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Intraocular Pressure Lowering Activities of Endogenous Cannabinoids, and Their Uptake and Enzyme Hydrolysis Inhibitors in Normotensive Rabbits

Doctoral dissertation

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ABSTRACT

Existence of an endogenous cannabinoid system has been confirmed in various mammalian organs, including the eye. Discovery of specific molecular targets for cannabinoid action has revealed a possibility to develop novel cannabinoid analogues with various therapeutic actions. The intraocular pressure (IOP) reducing properties of cannabinoids were first reported in 1970's by human's smoking marijuana. Since then, numerous studies have explored cannabinoids as novel anti-glaucoma agents. The present study investigated the IOP effects of endogenous cannabinoids, their uptake and fatty acid amide hydrolase (FAAH) inhibitors in normotensive rabbits in order to obtain preliminary data for the possible utilization of the ocular endocannabinoid system in the development of novel IOP lowering agents.

Topical unilateral administration of endocannabinoids N-arachidonylethanolamide (AEA), 2-arachidonylglycerol (2-AG) and noladin ether decreased IOP in the treated eyes of normotensive rabbits. The IOP profiles of AEA and 2-AG were bi-phasic (i.e., an initial increase followed by IOP reduction in treated eyes), whereas noladin ether decreased IOP directly in rabbits, suggesting that they act via separate mechanisms. In fact, AEA and 2-AG were shown to rapidly degrade to arachidonic acid in bovine iris-ciliary body and cornea homogenates, while noladin ether was stabile. The studies with CB1 receptor antagonist further indicated that the IOP effects of noladin ether were mediated via CB1 receptors in rabbits, whereas the effects of 2-AG were mediated by its arachidonic acid metabolites. The topical unilateral administration of AEA uptake inhibitors, AM404 and olvanil, both reduced IOP in the treated eyes of rabbits. Neither AM404 nor olvanil exhibited CB1 receptor -dependent G-protein activity when monitored using $[^{35}S]GTP\gamma S$ autoradiography of rat brain sections, indicating that the IOP effects of transport inhibitors were not likely mediated through direct agonism at the CB1 receptors. The FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF) failed to have any significant effect on rabbit IOP, compared to vehicle treatment, when administered subcutaneously without any topical cannabinoid drug. In contrast, PMSF (s.c.) eliminated the initial hypertensive phase from the bi-phasic IOP profiles of topically applied AEA and its synthetic analogue archidonylpropionitrileamide (APN) in normotensive rabbits. The IOP reduction induced by AEA in the presence of PMSF was mediated via CB1 receptors.

Finally, to evaluate the role of CB2 receptors on the IOP lowering effects of cannabinoids, the IOP effects of topically administered CB2 specific agonist JWH-133 was measured and compared to the IOP effects of the non-selective cannabinoid CP55,940. JWH-133 failed to generate IOP reduction in the treated eyes of rabbits, whereas CP55,940 at the same dose range produced statistically significant IOP reductions in treated eyes, suggesting that CB2 receptor agonists may not decrease IOP at the dose range used.

These results suggest that the IOP of normotensive rabbits can be lowered with topically administered CB1 receptor agonists but not with CB2 receptor agonist JWH-133. The inhibitors of FAAH and AEA transporter may potentiate the action of endogenous cannabinoids, if their endogenous levels are high enough to produce this pharmacological action. These preliminary data suggest that the endogenous cannabinoid system may act as a potential target for the development of novel IOP lowering therapeutics.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AA	Arachidonic acid
AA-5-HT	Arachidonyl serotonin
AcAHA	O-Acetyl-arachidonyl-hydroxamate
ACEA	Arachidonyl-2'-chloroethylamide
AchE	Acetylcholinesterase
ACMK	Arachidonyl-chloro-methylketone
ADMK	Arachidonyl-diazo-methylketone
AEA	N-Arachidonylethanolamide
AEG	Arachidonyl ethylene glycol
2-AG	2-Arachidonylglycerol
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AM374	Palmityl sulfonyl fluoride
AM403	N-(3-hydroxyphenyl)-arachidonylamide
AM404	N-(4-hydroxyphenyl)-arachidonylamide
APN	Arachidonyl propionitrileamide
ATFMK	Arachidonyl trifluoromethyl ketone
ATP	Adenosine triphosphate
AtT-20	Mouse pituitary cell line
BEL	Bromoenol lactone
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor type-1
CB1A	Splice variant of cannabinoid receptor type-1
CB2	Cannabinoid receptor type-2
CBD	Cannabidiol
CBN	Cannabinol
CD	Cyclodextrin
CHO	Chinese hamster ovary cell line
CNS	Central nervous system
COS-7	African green monkey kidney cell line
COX-1	Cyclo-oxygenase type-1
COX-2	Cyclo-oxygenase type-2
CP55,940	(-)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclo-hexyl]-phenol
cPLA$_2$	Cytosolic phospholipase A$_2$

CREAE	Chronic relapsing experimental allergic encephalomyelitis
DAG	Diacylglycerol
DHEA	N-Docosahexaenoylethanolamine
DMSO	Dimethylsulfoxide
FAAH	Fatty acid amide hydrolase
GABA	γ-Aminobutyric acid
GC/MS	Gas chromatography / mass spectrometry
$[^{35}S]GTP\gamma S$	Guanosine-5'-O-(3-[^{35}S]-thio)-triphosphate
HD	Huntington's disease
HEA	Hydroperoxyeicosatetraenoylethanolamide
HeLa	Human cervix carcinoma cell line
HETE	Hydroxyeicosatetraenoic acid
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HPLC	High performance liquid chromatography
5-HT	Serotonin (5-hydroxytryptamine)
HU-211	11-Hydroxy-Δ^8-tetrahydrocannabinol, dimethylheptyl (dexanabinol)
IC_{50}	Inhibition concentration
INL	Inner nuclear layer of retina
IOP	Intraocular pressure
IPL	Inner plexiform layer of retina
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
JWH-133	3-(1',1'-dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol
K_i	Inhibition constant
LC/MS/MS	Liquid chromatography / tandem mass specrometry
LOX	Lipoxygenase
MAFP	Methyl arachidonyl fluorophosphonate
MGL	Monoacylglycerol lipase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
N18TG2	Mouse neuroblastoma cell line
NArPE	N-Arachidonylphosphatidyl-ethanolamide
NDHPE	N-Docosahexaenoyl-phosphatidylethanolamine
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
O-1624	Methyl eicosanyl fluorophosphonate
O-1778	Methyl dodecyl fluorophosphonate

