$ = 63 h and 50 h, respectively) and at pH 5.0 ($t_{½}$ = 550 h and 490 h, respectively) although in theory **12** should be more stable because of the longer chain between the ester bond and the methyl piperazine ring. However, the degree of ionization affects the apparent stability and because of the higher pK_a values, prodrug **12** is more ionized than **11** at pH 7.4 and at pH 5.0. The stability of the shorter quaternary prodrug **13** was not very good at pH 7.4 but it was surprisingly stable at pH 5.0. The diquaternary prodrug **15** was the most unstable prodrug. The most stable prodrug at pH 7.4 proved to be quaternary prodrug **14** with a half-life of 150 hours. At pH 5.0, the most stable prodrug was **13** although it was not very stable at pH 7.4. This is partly explained by the fact

that ionization does not affect the monoquaternary compounds as much as the tertiary prodrugs. There is only one ionisable group in monoquaternary compounds **13** and **14** and the pK_a value of this group is so low that it is mostly unionized between pH 7.4 and 5.0. Tertiary prodrugs have two ionisable groups and at pH 5.0 at least one of the groups is almost fully ionized. Quaternary compound **13** is more stable than **14** at pH 5.0 because of the lower pK_a of tertiary amine group in **13** and thus the lesser degree of ionization. All the prodrugs were more stable at acidic pH 5.0 than pH 7.4. The stability order of prodrugs at pH 7.4 was **14>11>12>16>13>15** and at pH 5.0 **13>16>14>11>12** (table 2).

5.2.5 Enzymatic hydrolysis

Tertiary prodrug **12** was readily hydrolysed both in serum ($t_{½}$ = 16 ± 0 min) (Figure 3) and liver homogenate ($t_{½}$ = 3 ± 1 min) to release AEA. The quaternary prodrug **14** behaved very similarly to **12** in hydrolysis studies, showing that the positive charge is far enough from the ester group to enable efficient enzymatic hydrolysis. Shorter tertiary prodrug **11** was much more stable (Table 2). In compound **11,** the methyl piperazine ring is close to the ester bond and esterases cannot attack the ester bond because of steric reasons. In fact, it was actually observed that degradation of prodrug **11**, especially in liver homogenate, occurred partly via amide hydrolysis, releasing arachidonic acid instead of AEA. The situation is similar with short quaternary prodrugs **13** and **15,** and also with the longer diquaternary prodrug **16**. The reason why prodrug **16** was such a poor substrate for esterases could be attributable to the two positive charges in the piperazine ring since the quaternary prodrug **14** with only one positive charge is readily hydrolysed in serum and liver homogenate.

Table 2. Half-lives (mean ± S.D.; n = 2-3) of prodrugs in 80% human serum (pH 7.4), 10% rabbit liver homogenate (pH 7.4) and phosphate buffer (pH 5.0 and 7.4; hours) at 37 °C.

Compound	$t_{½}$ (min) 80 % human serum	$t_{½}$ (min) liver homogenate	$t_{½}$ (hours) phosphate buffer pH 7.4	$t_{½}$ (hours) phosphate buffer pH 5.0
11	640 ± 120	46 ± 2	63	550
12	16 ± 0	3 ± 1	50	490
13	820 ± 280	210 ± 11	19	2000
14	15 ± 0	4 ± 0	150	1170
15	-[a]	70 ± 2[b]	0.1	-[a]
16	720 ± 120	870 ± 7	23	1570

[a] Not determined due to chemical instability of the compound

[b] Mostly chemical hydrolysis

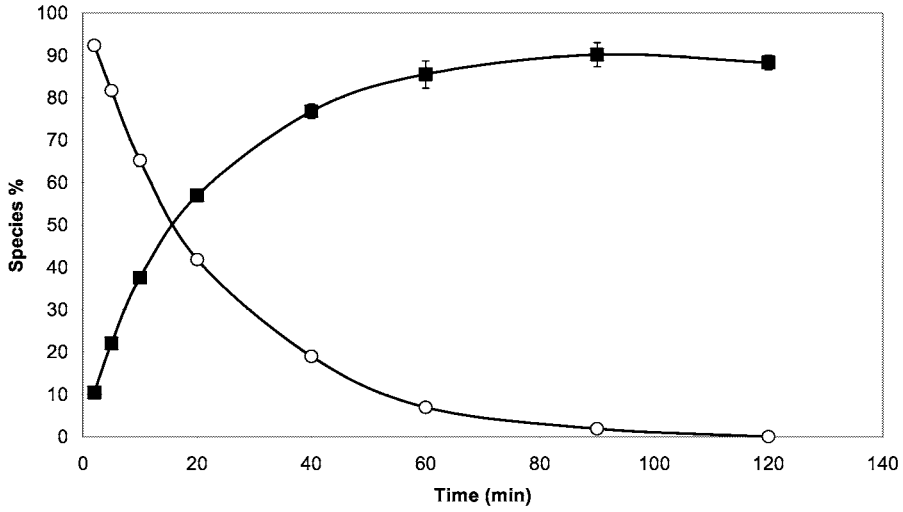


Figure 3. Time courses for the disappearance of **12** (○) and the appearance of AEA (■) during hydrolysis of the prodrug in 80 % human serum at 37 °C.

5.3 Conclusions

All of the synthesized prodrugs were more water-soluble than the parent compound, AEA. The most promising prodrugs, **12** and **14,** were chemically stable, rapidly released the parent compound AEA after enzymatic hydrolysis and thus are useful prodrugs of AEA.

5.4 Experimental

General procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer (Bruker, Rheinstetter, Germany) operating at 500.13 and 125.76 MHz, respectively, and using TMS as an internal reference. The letter 'J' indicates normal $^{3}J_{HH}$ couplings and all J values are given in Hz. The chemical shifts of the compounds were assigned from standard ^{1}H-^{1}H (COSY) and ^{1}H-^{13}C (HMQC, HMBC) correlated spectra.

Mass spectra were recorded using a LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). The spray needle was set at 4.5 kV in the positive ion mode and to –4.5 kV in the negative ion mode. The spray was stabilized by a nitrogen sheath flow, the value set to 100. The inlet capillary temperature was 225 °C. The samples were dissolved in 50 % methanol/water or in 100 % methanol (100 μg/ml) and 5 μl samples were injected. The mobile phase consisted of acetonitrile/water-mixture (50:50) and the flow was set to 200 μl/min. The preparative HPLC used for purification of quaternary compounds consisted of Shimadzu SLC-10Avp system controller, SPD-10Avp uv-vis detector, FRC-10A fraction collector and two LC-8A preparative pumps (Shimadzu, Kyoto, Japan)

Bromoacetic acid ethyl ester (3). Bromoacetic acid (13.9g, 100 mmol) (**1**), ethanol (27.6g, 60 mmol) and sulphuric acid (0.15g) were dissolved in 18ml of toluene. The mixture was distilled until the temperature of the distillate reached 78°C. The distillate was dried over potassium carbonate and poured back into the flask. The mixture was distilled again until the temperature reached 78°C, the solvents were evaporated and the product was distilled under reduced pressure (100mbar / 95°C) to obtain 9.5g (57%) of **3** as clear liquid. ^{1}H NMR ($CDCl_3$) δ: 1.29 (t, J = 7.2 Hz, 3H), 3.81 (s, 2H), 4.22 (q, J = 7.2 Hz, 2H)

4-Bromobutyric acid ethyl ester (4). Compound **4** was synthesized from bromobutyric acid (**2**) (10g, 60 mmol) and ethanol (16.6g, 360 mmol) as described for **3**. The product was distilled under reduced pressure (25mbar / 95°C) to give 9.65g (82.6%) of **4** as a clear liquid. ^{1}H NMR ($CDCl_3$) δ: 1.27 (t, J = 7.1 Hz, 3H), 2.18 (tt, J = 7.2 Hz, J = 6.5 Hz, 2H), 2.49 (t, J = 7.2 Hz, 2H), 3.47 (t, J = 6.5 Hz, 2H), 4.15 (q, J = 7.1 Hz, 2H)

(4-Methyl-piperazin-1-yl)-acetic acid ethyl ester (5). Methylpiperazine (5.5g, 55 mmol) was dissolved in 20 ml of benzene. Bromoacetic acid ethyl ester (**3**) was added dropwise and the reaction mixture was stirred for 6 hours. A white precipitate was removed by filtration and the solvent was removed from the filtrate *in vacuo* to give 3.5g (68%) of **5** as a slightly yellow liquid. ^{1}H NMR ($CDCl_3$) δ: 1.27 (t, J = 7.1 Hz, 3H), 2.30 (s, 3H), 2.42-2.56 (m, 4H), 2.56-2.71 (m, 4H), 3.21 (s, 2H), 4.19 (q, J = 7.1 Hz, 2H)

4-(4-Methyl-piperazin-1-yl)-butyric acid ethyl ester (6). Compound **6** was synthesized from methylpiperazine (4.6g, 46 mmol) and **4** (4.5g, 23 mmol) as described for **5** to give 1.88g (38.1%) as a slightly yellow liquid. ^{1}H NMR ($CDCl_3$) δ: 1.26 (t, J = 7.1 Hz, 3H), 1.82 (tt, J = 7.3 Hz, J = 7.4 Hz, 2H), 2.28 (s, 3H), 2.30-2.60 (m, 12H), 4.13 (q, J = 7.1 Hz, 2H)

(4-Methyl-piperazin-1-yl)-acetic acid (7). Compound **5** (3.5g) was dissolved in 100ml of water and the mixture was refluxed for 2 hours. Water was evaporated *in vacuo* and the product was recrystallized from diethylether/methanol to give 1.86g (63%) of **4** as an off-white solid. ^{1}H NMR ($CDCl_3$) δ: 2.53 (s, 3H), 2.82-2.98 (m, 4H), 3.06-3.22 (m, 4H), 3.41 (s, 2H)