O-1887	Methyl octyl fluorophosphonate
OEA	*N*-Oleylethanolamide
OMDM-1	(S)-*N*-Oleoyl tyrosinol
OMDM-2	(R)-*N*-Oleoyl tyrosine
OPL	Outer plexiform layer of retina
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEA	*N*-Palmitoylethanolamide
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PMSF	Phenylmethylsulfonyl fluoride
PPAR-α	Nuclear peroxisome-proliferator-activated receptor-α
PVA	Poly(vinylalcohol)
RBL-1	Rat basophilic leukemia cell line (original)
RBL-2H3	Rat basophilic leukemia cell line
SR141616A	*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride
SR144528	*N*-[(1S)-*endo*-1,3,3,-trimethyl-bicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
Δ^9-THC	Δ^9-Tetrahydrocannabinol
Δ^8-THC	Δ^8-Tetrahydrocannabinol
TMEV	Theiler's murine encephalomyelitis virus
UCM707	*N*-(3-furylmethyl)-arachidonylamide
URB52	Cyclohexylcarbamic acid biphenyl-3-yl ester
VDM11	*N*-(1-methyl-4-hydroxyphenyl)arachidonylamide
VDM12	*N*-(1-hydroxy-4-hydroxyphenyl)-arachidonylamide
VDM13	Arachidonoyl-5-methoxytryptamine
WIN55,212-2	*R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate
WIN55,212-3	*S*-(-)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate
VR1	Vanilloid receptor type-1

LIST OF ORIGINAL PUBLICATIONS

This doctoral dissertation is based on the following publications, which are referred to in the text as Roman numerals **I-IV**.

I   Krista Laine, Kristiina Järvinen, David W. Pate, Arto Urtti, Tomi Järvinen: Effect of the enzyme inhibitor, phenylmethylsulfonyl fluoride, on the IOP profiles of topical anandamides.
Investigative Ophthalmology & Visual Science 43: 393-397, 2002.

II   Krista Laine, Tomi Järvinen, Juha Savinainen, Jarmo T. Laitinen, David W. Pate, Kristiina Järvinen: Effects of topical anandamide-transport inhibitors, AM404 and olvanil, on intraocular pressure in normotensive rabbits.
Pharmaceutical Research 18: 494-499, 2001.

III   Krista Laine, Kristiina Järvinen, Aviva Breuer, Raphael Mechoulam, Tomi Järvinen: Comparison of the enzymatic stability and intraocular pressure effects of 2-arachidonylglycerol and noladin ether, a novel putative endocannabinoid.
Investigative Ophthalmology & Visual Science 43: 3216-3222, 2002.

IV   Krista Laine, Kristiina Järvinen, Tomi Järvinen: Topically administered CB2-receptor agonist, JWH-133, does not decrease intraocular pressure (IOP) in normotensive rabbits.
Life Sciences 72: 837-842, 2003.

CONTENTS

1 INTRODUCTION

Glaucoma is one of the leading causes of irreversible blindness worldwide (Quigley 1996). Currently, a number of commercial drugs such as cholinergic and adrenergic agents, carbonic anhydrase inhibitors and prostaglandin analogs are available to lower the high intraocular pressure (IOP) from glaucoma. However, these anti-glaucoma medications may sometimes prove ineffective or lose their efficacy during chronic use, indicating that there is a constant need for the development of novel therapeutic agents. The IOP reducing properties of cannabinoids were first reported in 1970's by humans smoking marijuana (Hepler and Frank 1971). Since then, numerous studies have explored cannabinoids as novel anti-glaucoma agents (Järvinen et al. 2002). However, the major drawback for the use of plant-derived cannabinoids has revealed to be their tendency to generate unwanted psychoactive adverse effects, particularly after their systemic administration (ElSohly et al. 1981; Flach 2002). In addition, the hydrophobic cannabinoids have remarkably poor water solubility, which hinders their development as aqueous eye drop formulations.

Recently, the discovery of endogenous cannabinoids and their specific binding sites, namely cannabinoid CB1 and CB2 receptors, has been confirmed by studies from various mammalian organs, including the eye (Porcella et al. 1998; Straiker et al. 1999a; Lu et al. 2000; Stamer et al. 2001). Endogenous cannabinoids are produced in neurons "on demand" after depolarisation, and immediately released after their production. After release, they are rapidly removed from the extracellular space by transport into cells, followed by enzymatic hydrolysis. The most important and extensively studied endogenous cannabinoids include N-arachidonylethanolamide (anandamide, AEA) (Devane et al. 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995). Additionally, a few other novel cannabimetic lipids, that are able to bind and activate cannabinoid receptors, such as noladin ether (Hanus et al. 2001) and virodhamine (Porter et al. 2002) have recently been identified in mammalian tissues.

The main objective of the present study was to obtain preliminary data on whether the ocular endocannabinoid system might act as a possible target for the development of novel topically applied IOP lowering agents. The low aqueous solubility of the studied compounds was overcome by cyclodextrins. Compounds affecting the endogenous cannabinoid system most probably lower IOP through mechanisms independent of current medications, suggesting that these compounds might be an entirely new class of IOP lowering drugs.

2 REVIEW OF LITERATURE

2.1 ENDOGENOUS CANNABINOID SYSTEM

2.1.1 Cannabinoid receptors

The cellular effects of cannabinoids were originally thought to be mediated via their lipophilic nature, by perturbing the cellular membrane. Discovery and subsequent cloning of the CB1 receptor from rat brain (Devane et al. 1988; Matsuda et al. 1990) and later the CB2 receptor from adult rat spleen (Munro et al. 1993) revealed that the biological effects of cannabinoids are exerted via specific receptors. Both of these cannabinoid receptor subtypes belong to the G-protein coupled receptor superfamily, and the overall homology between CB1 and CB2 receptor proteins is shown to be approximately 44 %, although it is around 68 % in the transmembrane protein regions that are assumed to contribute to ligand binding (Munro et al. 1993) (Fig. 1). For a CB1 receptor, the alternatively spliced receptor isoform CB1A mRNA has been isolated in minor quantities together with CB1 from human central nervous and peripheral tissues (Shire et al. 1995). The short CB1A variant has been shown to possess similar, but slightly attenuated, pharmacological characteristics than CB1 (Rinaldi-Carmona et al. 1996). Furthermore, recent pharmacological studies suggest the possible existence of additional G-protein coupled cannabinoid receptor subtypes (Breivogel et al. 2001; Fride et al. 2003).

```
                *         20        *         40        *         60        *         80
CB1R_HUMAN : MKSILDGLADTTFRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQEKMTAGDNPQLVPADQVNITEFYN :  83
CB2R_HUMAN : --------------------------------------------------------------------------MEECWVTEIAN :  11
                                                                                        TE   N

                *        100        *        120        *        140        *        160
CB1R_HUMAN : KSLSSFKENEENIQCGENFMDIECFMVINPSQQLAIAVLSLTLGTFTVLENLLVLCVTLHSRSLRCRPSYHFIGSLAVADLLG : 166
CB2R_HUMAN : GSKDGLDSNP----------MKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFIGSLAGADFLA :  83
              S      N             M L  Q  A AVL   LG    LEN  VL   IL S  LR  PSY FIGSLA AD L

                *        180        *        200        *        220        *        240
CB1R_HUMAN : SVIFVYSFIDFHVFHRKDSRNVFLFKLGGVTASFTASVGSLFLTAIDRYISIHRPLAYKRIVTRPKAVVAFCLMWTIAIVIAV : 249
CB2R_HUMAN : SVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIMWVLSALVSY : 166
              SV F  SF    FHVFH   DS   VFL K G VT   FTASVGSL LTAIDRY      P   YK   TR  A V     MW

                *        260        *        280        *        300        *        320        *
CB1R_HUMAN : LPLLGWNCEKLQSVCSDIFPHIDETYLMFWIGVTSVLLLFIVYAYMYILWKAHSHAVRMIQRGTQKSIIHTSEDGKVQVTRP : 332
CB2R_HUMAN : LPIMGWTCCPRP--CSELFPLIPNDYLLSWLLFIAFLFSGIIYTYGHVLWKAHQHVASLSGHQDR------------QVPGM : 234
              LPL GW C      CS  FP I   YL  W       L   I Y Y   LWKAH H                      QV

                340        *        360        *        380        *        400        *
CB1R_HUMAN : DQARMDIRLAKTLVLILVVLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPIIYALRSKDLRHAFRSMFPSC : 415
CB2R_HUMAN : ARMRLDVRLAKTLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYALRSGEIRSSAHHCLAHW : 317
              R D RLAKTL L L VL ICW P LA M       K  FAFCSMLCL NS VNP IYALRS  R

                420        *        440        *        460        *
CB1R_HUMAN : EGTAQPLDNSMGDSDCLHKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL : 472
CB2R_HUMAN : KKCVRGLG----------SEAKEEAPKSSVTETEADGKITPWPDSRDLDLSDC-- : 360
                     L                        R        KI   S   D S
```

Fig. 1. Amino acid sequence alignment of human CB1 and CB2 receptors (Horn et al. 2001). Shading with grey indicates the matching amino acids.

The CB1 and CB2 receptors are negatively coupled through heterotrimeric $G_{i/o}$ proteins to adenylyl cyclase, of which activation leads to the consequent reduction of intracellular cyclic AMP (cAMP) levels (Howlett 1984; Matsuda et al. 1990; Bouaboula et al. 1996). Activation of CB1 receptors by a cannabinoid agonist induces $G_{i/o}$ protein mediated inhibition of the N- and P/Q-type voltage-dependent Ca^{2+}-channels (Mackie and Hille 1992; Twitchell et al. 1997) and the activation of A-type and inwardly rectifying K^+-channels (Deadwyler et al. 1993; Mackie et al. 1995). Under certain conditions, CB1 receptor may also stimulate intracellular cAMP formation through G_s-type G-proteins (Glass and Felder 1997). Other cellular signalling pathways that may be involved in cannabinoid effects include stimulation of *tyr*-phosphorylation of focal adhesion kinase (Derkinderen et al. 1996), induction of immediate-early gene *Krox-24* and activation of mitogen-activated protein kinases (Wartmann et al. 1995; Bouaboula et al. 1995a; 1995b; 1996) and of c-Jun-N-terminal kinase (Rueda et al. 2000). In addition, cannabinoids are shown to activate protein kinase B (Gomes del Pulgar et al. 2000), induce breakdown of sphingomyelin and increase the levels of second messenger ceramide (Sanchez et al. 1998a; Galve Roperh et al. 2000).

The tissue distribution of CB1 receptors in the central nervous system (CNS) correlates well with the known effects of cannabinoids on memory, perception and the control of movement. Based on quantitative autoradiography, the amount of CB1 receptor protein in the brain is suggested to be higher than that of most other known G-protein coupled receptors (Herkenham et al. 1990). Particularly high CB1 receptor densities are found in the pars reticulata of substantia nigra, globus pallidus, endopeduncular nucleus, hippocampus and cerebellum of the human brain, with low to moderate levels in the cerebral cortex, caudate nucleus and putamen of striatum (Herkenham et al. 1990; Mailleux and Vanderhagen 1992; Tsou et al. 1998). The localization of CB1 receptors is suggested to be presynaptic in inhibitory axon terminals (Katona et al. 1999; 2000). Additionally, the CB1 receptor transcripts are found in several human peripheral tissues, such as adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, tonsils and thymus, as analysed by PCR-based method (Galiegue et al. 1995), and evidence for functional CB1 receptors has been obtained from human vascular endothelial cells (Liu et al. 2000), prostate (Ruiz-Llorente et al. 2003), keratinocytes (Maccarone et al. 2003) and longitudinal smooth muscle of the ileum (Croci et al. 1998). The CB2 receptor is absent in CNS, and mainly localized in peripheral immune-related tissues such as spleen, tonsils, B-cells, natural killer cells and monocytes (Galiegue et al. 1995).

The physiological role of cannabinoid receptors in the CNS is not yet completely understood, although, there is evidence that CB1 receptors may modulate the release of

other neurotransmitters in an inhibitory manner, such as γ-aminobutyric acid (GABA), dopamine, glutamate, acetylcholine, noradrenalin and serotonin (5-HT) (Schlicker and Kathmann 2001). Very little is also known about the physiological role of CB2 receptors, but one function of immune modulation has been suggested (Buckley et al. 2000). In the periphery, cannabinoid receptors may also participate in the regulation of nociceptive, cardiovascular, respiratory, digestive, reproductive and ocular systems.

2.1.2. Endocannabinoids

The first endogenous cannabinoid, N-arachidonylethanolamide (anandamide, AEA), was identified in porcine brain in 1992 (Devane et al. 1992), and is known to exert a wide range of pharmacological effects similar to Δ^9-THC; i.e., analgesia, inhibition of locomotor activity, catalepsy and hypothermia (Fride and Mechoulam 1993). Structurally, AEA is an ethanolamide of arachidonic acid and belongs to the C20:4 n6 series of fatty acids (**Fig. 2**). AEA is considered to act as a partial agonist at CB1 receptors (Mackie et al. 1993; Savinainen et al. 2001), and exhibits less activity towards CB2 receptors (**Table 1**). Furthermore, AEA is reported to act as a partial agonist at native vanilloid receptors (VR1) of mouse trigeminal neurons (Roberts et al. 2002) and share similar binding site with capsaicin on mammalian VR1 (Jordt and Julius 2002). The basal levels of AEA have been reported to be 10 - 90 pmol/g wet weight of tissue in various rat brain regions, as measured by isotope-dilution GC/MS (Bisogno et al. 1999a). The levels of AEA detected from human brain were at least 10-fold lower than those reported for glutamate and GABA, and ranged from 25 pmol/g in the cerebellum to 148 pmol/g in the hippocampus of human brain, as measured by LC/MS/MS (Felder et al. 1996).