(4-methyl-piperazin-1-yl)-butyric acid (8). Compound **8** was synthesized from **6** (1.88g, 8.8 mmol) as described for **7**. The product was purified by flash chromatography to give 1.16g (71%) of **8** as a light brown solid. ^{1}H NMR (DMSO) δ: 1.63 (tt, J = 7.0 Hz, J = 7.2 Hz, 2H), 2.15 (s, 3H), 2.20 (t, J = 7.2 Hz, 2H), 2.29 (t, J = 7.0, 2H), 2.24-2.52 (m, 8H)

(4-methyl-piperazin-1-yl)-acetic acid 2-((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoyl-amino)-ethyl ester (11). Arachidonylethanolamide (**10**) (1.4g, 4.0 mmol), (4-methyl-piperazin-1-yl)-acetic acid (**7**) (1.28g, 8.0 mmol), N,N'-dicyclohexylcarbodiimide (1.66g, 8.0 mmol) and 4-dimethylaminopyridine (985mg, 8.0 mmol) were dissolved in 100 ml of dry dichloromethane. The mixture was stirred for 2 days under a nitrogen atmosphere. Cyclohexylurea produced in the reaction was filtered and solvent was removed *in vacuo*. The crude product was purified

using flash chromatography (17 % methanol/ 83% dichloromethane) to give 1.5g (76%) of **11** as a brown oil. The purification was conducted as fast as possible with small amount of silica to avoid breakdown of **11**. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, J = 6.9 Hz, 3H), 1.21-1.42 (m, 6H), 1.68-1.75 (m, 2H), 2.02-2.14 (m, 4H), 2.16-2.21 (m, 2H), 2.30 (s, 3H), 2.42-2.56 (m, 4H), 2.57-2.70 (m, 4H), 2.77-2.86 (m, 6H), 3.24 (s, 2H), 3.50-3.55 (m, 2H), 4.22 (t, J = 5.3 Hz, 2H), 5.27-5.47 (m, 8H), 5.85 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.05, 22.56, 25.43, 25.64 (2C), 25.67, 26.67, 27.22, 29.31, 31.51, 35.97, 38.71, 45.94, 52.89 (2C), 54.81 (2C), 59.28, 63.58, 127.52, 127.84, 128.14, 128.27, 128.62, 128.81, 129.06, 130.53, 170.39, 172.93. ESI-MS: 488.2

(4-Methyl-piperazin-1-yl)-butyric acid 2-((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoylamino)-ethyl ester (12). Compound **12** was synthesized from arachidonylethanolamide (**10**) (1g, 2.9 mmol), (4-Methyl-piperazin-1-yl)-butyric acid **(8)** (1.1g, 5.9 mmol), N,N'-dicyclohexylcarbodiimide (1.2g, 5.8 mmol) and 4-dimethylaminopyridine (700mg, 5.73 mmol) as described for **11** to give 1.29g (87%) of **12** as a brown oil. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, J = 6.9 Hz, 3H), 1.22-1.40 (m, 6H), 1.67-1.76 (m, 2H), 1.79-1.87 (m, 2H), 2.02-2.08 (m, 2H), 2.09-2.14 (m, 2H), 2.16-2.22 (m, 2H), 2.31 (s, 3H), 2.34-2.42 (m, 4H), 2.42-2.62 (m, 8H), 2.78-2.86 (m, 6H), 3.49-3.54 (m, 2H), 4.17 (t, J = 5.2 Hz, 2H), 5.30-5.44 (m, 8H), 5.77 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.07, 21.95, 22.58, 25.47, 25.67 (3C), 26.69, 27.24, 29.33, 31.53, 32.03, 36.02, 38.79, 45.74, 52.70 (2C), 54.81 (2C), 57.39, 63.32, 127.54, 127.86, 128.17, 128.28, 128.64, 128.82, 129.09, 130.54, 172.91, 173.50. ESI-MS: 516.3

[2-((5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoylamino)-ethoxycarbonylmethyl]-1,1-dimethyl-piperazin-1-ium iodide (13) and 4-[2-((5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoylamino)-ethoxycarbonylmethyl]-1,1,4-trimethyl-piperazinedium diiodide (15). Compound **11** (300 mg, 0.582 mmol) and methyl iodide (825 mg, 5.82 mmol) were dissolved in 15 ml of dry acetonitrile. The solution was stirred for two days under an argon atmosphere and the products were purified using preparative HPLC to give 280 mg (72%) of **13** and 60 mg (12%) of **15** as yellow solids. NMR and MS-data for compound **13**: ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, J = 6.9 Hz, 3H), 1.25-1.39 (m, 6H), 1.65-1.77 (m, 2H), 2.02-2.17 (m, 4H), 2.20-2.32 (m, 2H), 2.76-2.87 (m, 6H), 3.43-3.55 (m, 6H), 3.60 (s, 6H), 3.87-3.97 (m, 6H), 4.25 (t, J = 5.1 Hz, 2H), 5.29-5.43 (m, 8H), 6.83 (bs, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.10, 22.58, 25.66, 25.67, 25.68, 25,69, 26.77, 27.24, 29.33, 31.52, 35.97, 38.17, 45.70, 52.34, 56.56, 61.23, 64.50, 127.55, 127.89, 128.25, 128.31, 128.67, 128.69, 129.27, 130.56, 168.26, 173.65. ESI-MS: 502.4. NMR and MS-data for compound **15**: ^{1}H NMR ($CDCl_3/CD_3OD$ 95/5) δ: 0.86 (t, J = 6.9 Hz, 3H), 1.20-1.38 (m, 6H), 1.66 (m, 2H), 2.00-2.45 (m, 6H), 2.72-2.85 (m, 6H), 3.50 (bs, 2H), 3.77 (bs, 3H), 3.80 (bs, 3H), 3.86 (bs, 3H), 4.24-4.68 (m, 12H), 5.45-5.25 (m, 8H), 7.58 (bs, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$ 95/5) δ: 14.07, 22.56, 25.62, 25.63, 25.64, 25.73, 26.75, 27.21, 29.31, 31.49, 35.91, 42.16, 55.01, 55.23, 62.05, 127.53, 127.85, 128.18, 128.35, 128.71, 128.84, 129.19, 130.53, 164.20, 172. 44.

4(3-[2-((5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoylamino)-ethoxycarbonylmethyl]-1,1-dimethyl-piperazin-1-ium iodide (14) and 4-{3-[2-((5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoylamino)-ethoxycarbonyl]-propyl}-1,1,4-trimethyl-piperazinediium diiodide (16). Compounds **14** and **16** were synthesized from methyl iodide (825mg, 5.82 mmol) and **12** (300mg, 0.582 mmol) as described for compounds **13** and **15**. The compounds were purified by preparative HPLC to give 140mg (37%) of **14** and 140mg (30%) of **16** as yellow solids. NMR and MS-data for compound **14**: ^{1}H NMR ($CDCl_3/CD_3OD$ 95/5) δ: 0.88 (t, J = 6.9 Hz, 3H), 1.24-1.39 (m, 6H), 1.62-1.75 (m, 2H), 2.00-2.20 (m, 6H), 2.22-2.32 (m, 2H), 2.54 (bs, 2H), 2.76-2.86 (m, 6H), 3.41 (bs, 2H), 3.44-3.52 (m, 2H), 3.70 (bs, 6H), 3.74-3.95 (m, 4H), 4.05-4.28 (m, 6H), 5.29-5.43 (m, 8H), 6.84 (bs, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$ 95/5) δ: 14.01, 19.83, 22.58, 25.66, 25.68, 25.69, 25.70, 26.76, 27.24, 29.33, 31.12, 31.52, 35.98, 38.35, 45.93, 55.55, 59,69, 63.65, 127.54, 127.87, 128.21, 128.34, 128.69, 128.75, 129.21, 130.56, 172.56, 173.69. ESI-MS: 530.5. NMR and MS-data for compound **16**: ^{1}H NMR ($CDCl_3/CD_3OD$ 95/5) δ: 0.88 (t, J = 6.9 Hz, 3H), 1.24-1.39 (m, 6H), 1.62-1.72 (m, 2H), 2.00-2.15 (m, 4H), 2.15-2.35 (m, 4H), 2.62 (bs, 2H), 2.77-2.85 (m, 6H), 3.48 (bs, 2H), 3.72 (s, 3H), 3.80 (s, 3H), 3.88 (s, 3H), 4.09 (bs, 2H), 4.18 (m, 2H), 4.26-4.53 (m, 8H), 5.29-5.43 (m, 8H), 7.20 (t, J = 5.2 Hz, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$ 95/5) δ: 14.11, 17.83, 22.61, 25.67, 25.69, 25.70, 25.78, 26.78, 27.26, 29.36, 30.32, 31.55, 35.97, 38.18, 54.27, 55.32, 63.93, 127.57, 127.88, 128.20, 128.39, 128.72, 128.82, 129.16, 130.58, 172.34, 174.21. ESI-MS: 544.3.

HPLC analysis. Prodrugs and parent compound were analysed and quantified by HPLC. Analytical HPLC determinations were performed with a Merck LaChrom HPLC system consisting of L-7250 programmable autosampler, L-7100 intelligent pump, D-7000 interface, L-7455 diode array detector and D-7000 HPLC system manager software (Hitachi, Tokyo, Japan). Purospher RP-18e or RP-8e columns (125 mm x 4.0 mm, 5μm) (Merck, Darmstadt, Germany) were used for the tertiary prodrugs and Supelcogel ODP-50 column (Supelco, Bellefonte, USA) for quaternary prodrugs.

Aqueous solubility. The aqueous solubilities of prodrugs were determined in 50 mM phosphate buffer at pH 5.0 and pH 7.4 by adding 5mg of the prodrug into 1 ml of buffer and shaking for 30 minutes (due to limited stability of some prodrugs) during which the pH was measured and adjusted (if necessary) . The solutions were then centrifuged (10000 rpm, 10 min) and the concentration of the prodrug was analysed from the supernatant by HPLC.

***pK$_a$* values.** pK_a values were determined for tertiary prodrugs **11** and **12** by the pH-metric technique (Avdeef 1993) using Sirius PCA200 computerized titration system. Measurements were performed at 25 ± 1 °C under an argon stream to minimize CO_2 absorption. The HCl and KOH solutions used in titrations were made using standardized ampoules (Merck, Darmstadt, Germany). The ionic strength during the titrations was 0.151-0.168 M (adjusted with 0.15M KCl). Sample concentrations ranged from 0.6 mM to 1.4 mM. At least four independent

measurements were made for each compound with different methanol ratios (20-60 wt %) and the results were combined into a multiset. Aqueous pK_a was extrapolated from co-solvent titration data using the Yasuda-Shedlovsky method (Avdeef et al. 1999).

Distribution coefficient. Distribution coefficients for tertiary prodrugs were determined using Sirius PCA200 computerized titration system. Three separate titrations were performed with three different amounts of octanol (0.1 ml, 1.1 ml, 11.1 ml) and the titrations were combined into a multiset. The lipophilicity profile was constructed for the tertiary prodrugs. Partition coefficients for quaternary prodrugs were determined at pH 5.0 and pH 7.4 (50 mM phosphate buffer, $\mu = 0.15$) using the shake-flask method.

Hydrolysis in aqueous solution. Chemical stabilities of prodrugs were determined in 50 mM phosphate buffer at pH 5.0 and pH 7.4 (ionic strength 0.15 and temperature 37 °C). An appropriate amount of prodrug was dissolved in 8 ml of buffer and the solutions were placed in a thermostatically controlled water bath at 37 °C. Samples were taken at appropriate intervals and analysed for remaining prodrug by HPLC. The pseudo-first-order half-life ($t_{½}$) for the hydrolysis of prodrug was calculated from the slope of logarithm of remaining prodrug versus time.

Hydrolysis in human serum. The rates of hydrolysis for prodrugs were studied in 80% human serum at 37 °C. Prodrugs were dissolved into 50 mM isotonic phosphate buffer (pH 7.4). Both serum and sample solution were placed into a water bath and 1ml of sample solution was mixed with 4 ml of serum. The solution was kept in a water bath at 37 °C, and 200μl samples were withdrawn and added to 400μl of acetonitrile to stop the enzymatic activity in serum. After mixing and centrifugation for 5 minutes at 14000rpm, the supernatant was analysed for remaining prodrug and released AEA.

Hydrolysis in liver homogenate. The hydrolysis of prodrugs was studied in 10% rabbit liver homogenate at 37 °C. The 20% liver homogenate was prepared by homogenizing rabbit liver with four equivalents of isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000×g at 4 °C and the supernatant was stored at -80 °C until used. One volume of prodrug solution in phosphate buffer (pH 7.4) was added to one volume of stirred, pre-incubated (37 °C) 20% liver homogenate to give 10% liver homogenate. The solution was kept at 37 °C and 200 μl samples were withdrawn and added to 400 μl of cold acetonitrile. After mixing and centrifugation (14000rpm, 5min), the samples were analyzed for the remaining prodrug and for released AEA.

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6 SYNTHESIS, *IN VITRO* EVALUATION AND INTRAOCULAR PRESSURE EFFECTS OF WATER-SOLUBLE PRODRUGS OF ENDOCANNABINOID NOLADIN ETHER*

Abstract. The poor aqueous solubility of 2-arachidonyl glyceryl ether (noladin ether) **2** hinders both its pharmacological studies and future pharmaceutical development. The synthesized mono- and diphosphate esters of noladin ether (**4** and **6**, respectively) considerably increased the aqueous solubility of noladin ether (> 40 000-fold), showed high stability against chemical hydrolysis in buffer solutions and were rapidly converted to the parent drug via enzymatic hydrolysis. The monophosphate ester of noladin ether (**4**) (or the released parent compound) reduced IOP in normotensive rabbits.

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6.1 Introduction

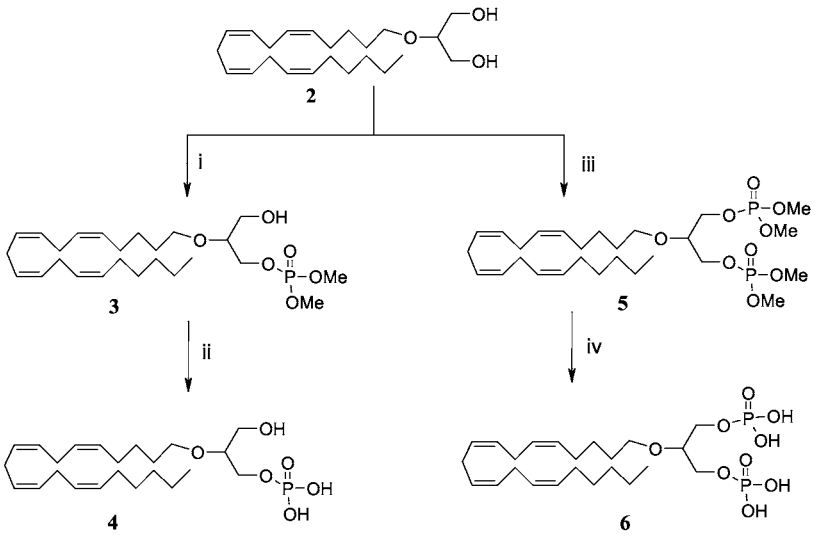
Noladin ether (2-arachidonyl glyceryl ether, HU-310) is a newly discovered endogenous cannabinoid CB_1 receptor agonist (Hanuš et al. 2001). Previously identified endocannabinoids are amides or esters, of which the best known are arachidonylethanolamide (AEA) (Devane et al. 1992) and 2-arachidonyl glycerol (2-AG) (Mechoulam et al. 1995), respectively. AEA and 2-AG have a short duration of action *in vivo* due to rapid metabolism, but noladin ether has higher metabolic stability and thus it may offer advantages.

There has been a growing interest in the search for potential therapeutic applications of endocannabinoids, and there are several excellent reviews covering this topic (Goutopoulos and Makriyannis 2002; Piomelli et al. 2000; Porter and Felder 2001). Noladin ether has been shown to reduce IOP and this effect is mediated through the ocular CB_1 receptor (Laine et al. 2002). Although much research has focussed around endocannabinoids, not much attention has been paid to their unfavorable physicochemical properties. Endocannabinoids are very lipophilic and poorly water-soluble compounds, which hinders their pharmaceutical development and possible clinical use. Various approaches such as non-aqueous solvents (Wenger et al. 1995), emulsifiers (Cabral et al. 1995) and cyclodextrins (Jarho et al. 1996) have been used to overcome this problem. Another approach is to increase aqueous solubility of endocannabinoids by synthesizing water-soluble prodrugs with ionizable or permanent charge-containing groups. In this study, we describe the synthesis of mono- and diphosphate esters of 2-arachidonyl glyceryl ether and their *in vitro* and *in vivo* evaluations as water soluble endocannabinoid prodrugs.

6.2 Results and discussion

6.2.1 Synthesis

2-Arachidonyl glyceryl ether was synthesized from arachidonyl alcohol using the method of Hanuš et al. (Hanuš et al. 2001). Mono- and diphosphate esters of 2-arachidonyl glyceryl ether were synthesized using dimethyl chlorophosphate as described in Scheme 1.



(i) Pyridine, Cl-P(O)(OMe)$_2$ (1.5 equiv.), 0 °C 2 h, 20 °C overnight. (ii) NaI (2.2 equiv.), Me_3SiCl (2.2 equiv.), 40 °C 1h, MeOH, 20 °C 1h. (iii) Pyridine, Cl-P(O)(OMe)$_2$ (3 equiv.), 0 °C 2 h, 20 °C overnight. (iv) NaI (4.4 equiv.), Me_3SiCl (4.4 equiv.), 40 °C 1h, MeOH, 20 °C 1h.

Scheme 1. Synthesis of phosphate ester prodrugs of noladin ether

6.2.2 Physicochemical properties

The aqueous solubility of the phosphate esters was over 5 mg/ml, which is over 40000 times the solubility of noladin ether (< 0.1 μg/ml). The exact prodrug solubility was not determined, due to the small amount of phosphate esters available.

Partition or distribution coefficient has been the most frequently used physicochemical parameter to estimate the permeability of drugs or drug-like molecules through biological membranes. Optimal log distribution coefficients for membrane permeability are in the range of 2-3 (Schoenwald and Ward 1978). The calculated log distribution coefficient for noladin ether at pH 7.4 was 7.05 (calculated using ACDLabs log D suite), which shows that the molecule is too lipophilic for optimal membrane penetration. Phosphate esters exist in their ionized form at pH 7.4, and prodrugs **4** and **6** have significantly lower log D values (Table 1). However, the phosphate prodrug strategy does not necessarily involve membrane permeation of the prodrug. In many cases it is actually feasible that prior to absorption the water-soluble prodrug undergoes enzymatic degradation to the highly permeable parent drug to ensure high absorption fluxes (Amidon et al. 1980).