Few years after the discovery of AEA, a second endogenous cannabinergic lipid, 2-arachidonylglycerol (2-AG) (**Fig. 2**), the 2-glyceryl ester of arachidonic acid, was isolated almost simultaneously from the canine gut (Mechoulam et al. 1995) and rat brain (Sugiura et al. 1995). 2-AG has been reported to act as a full agonist towards rat cerebellar CB1 receptors (Savinainen et al. 2001). The binding affinity of 2-AG seems to be lower for the CB1 receptor than AEA (Sugiura et al. 1995) (**Table 1**), but it has greater efficacy at the CB1 receptor (Sugiura et al. 1999) and is more abundant in brain than AEA. The endogenous levels of 2-AG were observed to be 55 - 1000 fold higher than concentrations of AEA (Sugiura et al. 1995; Stella et al. 1997; Stella et al. 2001). The 2-AG levels in various rat brain regions were 2 – 14 nmol/g wet weight of tissue, as measured by isotope-dilution GC/MS, and the highest levels were found in the rat brainstem and hippocampus (Bisogno et al. 1999a).
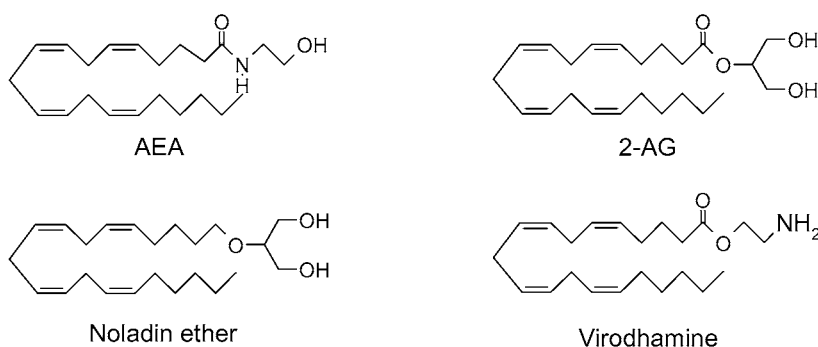
Fig. 2. Chemical structures of *N*-arachidonylethanolamide (AEA) and 2-arachidonylglycerol (2-AG), and the novel putative endocannabinoids noladin ether and virodhamine.

Recently, a third putative endocannabinoid, noladin ether (**Fig. 2**), was identified in porcine (Hanus et al. 2001) and rat brains (Fezza et al. 2002). Structurally, noladin ether is the 2-glyceryl ether of arachidonyl alcohol and, therefore, differs from the enzymatically more susceptible structures of AEA and 2-AG. The binding affinity of noladin ether was demonstrated to be significantly higher towards CB1 than CB2 receptors (Hanus et al. 2001) (**Table 1**). Noladin ether is reported to exhibit a functional agonist activity towards cells expressing CB1 and CB2 receptors, as evaluated by estimating its ability to induce Ca^{2+} -currents (Suhara et al. 2001). Noladin ether has been shown to induce typical cannabinergic behavioural responses *in vivo*, including sedation, hypothermia and mild analgesia in mice (Hanus et al. 2001). The noladin ether levels detected in rat brain ranged from 1.4 pmol/g of the spinal cord to 64.5 pmol/g of the thalamus, being 25.4 pmol/g in whole brain wet tissue, as measured by GC/MS (Fezza et al. 2002). In porcine brain, the noladin ether levels were found to be 0.6 nmol/g of whole brain as detected by HPLC (Hanus et al. 2001), whereas recently Oka et al. (2003) failed to found noladin ether in brains of various animal species. In contrast, Oka et al. (2003) confirmed the presence of a 1(3)-arachidonyl glyceryl ether-like molecule in the brains of pig, rat, mouse, hamster and quinea-pig.

In addition, another putative endogenous lipid, virodhamine (**Fig. 2**), has recently been isolated from rat brain and peripheral tissues, as well as, the human hippocampus (Porter et al. 2002). The concentrations of virodhamine were found to be 2- to 9-fold higher than those of AEA in rat peripheral tissues, including spleen, skin, kidney and heart. In human hippocampus and rat hippocampus, cortex and cerebellum the virodhamine levels were similar to those of AEA (4.2 - 20.6 pmol/g tissue) (Porter et al. 2002).

Table 1: Binding affinities of endocannabinoids to CB1 and CB2 receptors (COS-7 = African green monkey kidney cell line; L-cells = mouse embryo fibroblasts; AtT-20 = mouse pituitary cell line; CHO = Chinese hamster ovary cell line).

Endocannabinoid	Binding affinity (Ki)		Transfected cell line / membranes used	Radioligand	Reference
	CB1	CB2			
N-Arachidonyl-ethanolamide (AEA)	252 nM	581 nM	COS-7 (rCB1 and hCB2)	[^{3}H]HU-243	Mechoulam et al. 1995
	89 nM	---	Rat brain synaptosomes	[^{3}H]CP55,940	Sugiura et al. 1995
	543 nM	1940 nM	Murine L-cells (hCB1), AtT-20 (hCB2)	[^{3}H]CP55,940	Felder et al. 1995
	89 nM^A	371 nM^A	CHO (hCB1 and hCB2)	[^{3}H]CP55,940	Showalter et al. 1996
	71 nM^A	279 nM^A	Rat cerebellum (CB1), Rat spleen (CB2)	[^{3}H]CP55,940	Hillard et al. 1999
2-Arachidonylglycerol (2-AG)	472 nM	1400 nM	COS-7 (rCB1 and hCB2)	[^{3}H]HU-243	Mechoulam et al. 1995
	48 μM	---	Rat brain synaptosomes	[^{3}H]CP55,940	Sugiura et al. 1995
	58 nM	145 nM	COS-7 (rCB1 and hCB2)	[^{3}H]HU-243	Ben-Shabat et al. 1998
Noladin ether	21 nM	> 3 μM	Rat brain synaptosomes (CB1), COS-7 (hCB2)	[^{3}H]HU-243	Hanus et al. 2001

^{A}Treated with 50-150 μM of phenylmethylsulfonyl fluoride (PMSF)

2.1.3 Biosynthesis of endocannabinoids

The formation of endogenous AEA in neurons is suggested to occur by two alternative pathways. The first pathway involves an ATP-independent condensation of arachidonic acid with ethanolamine, which is catalysed by FAAH activity (Deutsch and Chin 1993). Because of the high ethanolamine requirements of FAAH, and low intracellular concentrations of arachidonic acid in resting neurons, the second and more apparent biosynthetic pathway for AEA is its phospholipase D -catalyzed hydrolysis from N-arachidonoylphosphatidyl-ethanolamide (NArPE), which serves as a precursor for AEA and other N-acylethanolamides (Di Marzo et al. 1994) (Fig. 3). The synthesis and release of AEA can be stimulated in rat intact cortical and striatal neurones (but not astrocytes) by membrane depolarizing agents, such as ionomycin, high levels of K$^+$, 4-aminopyridine and kainate. After depolarization, AEA is produced and released from neurons in a Ca^{2+}-dependent manner (Di Marzo et al. 1994). The formation of AEA has been shown to be enhanced by 5-times through simultaneous activation of N-methyl-D-glutamate (NMDA) and acetylcholine receptors in primary cultures of rat cortical neurons (Stella et al. 2001).