6.2.3 Chemical stability

Both prodrugs **4** and **6** showed good chemical stability in tris and phosphate buffers at pH 7.4 at 37 °C. No degradation of diphosphate **6** was observed after 10 days. The structures of the prodrugs **4** and **6** resemble surface active agents, bearing a highly lipophilic (straight hydrocarbon chain) and also a highly hydrophilic portion (phosphate group) on the molecule. This amphiphilic nature allows the formation of micelles in aqueous solutions, which may protect the hydrolytically labile prodrug linkage and unstable double bonds within the micelle interior (Schreier et al. 2000).

Table 1. Chemical stability, aqueous solubility and distribution coefficient (log *D*) of noladin ether **2** and its phosphate esters **4** and **6**.

Compound	Chemical stability phosphate buffer pH 7.4 (t½, days)	Chemical stability tris buffer pH 7.4 (t½, days)	Solubility tris buffer pH 7.4	log $D_{7.4}$ (pH 7.4) (mean ± SD ; n =3)
2	n.d.	n.d.	<0.1 μg/ml	7.05[a]
4	17.9	9.0	>5 mg/ml	1.98 ± 0.06
6	Stable[b]	Stable[b]	>5 mg/ml	0.65 ± 0.07

n.d., not determined.

[a] Calculated using ACDLabs log *D* suite

[b] No degradation was observed after 10 days

6.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis of **4** and **6** was studied in alkaline phosphatase solution, liver homogenate and cornea homogenate to confirm the formation of the parent active compound. Alkaline phosphatase quantitatively hydrolyzed **4** and **6** to noladin ether as presented in Figure 1 showing the hydrolysis of **6**, where release of noladin ether is preceded by the formation of monophosphate ester **4**. The hydrolysis reactions can be described by pseudo-first-order rate constants k_1 and k_2, which were calculated to be 0.36 min^{-1} and 0.065 min^{-1}, respectively. The solid lines in Figure 1 were constructed by calculated constants according to the proposed reaction scheme, and show good agreement with experimental data. The somewhat higher k_2 (0.11 min^{-1}) in the hydrolysis experiment of **4** is probably due to the inhibition of alkaline phosphatase by the higher amount of released phosphate in the hydrolysis experiment of **6**.

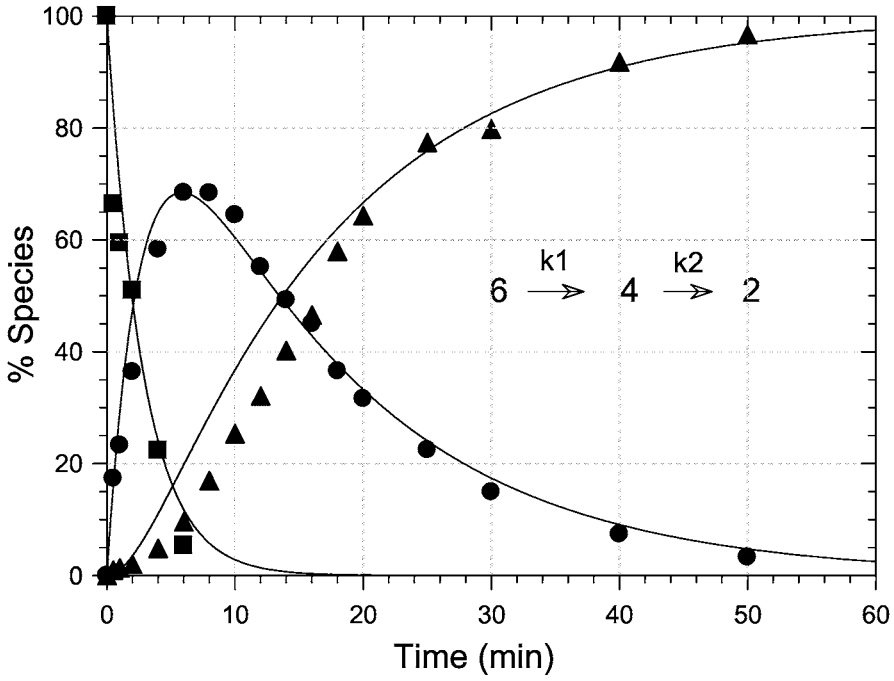


Figure 1. Time courses for noladin ether diphosphate ester **6** (■), noladin ether monophosphate ester **4** (●) and noladin ether **2** (▲) during hydrolysis of **6** in alkaline phosphatase solution. The solid lines are constructed by the proposed reaction scheme shown and calculated first-order rate constants.

Hydrolysis rates in 4% bovine cornea homogenate were studied in order to clarify whether or not these phosphate esters can be hydrolyzed during permeation of the cornea. Both 4 and 6 hydrolyzed to noladin ether in cornea homogenate (Table 2). These results suggest that phosphate esters of noladin ether are at least partly hydrolyzed during permeation of the cornea.

Table 2. Half-lives (min) (mean ± SD) of mono- (**4**) and diphosphate esters (**6**) of noladin ether **2** in alkaline phosphatase solution (pH 7.4), liver homogenate (pH 7.4) and in cornea homogenate (pH 7.4) at 37 °C.

Compound	$t_{½}$ (min), n = 2 alkaline phosphatase	$t_{½}$ (min), n = 3 10% liver homogenate	$t_{½}$ (min), n = 3 4% cornea homogenate
4	4.6 ± 0.7	7.1 ± 0.1	18.1 ± 1.4
6	1.6 ± 0.0	6.4 ± 0.7	17.2 ± 1.7

6.2.5 Intraocular pressure effects

IOP study was performed to confirm that these phosphate ester prodrugs also work *in vivo*. *In vitro* studies already showed that prodrug **6** is rapidly converted to **4** and therefore, only compound **4** was tested. Both noladin ether (dissolved in HP-*β*-CD solution) and its phosphate ester **4** caused a statistically significant ($p < 0.05$) fall in IOP of the treated eye when compared to a buffer solution (Figure 2). The maximal observed decrease in IOP was 8.5 ± 4.5 % at 2 hours after the topical administration of 172 nmol of noladin ether and 9.7 ± 4.4 % at 3 hours after the topical administration of 172 nmol of phosphate ester **4.** No statistically significant difference between noladin ether in HP-*β*-CD formulation and **4** could be observed. The reduction of IOP was most probably a local effect in the eye since no statistically significant decrease of IOP was observed in the corresponding untreated eyes when compared to the buffer solution (data not shown).

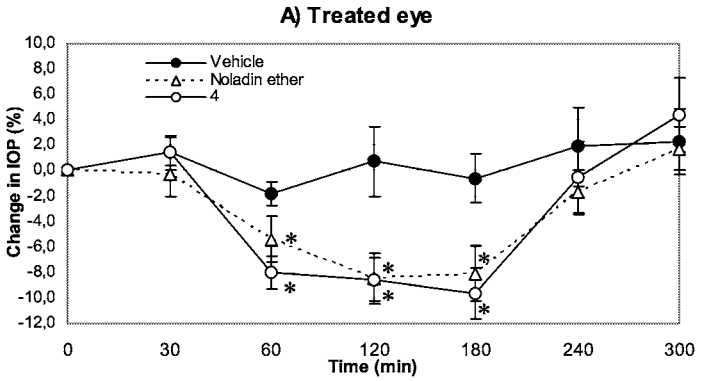


Figure 2. IOP changes (mean ± S.E., n = 5) in treated eyes of normotensive rabbits after unilateral ocular administration of isotonic citrate buffer (•), 172 nmol noladin ether **2** in HP-β-CD (Δ) or 172 nmol phosphate ester **4** (O). Symbols (*) indicate data significantly different from values for the citrate buffer.

6.3 Conclusions

The physicochemical properties of noladin ether **2** were successfully improved by introducing a phosphate moiety into the structure. High water solubility and chemical stability, together with a rapid and quantitative enzymatic hydrolysis *in vitro* and the ability to reduce IOP *in vivo*, prove that these phosphate esters are promising prodrug candidates of the endocannabinoid, noladin ether.

6.4 Experimental

General procedures. ^{1}H, ^{31}P, ^{13}C NMR, spectra were recorded on a Bruker Avance (Bruker, Rheinstetter, Germany) spectrometer operating at 500.13, 202.45 and 125.76 MHz, respectively. TMS was used as an internal standard for ^{1}H and ^{13}C, and 85% H_3PO_4 as an external standard for ^{31}P. Mass spectra were recorded using a LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer. HPLC determinations were performed with a Merck LaChrom HPLC system equipped with a diode array detector (Hitachi, Tokyo, Japan). A Purospher RP-8e (125 mm x 4.0 mm, 5μm) (Merck, Darmstadt, Germany) reversed phase column was used for all analytical HPLC determinations.