Phosphatidylethanolamine

N-Acyltransferase

+ Ca²⁺

SN-1-Arachidonyl-phospholipid

N-Arachidonyl-phosphatidylethanolamine

Phosphodiesterase

N-Arachidonylethanolamine (AEA)
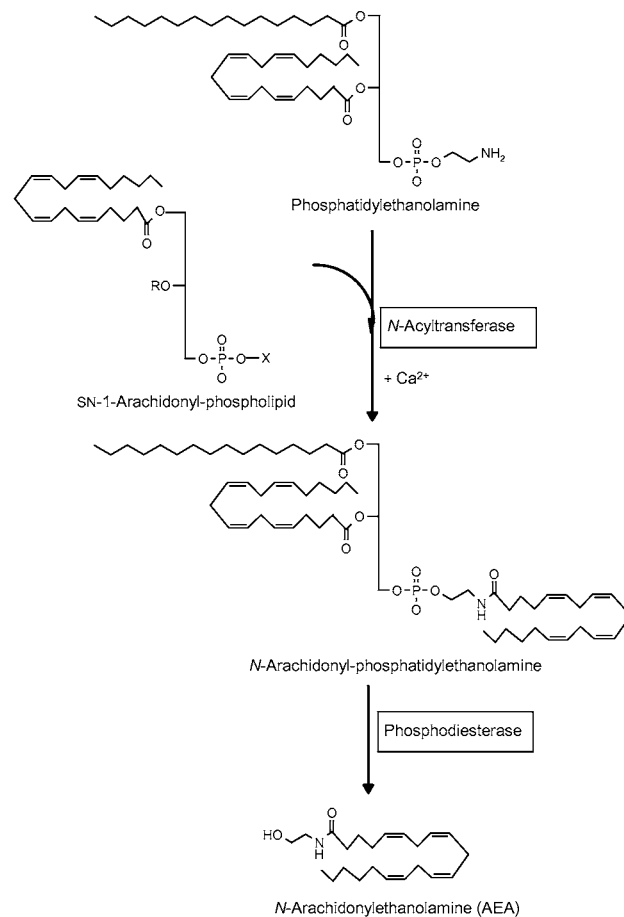
Fig. 3. Suggested main pathway for the biosynthesis of N-arachidonylethanolamide (AEA), which involves the synthesis of phosphatidylethanolamine by N-acyltransferase activity and its subsequent hydrolysis by phospodiesterase activity (such as phospholipase D) to yield AEA (Modified from Piomelli et al. 1998) (X = Polar headgroup).

The neuronal stimulation and cellular Ca^{2+} -entry may evoke the biosynthesis and release of 2-AG in mouse neuroblastoma cells, macrophages and in rat brain neurons (Bisogno et al. 1997b; Stella et al. 1997; Di Marzo et al. 1999). The most likely route for 2-AG formation includes phospholipase C –mediated formation of diacylglycerol lipase, which is further converted to 2-AG by diacylglycerol lipase activity (Stella et al. 1997) (**Fig.** 4). Alternatively, 2-AG may be formed by the hydrolysis of lysophospholipids or triacylglycerols (see review by Piomelli et al. 1998). Activation of ionotropic NMDA receptors, and following Ca^{2+} -entry into neurons, has been reported
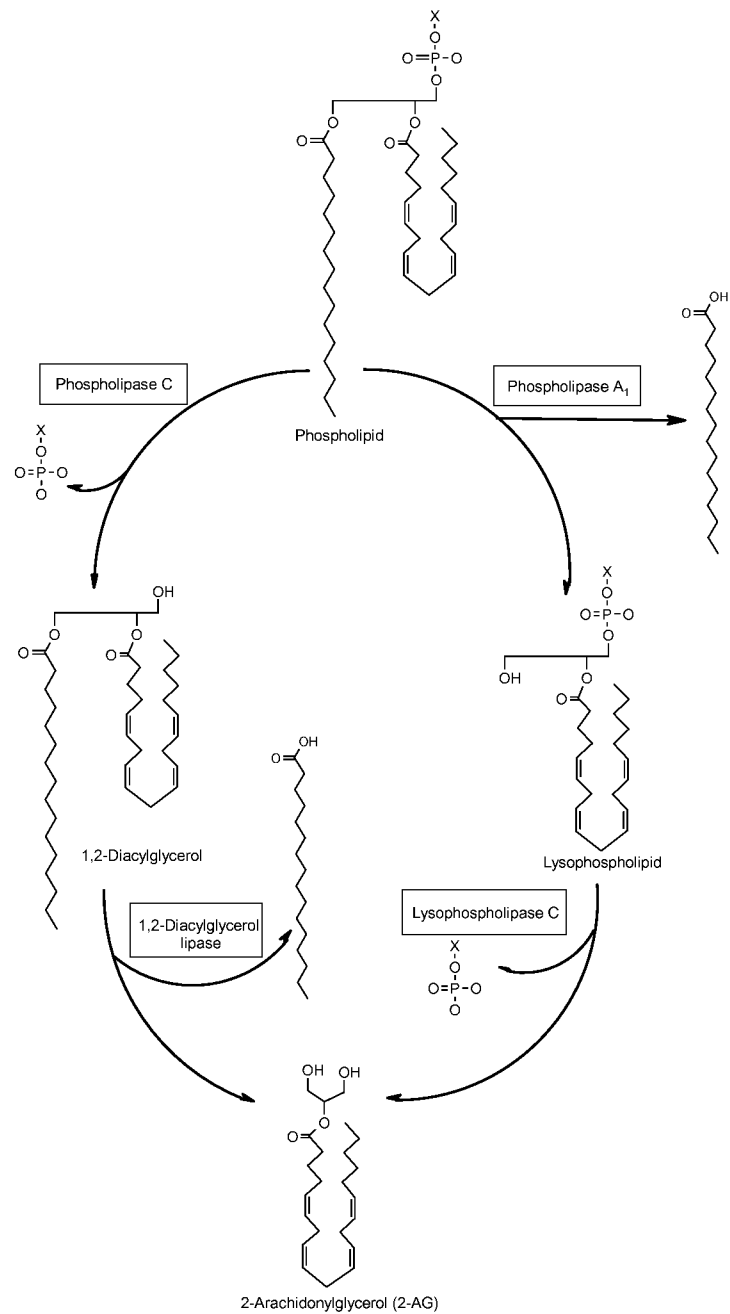
Fig. 4. Two possible pathways for the formation of 2-arachidonylglycerol (2-AG) in neurons. Phospholipase C –mediated synthesis of diacylglycerol lipase, which is converted to 2-AG by diacylglycerol lipase activity (left), is suggested to be the primary route (Modified from Piomelli et al. 1998) (X = Polar headgroup).

to be sufficient to stimulate 2-AG synthesis in primary cell cultures of rat cortical neurons, suggesting that cortical neurons may produce endocannabinoids in a receptor-dependent manner (Stella et al. 2001). The biosynthetic pathways for putative endocannabinoids noladin ether and virodhamine are currently unknown.