Phosphoric acid 2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenyloxy)-3-hydroxy-propyl ester dimethyl ester (3). 2-((5Z,8Z,11Z,14Z)-Eicosa-5,8,11,14-tetraenyloxy)-propane-1,3-diol **(2)** (300 mg , 0.82 mmol) was dissolved in 15 ml of pyridine and cooled to 0 °C. Dimethyl chlorophosphate (Müller 1964) (**1**) (178 mg, 1.23 mmol), was dissolved in 2 ml of diethyl ether, then added dropwise to the solution and the mixture was stirred for 2 hours at 0 °C. The reaction mixture was stirred at 20 °C overnight, and water (2 ml) was added to dissolve pyridinium chloride and the mixture was extracted with diethyl ether (3 x 30 ml). The combined ether fractions were washed with 0.1 M H_2SO_4 (2 x 20 ml), 5% $NaHCO_3$ (20 ml) and water (3 x 20 ml). The organic phase was dried over Na_2SO_4 and purified by flash chromatography to give 190 mg (49 %) of **3**. ^{1}H NMR ($CDCl_3$) δ: 5.45-5.30 (m, 8H), 4.17-4.10 (m, 2H), 3.785 (d, 3H, J= 11.1 Hz), 3.782 (d, 3H, J=11.1 Hz), 3.75-3.72 (m, 1H), 3.68-3.50 (m, 4H), 2.88-2.78 (m, 6H), 2.37 (br s, 1H), 2.14-2.00 (m, 4H), 1.64-1.56 (m, 2H), 1.50-1.23 (m, 8H), 0.89 (t, 3H, J=6.9 Hz)

Phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenyloxy)-3-hydroxy-propyl] ester (4). The protected phosphate ester **3** (190 mg, 0.402 mmol) and NaI (133 mg, 0.884 mmol) were placed in a round bottomed flask and 5 ml of dry acetonitrile was added. Trimethylsilyl chloride (96 mg, 0.844 mmol) was added and the mixture was stirred for 1 h at 40 °C. Solvent was evaporated *in vacuo*, MeOH (5 ml) was added and the mixture was stirred for 1h at room temperature. Solvents were evaporated and the product was purified using reversed phase preparative HPLC to give 70 mg (39 %) of **4** as a sticky semi-solid product. ^{1}H NMR ($CDCl_3$) δ: 6.66 (br s, 3H), 5.48-5.27 (m, 8H), 4.11 (br s, 2H), 3.83-3.67 (m, 2H), 3.65-3.48 (m, 3H), 2.87-2.72 (m, 6H), 2.12-2.00 (m, 4H), 1.64-1.53 (m, 2H), 1.45-1.21 (m, 8H), 0.89 (t, 3H, J= 8.0 Hz); ^{13}C NMR δ: 130.49, 129.85, 128.59, 128.34, 128.19, 128.15, 127.93, 127.59, 77.90 (d, J_{CP}=7.4 Hz), 70.61, 64.98 (d, J_{CP}=5.3 Hz), 60.83, 31.53, 29.46, 29.34, 27.24, 27.01, 26.01, 25.66, 22.59, 14.08; ^{31}P NMR δ: 1.52. ESI-MS: 443.7 (M-1). Anal. ($C_{23}H_{41}O_6P \cdot 0.33$ H_2O) C, H.

Phosphoric acid (dimethoxy-phosphoryloxy)-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetra-enyloxy)-propyl ester dimethyl ester (5). Compound **5** was synthesized from **2** (300 mg, 0.82 mmol) and **1** (357 mg, 2.47 mmol) as described for **3** to give 210 mg, (44 %) of **5**. ^{1}H NMR ($CDCl_3$) δ: 5.44-5.28 (m, 8H), 4.20-4.04 (m, 4H), 3.782 (d, 6H, J=11.1 Hz), 3.779 (d, 6H, J=11.1 Hz), 3.70 (qui, 1H, J=5.1 Hz), 3.59 (t, 2H, J=6.5 Hz), 2.87-2.77 (m, 6H), 2.12-2.02 (m, 4H), 1.64-1.54 (m, 2H), 1.48-1.23 (m,8H), 0.89 (t, 3H, J=6.9 Hz).

Phosphoric acid mono-[2-((5Z,8Z,11Z,14Z)-eicosa-5,8,11,14-tetraenyloxy)-3-phosphono-oxy-propyl] ester (6). Compound **6** was synthesized from **5** (210 mg, 0.36 mmol), NaI (237 mg, 1.58 mmol), trimethylsilyl chloride (172 mg, 1.58 mmol) and MeOH (5 ml) as described for **4** to give 30 mg (16 %) of **6**. ^{1}H NMR ($CDCl_3$) δ: 9.28 (br s, 4H), 5.49-5.24 (m, 8H), 4.16 (br s, 4H), 3.82-3.70 (m, 2H), 3.62 (br s, 1H), 2.89-2.71 (m, 6H), 2.13-1.95 (m, 4H), 1.60 (br s, 2H), 1.48-1.20 (m, 8H), 0.88 (t, 3H, J=6.9 Hz); ^{13}C NMR δ: 130.47, 129.88, 128.59, 128.37, 128.19, 128.13, 127.94, 127.60, 76.57, 70.98, 64.46, 31.53, 29.34, 29.25, 27.23, 26.99, 25.89, 25.66, 25.64, 22.59, 14.09; ^{31}P NMR δ: 0.98. ESI-MS: 523.8 (M-1). Anal. ($C_{23}H_{42}O_9P_2 \cdot H_2O$) C,H.

Distribution coefficient. The distribution coefficients (log D) of **4** and **6** were determined at 25 °C in a 1-octanol-tris buffer (50 mM, pH 7.4, ionic strength 0.15) system. A known amount of compound was dissolved in the buffer and the pH was checked and adjusted (if necessary). A 1 ml aliquot of this solution was shaken with 1 ml of 1-octanol for 1 hour. The phases were separated by centrifugation for 10 minutes at 4000 rpm. The concentration of the compound in the buffer, before and after the shaking, was determined by HPLC.

Hydrolysis in aqueous solution. The rates of chemical hydrolysis for phosphate esters were determined in phosphate buffer (50 mM, pH 7.4, ionic strength 0.15) and tris buffer (50 mM, pH 7.4, ionic strength 0.15) at 37 °C. Prodrugs **4** or **6** were each dissolved in buffer to achieve a final concentration of 225 μM. The solutions were placed in a water bath at 37 °C and samples were withdrawn at pre-determined time intervals. Samples were analyzed for remaining phosphate ester by HPLC.

Hydrolysis in enzyme solution. The rate of hydrolysis in alkaline phosphatase solution was determined at 37 °C. Alkaline phosphatase (Type VII-S: from bovine intestinal mucosa, 3150 units/mg protein) was purchased from Sigma (St. Louis, MO, USA). Prodrug **4** or **6** (0.9 μmol each) was added to 4 ml of 50 mM tris buffer (pH 7.4) and placed in a water bath at 37 °C followed by the addition of 2 μl (62 units) of alkaline phosphatase. At predetermined time intervals, 200 μl samples were removed, and 400 μl of acetonitrile was added to each sample to stop the enzymatic hydrolysis. After centrifugation (14000 rpm, 10 min), samples were analyzed for remaining phosphate ester and for released noladin ether **2** by HPLC.

Hydrolysis in liver homogenate. The hydrolysis of phosphate esters was studied in 10% rabbit liver homogenate at 37 °C. A 20% liver homogenate was prepared by homogenizing

rabbit liver with four equivalents of isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000 *g* at 4°C and the supernatant was stored at –80 °C until used. One volume of 1.1 mM phosphate ester solution in tris buffer (pH 7.4) was added to one volume of stirred, pre-incubated (37°C) 20% liver homogenate. The solution was kept at 37 °C and 200 μl samples were withdrawn and added to 400 μl of cold acetonitrile. After mixing and centrifugation (14000 rpm, 10 min), the samples were analyzed for remaining phosphate ester, and for released noladin ether **2** by HPLC.

Hydrolysis in cornea homogenate. Fresh bovine eyes were obtained from a local slaughterhouse. The eyes were washed with 0.9% NaCl solution and corneas were dissected, rinsed with 0.9% NaCl solution and stored at -80 °C until used. The corneas were cut into small pieces with scissors and placed into preweighed centrifuge tubes. Tris buffer (50 mM, pH 7.4 at 37 °C) was added to give 16.7 % (w/v) solutions. The corneas were then homogenized at 4 °C with a Ystral X-1020 homogenizer (Ystral gmbh, Germany). The homogenate was centrifuged for 90 min at 9000 *g* at 4 °C and the supernatant was stored at –80 °C until used. Phosphate esters (0.9 μmol) were dissolved into 3 ml of tris buffer (50mM, pH 7.4 at 37 °C). Each hydrolysis experiment was initiated by adding 1 ml of preincubated (37 °C) corneal supernatant. The solution was kept at 37 °C and 200 μl samples were withdrawn and added to 400 μl of cold acetonitrile. After mixing and centrifugation (14000 rpm, 10 min), the samples were analyzed for remaining phosphate ester, and for released noladin ether **2** by HPLC.

Eye drop formulation. Noladin ether was dissolved in aqueous 25 % hydroxypropyl-β-cyclodextrin (HP-β-CD). The pH of the noladin ether solution was adjusted to 7.4 with NaOH solution and the solution was made isotonic with NaCl. Due to its higher water solubility, the monophosphate ester of noladin ether (**4**) was dissolved in 10 mM citrate buffer at pH 5.0 and the solution was made isotonic with NaCl. Final drug concentrations of noladin ether and its monophosphate prodrug **4** in eye drop solutions were 2.5 mg/ml and 3.05 mg/ml (6.86 mmol/l), respectively. Isotonic 10 mM citrate buffer (pH 5.0) was used as a control vehicle.

Intraocular pressure (IOP) experiments. A single drop (25 µl) of the test solution or vehicle was instilled unilaterally into the left eye, on the upper corneoscleral limbus. Before each measurement, one or two drops of topical anesthetic (0.06 % oxybuprocaine) were applied to the cornea to reduce possible discomfort. IOP of the rabbits was measured using a pneumatonometer (Digilab Modular One, Bio-Rad, Cambridge, MA). For each determination, at least two readings were taken from the treated and untreated eye, and the mean of these readings was used. IOP of the rabbits was measured 1 hour before administration and then at 0, 0.5, 1, 2, 3, 4 and 5 hours after application of the eye drops. IOP at the time of eye drop administration (0 h) was used as a baseline value. Baseline IOPs ranged from 18.0 to 23.9 mm Hg. All studies were arranged using a randomised crossover design. At least a 72 h wash-out time was allowed for each rabbit between doses.

Data analysis. Results are given as a change in IOP (%) mean ± S.E. (standard error). A one-factor analysis of the variance (ANOVA) for repeated measurements was used to test for possible statistical differences between noladin ether **2**, prodrug **4** and vehicle treated groups. Significance in differences of the means was tested by the Fisher's Protected Least Significant Difference (PLSD) method at the 95 % confidence level. Experimental data fitting (non-linear regression analyses) of hydrolysis data was performed by Scientist Software (MicroMath Scientific Software, Salt Lake City, UT).

Animals. Normotensive pigmented Dutch rabbits (Harlan Netherlands B.V.) of both sexes (2.4 - 3.3 kg, n = 5) were used as experimental animals. The rabbits were housed singly in cages under controlled illumination (12-hour light-dark cycle, with lights-on at 7:00 AM) and environmental conditions (room temperature 20.0 ± 0.5 °C and 55 - 75 % relative humidity). Rabbits were given water and food *ad libitum* except during the experiments. Animals were treated in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research.

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7 *IN VITRO* CORNEAL PERMEATION OF CANNABINOIDS AND THEIR WATER-SOLUBLE PHOSPHATE ESTER PRODRUGS*

Abstract. Topically administered cannabinoids have been shown to reduce intraocular pressure by interacting with the ocular cannabinoid receptor. Most of the cannabinoids have very poor aqueous solubility, which limits their pharmaceutical development and usefulness. In this study, permeation of three cannabinoids (arachidonylethanolamide, *R*-methanandamide, noladin ether) and their water-soluble phosphate ester prodrugs across isolated rabbit cornea was investigated *in vitro*. Hydroxypropyl-β-cyclodextrin (HP-β-CD) was used to solubilize the parent cannabinoids in permeation studies to achieve the required concentration in donor and receiving cells. Highest fluxes were obtained with lipophilic parent compounds administered with HP-β-CD, and the fluxes of phosphate esters were 45-70% that of their corresponding parent compounds. Phosphate esters hydrolyzed on the surface of the cornea or during the permeation to release the lipophilic parent compound, which further permeated the cornea. No phosphate esters were detected on the endothelial side of the cornea. Although the phosphate esters had lower fluxes than their corresponding parent compounds in these HP-β-CD formulations, the results are promising and the fluxes of phosphate esters are significantly higher than the fluxes of parent compounds administered as a suspension (due to their low aqueous solubility) without HP-β-CD.

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7.1 Introduction

Hepler and Frank (Hepler and Frank 1971) reported in 1971 that smoked marijuana reduces intraocular pressure (IOP). This finding was later confirmed (Cooler and Gregg 1977) by intravenous administration of *delta*-9-tetrahydrocannabinol (THC), which is the main active ingredient in marijuana. Discovery of a cannabinoid receptor in 1988 (Devane et al. 1988), followed by the characterization of the first endogenous cannabinoid receptor ligand, arachidonylethanolamide (Devane et al. 1992), opened up new opportunities for the search of novel compounds as possible anti-glaucoma agents (Järvinen et al. 2002). In addition to their IOP lowering effects, cannabinoids also have neuroprotective actions, and both of these actions would be beneficial in the treatment of glaucoma. Pate et al. (1995) also observed that endocannabinoids, like arachidonylethanolamide (AEA), reduce IOP. This IOP decrease was shown to be a local effect, rather than mediated via the central nervous system, and the discovery of the ocular CB_1 receptor (Porcella et al. 1998) supports this finding. Therefore, topical administration of cannabinoids to the eye would be the preferred route of administration, as systemic administration of cannabioids has a higher risk of both unwanted psychoactive and cardiovascular effects.

Both classic and endogenous cannabinoids are very lipophilic, and thus poorly water-soluble, molecules. Because of the very low aqueous solubility, topical administration of cannabinoids to the eye requires an oily vehicle, or the use of excipients such as polyvinyl alcohol or cyclodextrins in aqueous formulations (Pate et al. 1995; Kearse and Green 2000). The aqueous solubility of a drug is very important in topical ophthalmic drug delivery, because the instilled volumes need to be kept to a minimum in order to minimize the loss of drug due to lacrimal drainage, which is directly proportional to the increase in lacrimal fluid volume. Other important drug factors that affect corneal permeability include partition coefficient, molecular size and degree of ionization.(Schoenwald 1990). We recently reported the development of phosphate ester prodrugs of arachidonylethanolamide (AEA), *R*-methanandamide and noladin ether (Juntunen et al. 2003a; Juntunen et al. 2003b). These prodrugs have high aqueous solubilities, and they can be formulated for topical administration at sufficient concentrations in buffered solutions without any solubilizing excipients.

Phosphate ester prodrugs have most commonly been used to increase the aqueous solubility of poorly water-soluble compounds intended for *i.v.* or *i.m.* administration. Also, applications involving their oral administration do exist. However, the topical ophthalmic administration route seems to be a practically unexplored possibility for such prodrugs. One of the few examples of the ophthalmic application of a phosphate ester prodrug is prednisolone phosphate (Musson et al. 1991).

Phosphate ester prodrugs exist in an ionized form near pH 7.4 which may hinder their permeation across the cornea. However, the IOP reducing effects of phosphate esters of both *R*-

methanandamide and noladin ether in rabbits were comparable to the effects of their parent compounds solubilized by HP-β-CD (Juntunen et al. 2003a; Juntunen et al. 2003b). This promising finding led us to study the corneal permeation of these phosphate ester prodrugs in more detail.

In the present study we have investigated the permeation of cannabinoids and their water-soluble prodrugs (Figure 1) through rabbit cornea *in vitro* using side-by-side diffusion cells to clarify whether or not these phosphate ester prodrugs can penetrate the cornea as such, or if they release the more lipophilic parent compound prior to permeating the cornea.

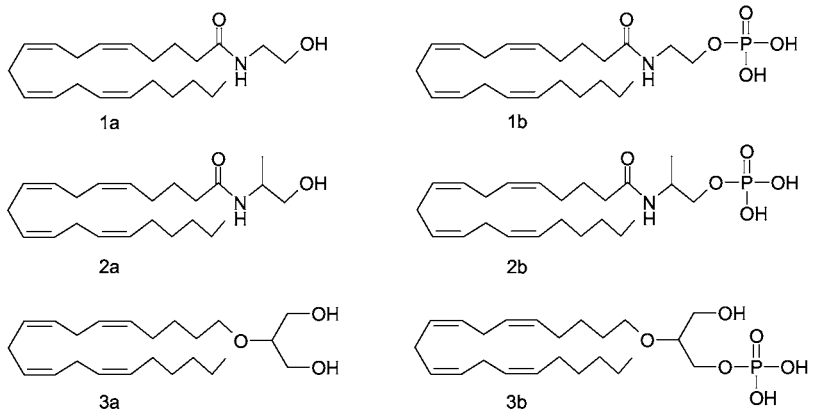

Figure 1. Structures of AEA (**1a**), *R*-methanandamide (**2a**), noladin ether (**3a**) and their phosphate esters (**1b**, **2b** and **3b**).

7.2 Results and discussion

7.2.1 *In vitro* corneal permeation

Our previous *in vivo* studies in rabbits had shown that phosphate ester prodrugs of both AEA and noladin ether were able to elicit an IOP decrease comparable to the effects of their corresponding parent compounds when dissolved in an aqueous 5% HP-β-CD solution. The question remained whether or not these prodrugs were able to permeate the cornea as such. It is worth noting, that although they are ionized at tear fluid pH, they still have apparent octanol-water partition coefficients near to optimal values for membrane permeation (Table 1).

Table 1. Physicochemical properties of cannabinoids (**1a-3a**) and their phosphate ester prodrugs (**1b-3b**)

Compound	Solubility at pH 7.4 (μg/ml)	Log $D_{7.4}$	pK_{a1}	pK_{a2}
1a	0.4[a]	5.97[b]	-	-
1b	> 5000	1.15 ± 0.09[c]	2.68[c]	6.88[c]
2a	-	6.31[b]	-	-
2b	> 5000	1.53 ± 0.02[c]	2.65[c]	6.73[c]
3a	< 0.1	7.05[b]	-	-
3b	> 5000	1.98 ± 0.06[d,e]	-[f]	-[f]

[a] From (Jarho et al. 1996a).

[b] Calculated with ACD labs log D suite.

[c] From (Juntunen et al. 2003a).

[d] From (Juntunen et al. 2003b).

[e] $p < 0.05$ compared to **1b** (Kruskall-Wallis test, Dunn's test)

[f] Not determided

In this study, highest fluxes across isolated rabbit cornea were achieved with cannabinoids solubilized with HP-β-CD, and flux-values of the corresponding water-soluble phosphate esters were 45-70% that of the parent compounds (Table 2, Figure 2 A, B and C). However, the differences in steady state flux-values between prodrugs (**1b** and **3b**) and the corresponding parent compounds (**1a** and **3a**) were not statistically significant. *R*-methanandamide (**2a**) had the highest flux of 54.9 ± 16.1 nmol/(cm^2·h), however, in comparison of the permeation characteristics for the parent compounds, one must bear in mind that the complexation with CDs also increases the fluxes (Table 2). To highlight the importance of both aqueous solubility and dissolution rate, **3a** was also administered as a suspension without HP-β-CD. The flux of **3a** in this case was reduced to 0.9 ± 0.6 nmol/(cm^2·h), which is only 3% of the flux of the same compound with HP-β-CD. The large difference between **3a** suspension and CD-formulation is due to the slow dissolution of free compound from the suspension compared to the fast equilibrium between the complexed and free form in the CD-formulation.

Table 2. Steady-state fluxes (J_{ss}) (mean ± SD; n = 3-6) for cannabinoids and their phosphate esters through isolated rabbit cornea *in vitro* (37 °C, pH 7.65).