2.1.4 Inactivation of endocannabinoids
2.1.4.1 Cellular uptake

The inactivation of AEA and 2-AG is assumed to occur by a two-step mechanism consisting of transport through cellular membranes and enzymatic hydrolysis (**Fig. 5**). The transport of AEA has been shown to meet several key criteria of a carrier-mediated process: saturability, fast rate, temperature dependence, and substrate selectivity (Di Marzo et al. 1994; Beltramo et al. 1997a; Hillard et al. 1997). Observations that the transport of AEA does not require cellular energy (ATP) or Na^+, but may occur in both directions and is dependent on a concentration gradient for AEA across the cell membrane, imply that the transport process may be mediated through facilitated diffusion (Hillard et al. 1997). A variety of compounds that structurally resemble endogenous cannabinoids, including arachidonic acid, palmitylethanolamide, prostaglandins, hydroxyeicosatetraenoic acids, epoxyeicosatrienoic acids and leukotrienes, as well as amino acids, biogenic amines and substrates or inhibitors of organic anion transport, fatty acid transport and P-glycoproteins, had no effect on [^{3}H]AEA accumulation in human astrocytoma cells (Piomelli et al. 1999). In contrast, [^{3}H]AEA accumulation was competitively inhibited by non-radioactive AEA and by the AEA analogue N-(4-hydroxyphenyl)-arachidonamide (AM404) (Piomelli et al. 1999).

2-AG has been shown to inhibit the cellular uptake of [^{3}H]AEA in human astrocytoma and in rat C6 glioma cells, indicating that these endocannabinoids apparently share similar or even the same membrane transport system (Piomelli et al. 1999; Beltramo et al. 2000; Bisogno et al. 2001). The AEA analogue AM404 prevents the cellular accumulation of 2-AG and AEA at similar potencies in rat glioma cells, with IC_{50} values of 10.2 μM for 2-AG and 7.5 μM for AEA, respectively (Bisogno et al. 2001). The uptake of 2-AG in rat glioma cells has been reported to be less efficacious than the uptake of AEA (Bisogno et al. 2001).

To date, the molecular characteristics of the transport protein(s) responsible for the cellular uptake of AEA and 2-AG are not known. Due to their lipid nature and relative small molecular size, passive diffusion may, however, be involved in the cellular accumulation of endocannabinoids. Recently, the cellular uptake of noladin ether was suggested to occur through endocannabinoid transporter (Fezza et al. 2002).
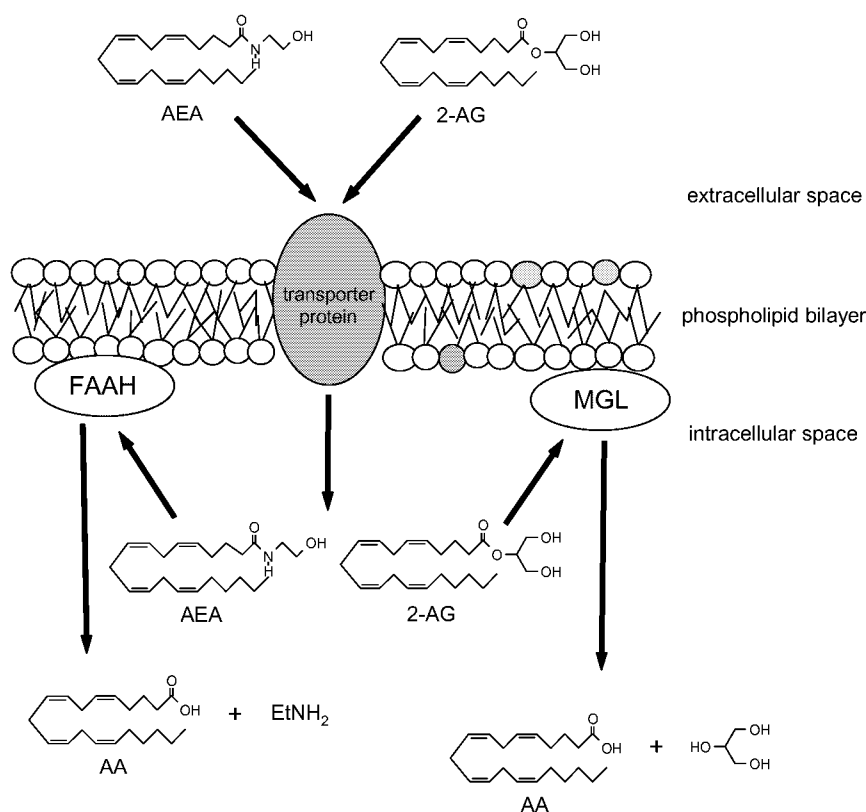
Fig. 5. Hypothetical model for the inactivation of endocannabinoids (AEA = *N*-arachidonylethanolamide; 2-AG = 2-arachidonylglycerol; AA = arachidonic acid; FAAH = fatty acid amide hydrolase and MGL = monoacylglycerol lipase).

2.1.4.2 Fatty acid amide hydrolase (FAAH)

Once transported into cells, AEA is rapidly hydrolysed to arachidonic acid and ethanolamine. The main enzyme responsible for terminating the cellular actions of AEA is recognized to be fatty acid amide hydrolase (FAAH, anandamide amidase, oleamide hydrolase) (Deutsch and Chin 1993; Desarnaud et al. 1995; Cravatt et al. 1996). FAAH is an intracellular membrane-bound protein with an amidase signature sequence (Cravatt et al. 1996). The mouse and rat FAAH share 91 % sequence identity, whereas human FAAH share 82 % and 84 % sequence identity with the rat and mouse FAAHs, respectively (Giang and Cravatt 1997). Results from the inhibition studies and site-directed mutagenesis have revealed that FAAH is a serine hydrolase that utilizes three

serine residues in catalysis; Ser-217, Ser-218 and Ser-241, of which the Ser-241 may act as the active nucleophile during the catalytic process (Desarnaud et al. 1995; Hillard et al. 1995; Ueda et al. 1995a; Omeir et al. 1999; Patricelli et al. 1999). In rat brain, the highest specific activity of FAAH is mainly located in microsomal, mitochondrial and myelin fractions (Desarnaud et al. 1995; Hillard et al. 1995).