Compound	Vehicle	J_{ss} (nmol/(cm^2·h))
1a	5% CD, tris buffer	39.30 ± 1.94
1b	tris buffer	26.66 ± 7.06[a]
2a	5% CD, tris buffer	54.89 ± 16.05[b]
2b	tris buffer	23.95 ± 6.70[c]
3a	5% CD, tris buffer	27.61 ± 5.81[d]
3a suspension	tris buffer	0.89 ± 0.58
3b	tris buffer	14.07 ± 4.06[d]
3b	Phosphate buffer	4.32 ± 0.69

[a] $p < 0.05$ compared to **3b** (tris buffer) (Kruskall-Wallis test, Dunn's test)

[b] $p < 0.05$ compared to **3a** (CD, tris buffer) (Kruskall-Wallis test, Dunn's test)

[c] $p < 0.05$ compared to **2a** (CD, tris buffer) (Kruskall-Wallis test, Dunn's test)

[d] $p < 0.05$ compared to **3a** (suspension) (Kruskall-Wallis test, Dunn's test)

Phosphate esters **1b** and **2b** had similar fluxes (Table 2) (26.7 ± 7.1 and 24.0 ± 6.7 nmol/(cm^2·h), respectively) but **3b** had a lower flux (14.1 ± 4.1), which corresponds with the lower flux of the parent compound **3a**. The HP-β-CD concentration used in this study had previously been determined to be optimal for corneal permeation of **1a** (Jarho et al. 1996b). Lower CD concentrations would have led to an incomplete dissolution of the lipophilic parent compounds, while higher concentrations would have produced excessive complexation of the compounds, thereby reducing the amounts of free compounds available for absorption. In this context, the results obtained with these phosphate ester prodrugs are promising.

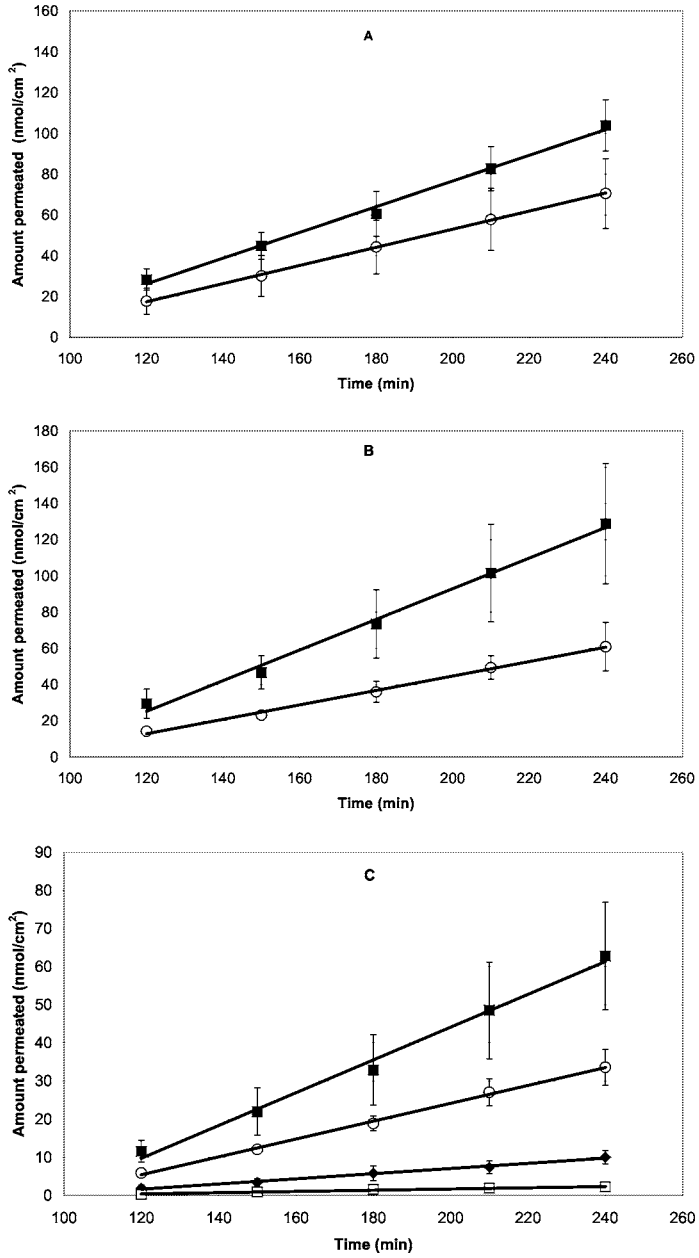

Figure 2: Linear portion of permeation profiles (mean ± SD; n= 3-4) for **A)** arachidonylethanolamide **1a** in 5% HP-β-CD (■) and prodrug **1b** (○), **B)** *R*-methanandamide **2a** in 5% HP-β-CD (■) and prodrug **2b** (○) and **C)** HU-310 **3a** in 5% HP-β-CD (■), prodrug **3b** (○), **3a** suspension (□) and **3b** in phosphate buffer (◆) through isolated rabbit cornea (pH 7.65).

7.2.2 Enzymatic hydrolysis

In the permeation experiments, no phosphate esters were detected on the receiver side, indicating that these prodrugs released the parent compound just prior to, or during, the absorption. Enzymatic hydrolysis experiments of prodrugs were performed to confirm hydrolysis of the prodrugs by corneal enzymes. All phosphate esters **1b**, **2b** and **3b** were hydrolyzed in the 4% cornea homogenate ($t_{½}$ = 20.2 ± 0.2, 14.6 ± 0.3 and 18.1 ± 1.4 min, respectively), and they quantitatively released the corresponding parent compounds (Figure 3). The possible hydrolysis of phosphate ester prodrugs can occur just before penetration on the surface of the cornea, during the penetration or after the prodrug has crossed the cornea. Histochemical studies of bovine and rabbit corneas have revealed that alkaline phosphatase is found in the corneal epithelium, endothelium and also in keratocytes (Lojda et al. 1976).

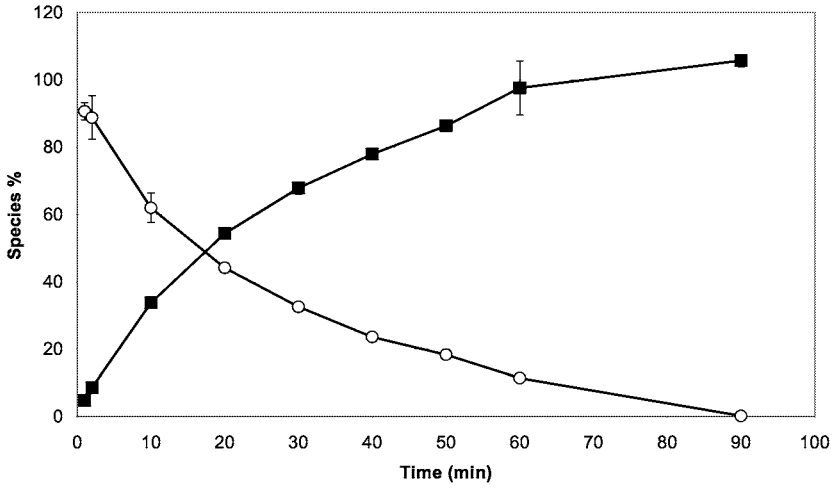

Figure 3: Time courses (mean ± SD; n= 2) for arachidonylethanolamide phosphate ester **1b** (○) and arachidonylethanolamide **1a** (■) during hydrolysis of the prodrug **1b** in 4% cornea homogenate (pH 7.65, 37 °C).

The effect of inhibiting alkaline phosphatase for the *in vitro* corneal permeation of **3b** was investigated in order to study the importance of enzymatic hydrolysis on the flux of phosphate ester prodrugs. Corneal alkaline phosphatase was inhibited simply by using phosphate buffer instead of tris buffer in the permeation experiments. Inhibition of alkaline phosphatase by phosphate was confirmed by observing phosphate ester hydrolysis in an alkaline phosphatase-containing solution, both in tris ($t_{½}$ = 4.5min) and phosphate ($t_{½}$ = 231.5 min) buffers (Figure 4). When the permeability experiment with **3b** was made using phosphate buffer, which is a known inhibitor of alkaline phosphatase, the flux was reduced by 70% to 4.3 ± 0.7 nmol/(cm^2·h). The decreased flux with phosphate buffer suggests that these phosphate ester prodrugs might be

enzymatically cleaved to release the lipophilic parent compound on the corneal surface, just prior to absorption.

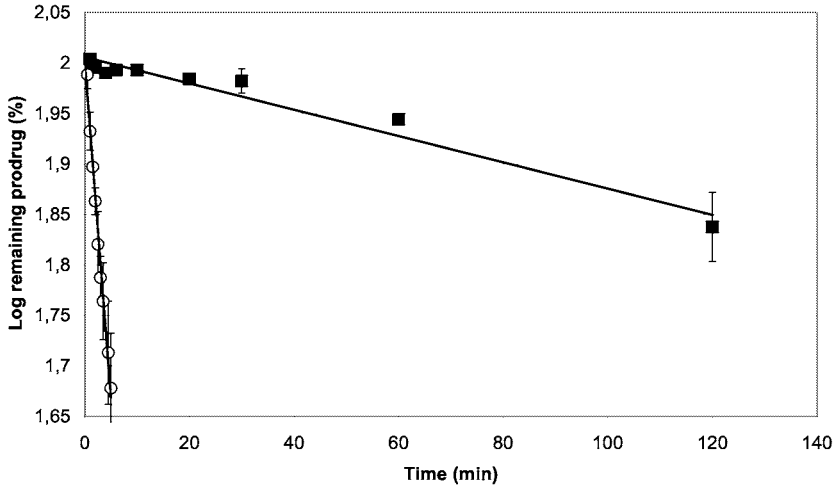

Figure 4: Pseudo-first order plots (mean ± SD; n= 2) for the hydrolysis of **3b** in an alkaline phosphatase-containing solution in tris (○) and phosphate buffers (■), showing the inhibitory effect of phosphate buffer (pH 7.65, 37 °C).