The anatomical distribution of FAAH activity, mRNA and protein seems to correlate well with the distribution of CB1 receptors in CNS (Desarnaud et al. 1995; Hillard et al. 1995; Thomas et al. 1997; Tsou et al. 1998). In rat brain homogenates, the hippocampus, cerebral cortex and cerebellum exhibit the highest FAAH activity, while the lowest activity was in rat striatum, hypothalamus and brain stem (Thomas et al. 1997). In rat peripheral tissues, the FAAH activity is found to be high in liver and kidney and low in heart, kidney, intestine, stomach, lung, spleen and skeletal muscle (Desarnaud et al. 1995). In addition to AEA, FAAH can hydrolyse various fatty acid amides or esters, including long chain unsaturated N-acyl ethanolamides, linoleyl-, oleoyl- and palmitoylethanolamide, oleamide (a novel sleep inducing factor) and 2-AG (Desarnaud et al. 1995; Ueda et al. 1995a; Cravatt et al. 1996; Goparaju et al. 1998). Compared to alternative substrates, FAAH appears to hydrolyse AEA at a higher rate (Desarnaud et al. 1995; Ueda et al 1995). Additionally, FAAH has been demonstrated to catalyse the reverse reaction, synthesising AEA from arachidonic acid in the presence of a high concentration of ethanolamine (Ueda et al. 1995a). Mice lacking the FAAH enzyme were observed to possess 15-fold enhanced endogenous brain AEA levels and display reduced pain sensation (Cravatt et al. 2001).

2.1.4.3 Monoacylglycerol lipase (MGL)

After its cellular uptake, 2-AG is rapidly inactivated through enzymatic hydrolysis of its ester bond, yielding arachidonic acid and glycerol. The main enzyme involved in the intracellular cleavage of 2-AG was recently reported to be monoglyceride lipase (MGL) (Dinh et al. 2002). MGL is a serine hydrolase that hydrolyse the 1(3)- and 2-ester bonds of monoglycerides at equal rates (Karlsson et al. 1997). The MGL mRNA has been detected in a variety of rat tissues, including adipose tissue, adrenal gland, ovary, heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis (Karlsson et al. 1997). In rat, MGL mRNA and protein are expressed throughout the brain tissue (Dinh et al. 2002). The expression levels of MGL mRNA are shown to be high in relatively few rat brain areas, such as cerebellum, cerebral cortex and hippocampus, intermediate in striatum and thalamus, and low in the brainstem and hypothalamus. Results from immunostaining of the rat hippocampus for MGL suggest that the MGL is localized

presynaptically in axon terminals (Dinh et al. 2002). MGL activity is blocked with a variety of serine hydrolase inhibitors, such as methyl arachidonylfluorophosphate, which were previously shown to inhibit the catalytic action of FAAH (Goparaju et al. 1999; Dinh et al. 2002). The MGL-infected HeLa cells hydrolysed 2-AG and 2-oleoylglycerol, but not AEA or palmitoylethanolamide, indicating that this enzyme preferentially catalyses the hydrolysis of 2-monoglycerides over fatty acid ethanolamides, such as AEA (Dinh et al. 2002).

In addition to MGL, FAAH has been shown to hydrolyse 2-AG efficiently (Goparaju et al. 1998; Lang et al. 1999). In rat FAAH transfected COS-7 cells, the hydrolysis rate for 2-AG was approximately 4-fold higher than that of AEA (Goparaju et al. 1998). However, the 2-AG hydrolysis was preserved in mice lacking the FAAH enzyme, which does not hydrolyse either endogenous or exogenous AEA, suggesting that an enzyme other than FAAH is primarily responsible for the catabolic regulation of 2-AG (Lichtman et al. 2002). Lipoprotein lipase is an enzyme that has 1(3)-monoglyceride hydrolysing activity, with absolute preference for the primary ester bond (Nilsson-Ehle et al. 1971; Karlsson et al. 1997). The role of this lipase in the hydrolysis of 1(3)-AG, which is formed through isomerization from 2-AG, has not been characterized.

To date, the main metabolic pathways for putative endocannabinoids noladin ether and virodhamine are unknown. However, noladin ether is assumed to be partly metabolised via direct esterification into membrane phospholipids (Fezza et al. 2002).

2.1.4.4 Oxidative metabolism

The structural similarity between polyunsaturated fatty acids and endocannabinoids suggests that endocannabinoids may serve as a substrate for the same cyclooxygenases and lipoxygenases that oxidase polyunsaturated fatty acids to important biologically active metabolites. In fact, the human recombinant cyclooxygenase 2 (COX-2) has been reported to metabolize AEA into a number of oxygenated products, including prostaglandin E_2 (PGE$_2$) –ethanolamide, whereas cyclooxygenase 1 (COX-1) was inactive (Yu et al. 1997). Purified human and mouse COX-2 oxygenated 2-AG as effectively as arachidonic acid, leading to the production of prostaglandin H_2 (PGH$_2$) glycerol ester and hydroxyeicosatetraenoic acid (HETE) glycerol esters (Kozak et al. 2000).

AEA has been demonstrated to be a substrate for porcine leukocyte 12-lipoxygenase (12-LOX) and rabbit reticulocyte 15-lipoxygenase (15-LOX) activities, which form 12-hydroperoxy-5,8,10,14-eicosatetraenoylethanolamide (12-HEA) and 15-hydroperoxy-5,8,11,13-eicosatetraenoylethanolamide (15-HEA), respectively (Ueda et al. 1995b).

Human platelet 12-LOX had a minor endocannabinoid oxygenase activity, whereas porcine leukocyte 5-lipoxygenase (5-LOX) was inactive (Ueda et al. 1995b). In contrast, intact human platelets have been shown to convert [^{14}C]AEA to 12-HEA, which binds to both CB1 and CB2 receptors with comparable affinity to AEA (Edgemond et al. 1998). The porcine leukocyte 12-LOX and soybean 15-LOX have been shown to oxygenate 2-AG to produce the 2-glyceryl esters of 12-hydroperoxy-5,8,10,14-eicosatetraenoic and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acids, respectively (Moody et al. 2001).

Mouse hepatic microsomal cytochrome P450s have been shown to oxidize AEA to at least 20 different polar lipids (Bornheim et al. 1995). Additionally, mouse brain microsomal P450s seem to catalyze the monooxygenation of AEA (Bornheim et al. 1995). Even though the oxygenation of endocannabinoids is not assumed to be their primary metabolic pathway, it is important to recognize that their oxygenated products, as with arachidonic acid, may display significant physiological and pharmacological activities. For example, prostamide $F_{2\alpha}$, which is apparently biosynthesised from AEA through COX-2 pathway, has been reported to bind to novel receptors and regulate ocular tension (Woodward et al. 2001).