7.3 Conclusions

Although the most effective corneal permeation was achieved with the lipophilic parent compounds administered with HP-β-CD, the results obtained with cannabinoid phosphate esters suggest that aqueous phosphate esters prodrug approach is a potential alternative to oil-based or cyclodextrin formulations in ophthalmic applications to overcome the delivery/formulation problem caused by poor aqueous solubility. Phosphate esters of these cannabinoids have adequate aqueous solubility, and they are enzymatically hydrolyzed by alkaline phosphatase to their lipophilic parent compounds, which subsequently permeate the cornea.

7.4 Experimental

Materials. Hydroxypropyl-β-cyclodextrin (Cavasol W7 HP Pharma) was obtained from Wacker-Chemie GmbH, Burghausen, Germany. Arachidonylethanolamide and *R*-methanandamide were synthesized from arachidonic acid and the corresponding amino alcohol using the method of Abadji et al.(Abadji et al. 1994). Noladin ether was synthesized from arachidonic alcohol as described by Hanuš et al.(Hanus et al. 2001). Phosphate esters of arachidonyl ethanolamide, *R*-methanandamide and noladin ether were prepared as previously reported (Juntunen et al.2003a; Juntunen et al. 2003b).

***In vitro* corneal permeation study.** The permeabilities of these cannabinoids and their phosphate esters were studied using glass diffusion cells at 37 °C. Corneas were obtained from albino rabbits (2-3 kg) of both sexes. Animals were sacrificed for other reasons than for this experiment. The study was performed according to ethical approval from the University of Kuopio. The eye was proptosed and the cornea with its scleral ring was carefully excised with scissors and placed into 37 °C BSS plus solution (Balanced salt solution, Alcon Laboratories, Fort Worth, USA). The lens, together with various tissues of the eye, were removed to leave only the cornea and scleral ring.

The isolated corneas were washed with tris buffer and mounted on a corneal holder, which was placed between the glass diffusion chambers. When phosphate buffer was used as a vehicle (one experiment), the isolated corneas were washed with phosphate buffer. Preheated (37 °C) 20 mM tris (or phosphate) buffer (3.4 ml) containing 5% HP-β-CD was first added to the receiver cell (endothelial side). HP-*β*-CD was used in the receiver cell to control for the solubility of penetrated cannabinoids. Then 3.2 ml of the buffered solution containing the test compound (1.4 mM) was added to the donor cell (epithelial side). AEA, *R*-methandamide and noladin ether were each dissolved separately in a 20 mM tris buffer (pH 7.65 at 37°C) containing 5% HP-β-CD. Phosphate esters were dissolved in 20 mM tris or phosphate buffer. Osmolality of solutions was adjusted to 290 mOsm using NaCl. A carbogen bubbling (95% O_2 : 5 % CO_2) was used to provide mixing of the solutions on both sides. At specified time intervals, 200 µl aliquots were withdrawn from the receiver side and replaced with fresh buffer. Determination of cannabinoid concentrations was made by HPLC.

Enzymatic hydrolysis in cornea homogenate. Fresh bovine eyes were obtained from a local slaughterhouse. The eyes were washed with 0.9% NaCl solution and corneas were dissected, rinsed with a 0.9% NaCl solution and stored at -80 °C until used. The corneas were cut into small pieces with scissors and placed into preweighed centrifuge tubes. Tris buffer (50 mM, pH 7.4 at 37 °C) was added to give 16.7% (w/v) solutions. The corneas were then homogenized at 4 °C with an Ystral X-1020 homogenizer (Ystral gmbh, Germany). The homogenate was centrifuged for 90 min at 9000 *g* and 4 °C, and the supernatant was stored at -80 °C until used. Phosphate esters were dissolved in 3 ml of tris buffer (50 mM, pH 7.4 at 37 °C). Each hydrolysis experiment was initiated by adding 1 ml of preincubated (37 °C) corneal supernatant. The solution was kept at 37 °C and 200 µl aliquots were withdrawn and then added to 400 µl of cold acetonitrile. After mixing and centrifugation, the supernatants were analyzed for remaining phosphate ester and for released parent cannabinoid by HPLC.

Hydrolysis in alkaline phosphatase containing solution. The rate of hydrolysis of **3b** in alkaline phosphatase solution, both in tris and phosphate buffer, was determined at 37 °C. Alkaline phosphatase (Type VII-S: from bovine intestinal mucosa, 3150 units/mg protein) was purchased from Sigma (St. Louis, MO, USA). A total of 0.9 µmol of **3b** in 4 ml of 50 mM tris

or phosphate buffer (pH 7.4) was placed in a water bath at 37 °C, and 2 µl (62 units) of alkaline phosphatase was added. At predetermined time intervals, 200 µl aliquots were removed and 200 µl of acetonitrile was added to stop the enzymatic hydrolysis. After centrifugation (14000 rpm, 10 min), samples were analyzed for remaining phosphate ester **3b** and for released noladin ether **3a** by HPLC.

HPLC analysis. HPLC determinations were performed with a Merck LaChrom HPLC system consisting of L-7250 autosampler, L-7100 pump, D-7000 interface, L-7455 diode array detector and a D-7000 HPLC system manager software (Hitachi, Tokyo, Japan). A Purospher RP-8e (125 mm x 4.0 mm, 5µm) (Merck, Darmstadt, Germany) reversed phase column was used for all analytical HPLC determinations. A mobile phase of acetonitrile and 20 mM phosphate buffer (pH 7.0) at a flow rate of 1.2 ml/min was used, with a gradient elution that began at 40% ACN and increased to 72% ACN.

Statistical analysis. Differences in steady state fluxes (n = 3-6) between the compounds were statistically compared using a Kruskal-Wallis test followed by a post-hoc comparison of the means between each compound using a Dunn's test. Prodrugs were compared to their corresponding parent compounds and comparison was also made between parent compounds and between prodrugs. The different formulations of **3a** and **3b** were also compared. A similar statistical analysis was performed on the measured log D-values (n=3). A significance level of $P < 0.05$ denoted significance in all cases.

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8 SUMMARY AND CONCLUSIONS

Advances in many areas of cannabinoid research have increased our understanding of the function of the endocannabinoid system. The endocannabinoid system offers new possibilities for the treatment of several diseases, including cancer, anxiety, control of appetite, glaucoma, pain and several neurodegenerative disorders. However, cannabinoids, like many new drug candidates, do not possess the optimal physicochemical properties to reach the site of action. Endocannabinoids are synthesized very near to their tissue receptors, so they easily reach the target. The situation is very different when these kinds of compounds are used as therapeutical agents. The poor aqueous solubility hinders the pharmaceutical usefulness of the current cannabinoids.

The present study describes the synthesis and *in vitro* evaluation of water-soluble prodrugs of endocannabinoids anandamide (AEA) and noladin ether, and also the prodrugs of a metabolically stable AEA analog, *R*-methanandamide. The ophthalmic usefulness of these prodrugs was studied in an *in vitro* corneal permeation study, and some of the prodrugs were also investigated *in vivo* for their ablility to reduce IOP after their topical administration. The following conclusions can be made based on the results of the present study:

1. Phosphate ester prodrugs of anandamide and *R*-methanandamide increased the aqueous solubility of the parent compounds considerably. The prodrugs were chemically relatively stable and rapidly released the parent compound in alkaline phosphatase-containing solution.

2. Tertiary and quaternary methylpiperazine prodrugs of anandamide were more water-soluble than the parent compound. The pK_a values of tertiary prodrugs were near 7.4 so the aqueous solubility was strongly pH-dependent at around neutral pH. The greatest advantage of the quaternary prodrugs of anandamide lies in their pH-independent solubility.

3. Some of the tertiary and quaternary prodrugs were not enzymatically cleaved. This is most probably due to steric reasons and also partly due to the precence of the permanent positive charge on the quaternary prodrugs. However, tertiary and monoquaternary prodrugs with a longer linker between the ester moiety and the methylpiperazine ring were readily cleaved by the enzymes in serum and liver homogenate. The chemical stabilities of tertiary and quaternary prodrugs were also dependent on the length of the linker between the solubilizing moiety and the ester bond.

4. Mono- and diphosphate esters of noladin ether offered good aqueous solubility enhancement and they were also good substrates for the alkaline phosphatase enzyme releasing the parent compound quantitatively.

5. The fluxes of phosphate prodrugs in the cornea permeation study were smaller than the fluxes of parent compounds solubilized with cyclodextrin. However, the flux of noladin ether prodrug was much better than the flux of noladin ether suspension without cyclodextrin. The phosphate esters were hydrolyzed during the permeation since no phosphate esters were detected on the receiver side.

6. The phosphate esters of *R*-methanandamide and noladin ether (or the released parent compunds) decreased intraocular pressure in normotensive rabbits after their topical administration and the effect was comparable to the parent compounds administered in a cyclodextrin formulation.

7. In general, the phosphate prodrug approach can be used to improve water-solubility of endocannabinoids and their derivatives. *In vitro* and *in vivo* studies indicate that phosphate ester prodrugs are suitable, for example, for topical ophthalmic delivery of endocannabinoids and similar lipophilic compounds.

8. Although the chemical stabilities of prodrug molecules were adequate to perform most of the experiments, the stabilities as such may not be good enough to enable formulation of liquid preparations with acceptable shelf lives. Further studies are needed to improve both the stability of parent cannabinoid compounds and the prodrug molecules.

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