2.2 INHIBITORS OF ENDOCANNABINOID METABOLISM
2.2.1 Uptake inhibitors
2.2.1.1 *N*-(4-Hydroxyphenyl)-arachidonylamide (AM404)

N-(4-Hydroxyphenyl)-arachidonylamide (AM404) (**Fig. 6**) is the first compound that was reported to competitively inhibit the cellular accumulation of AEA in cultured rat neurons and astrocytes at half maximal concentrations for inhibition (IC$_{50}$ values) of 1 μM in neurons and 5μM in astrocytes, respectively (Beltramo et al. 1997a). Its positional isomer, AM403 [*N*-(3-hydroxyphenyl)arachidonylamide] (**Fig. 6**), and prostaglandin E$_2$ (PGE$_2$) transport inhibitor bromcresol green (**Fig. 6**) were less effective than AM404 in inhibiting AEA accumulation (Beltramo et al. 1997a). In rat basophilic leukemia (RBL-2H3) and rat glioma cells, AM404 inhibited the re-uptake of [^{14}C]AEA with IC$_{50}$ values of 8 and 10 μM, respectively (De Petrocellis et al. 2000). AM404 was shown to bind to CB1 receptors with low affinity (K$_i$ = 1.8 μM) (Khanolkar et al. 1996), although it did not reduce intracellular cAMP concentrations when applied at 10 μM in forskolin-stimulated rat cortical neurons (Beltramo et al. 1997a). AM404 is reported to be resistant to FAAH hydrolysis, probably due to the
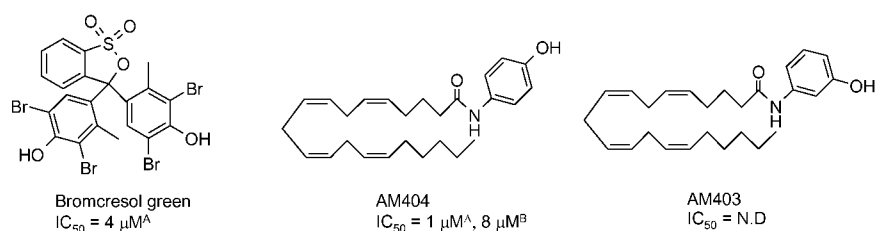
Fig. 6. Chemical structures and half-maximal concentrations for AEA uptake inhibition (IC_{50}) of PGE_2 transport inhibitor bromcresol green, AM404 and its positional isomer AM403. The IC_{50} values are measured either in rat neurons ([A]) or in RBL-2H3 cells ([B]) (N.D. = not determined) (Beltramo et al. 1997a; De Petrocellis et al. 2000).

electron-donating properties of its p-OH phenyl group, which leads to decreased susceptibility of the carbonyl group to nucleophilic attack by the FAAH catalytic site (Lang et al. 1999). AM404 is transported into cells as rapidly and effectively as AEA in a human astrocytoma cell line (Piomelli et al. 1999).

Administration of AM404 (10 mg/kg i.v.) significantly enhanced and prolonged the antinociceptive and hypotensive effects of AEA in mice and guinea pigs, respectively, but had no effect or a only minor effect when injected without AEA (Beltramo et al. 1997a; Calignano et al. 1997). The systemic administration of AM404 (10 mg/kg i.p.) has been reported to increase the circulating levels of AEA in rat plasma and cause a marked decrease in the motor activity of rats (Gonzales et al. 1999; Giuffrida et al. 2000). The effects of AM404 on immobility and locomotor activity are reversed with the CB1 receptor antagonist SR141716A (Giuffrida et al. 2000).

AM404 is reported to activate vanilloid VR1 receptors by provoking ion currents in excised inside-out membrane patches of *Xenopus* oocytes expressing VR1 (Zygmunt et al. 2000). In hepatic arteries of rat, AM404 is reported to induce vasodilatation that is reversed by capsazapine, a VR1 receptor antagonist, was but unaffected by SR141716A, suggesting that this effect is mediated by VR1 receptors (Zygmunt et al. 2000).

2.2.1.2 Vanilloids

The structural similarity between long chain vanilloids and aromatic inhibitors of AEA transport has suggested the possibility that vanilloids may interact with AEA transporter. Vanilloids include compounds that activate vanilloid receptors, which are ligand-gated non-selective ion channels present in nociceptors and result in a burning pain sensation when stimulated (Caterina et al. 1997; Szallasi and Blumberg 1999).

Olvanil (N-[(4-hydroxy-3methoxyphenyl)methyl]-9Z-octadecenamide) (**Fig. 7**) was initially reported to inhibit the cellular accumulation of [^{14}C]AEA in rat basophilic leukemia (RBL-2H3) cells at a potency higher than that detected for AM404 in the same study (IC$_{50}$ = 9 μM for olvanil) (Di Marzo et al. 1998). In rat C6 glioma cells, olvanil reduced the cellular uptake of [^{14}C]AEA with a lower potency (IC$_{50}$ = 20 μM) than AM404 (IC$_{50}$ = 10 μM) (De Petrocellis et al. 2000). In intact RBL-2H3 cells, olvanil inhibited the [^{14}C]AEA hydrolysis with an IC$_{50}$ value of 9 μM, but a higher concentration was needed to inhibit RBL-2H3 membrane preparations (IC$_{50}$ = 32 μM), suggesting that the inhibitory effect on [^{14}C]AEA hydrolysis observed in intact cells was due to the inhibition of [^{14}C]AEA uptake and not FAAH (Di Marzo et al. 1998). Indeed, in FAAH-containing N18TG2 cell membranes, olvanil affected [^{14}C]AEA hydrolysis only at high concentrations (IC$_{50}$ = 48 μM) (Melck et al. 1999). Olvanil binds to CB1 receptors with low affinity (K$_i$ = 1.6 - 7 μM), whereas its binding at the CB2 receptor is reported to be almost negligible (K$_i$ > 15 μM) (Di Marzo et al. 1998).

Thus far, arvanil (N-[(4-hydroxy-3-methoxyphenyl)methyl]-arachidonylamide) (**Fig. 7**) has been demonstrated to be the most potent vanilloid-type AEA re-uptake inhibitor. Arvanil is reported to inhibit the cellular accumulation of [^{14}C]AEA in RBL-2H3 cells and in rat C6 glioma cells with IC$_{50}$ values of 3.6 and 11.2 μM, respectively (Melck et al. 1999; De Petrocellis et al. 2000). In FAAH-containing N18TG2 cell membranes, arvanil inhibits the hydrolysis of [^{14}C]AEA at slightly lower concentrations than olvanil (IC$_{50}$ = 32 μM), itself being a poor substrate for FAAH (Melck et al. 1999). Arvanil has been demonstrated to be almost 4-fold more potent than AEA in binding at the CB1 receptor in N18TG2 cells (K$_i$ = 0.5 μM for arvanil and 1.9 μM for AEA, respectively), and exhibits at least a 30-fold selectivity for CB1 versus CB2 receptors (K$_i$ > 15 μM towards CB2 receptors), suggesting that arvanil may act as a potent agonist for both CB1 and VR1 receptors (Melck et al. 1999).

Linvanil (**Fig. 7**) inhibits the facilitated diffusion of [^{14}C]AEA with a slightly lower potency than arvanil in RBL-2H3 cells, and in rat C6 glioma cells (IC$_{50}$ values of 8 and 13 μM, respectively) (Melck et al. 1999; De Petrocellis et al. 2000). Linvanil has no significant effect on AEA hydrolysis in FAAH-containing N18TG2 cell membranes (IC$_{50}$ > 50 μM), and it exhibits low affinity for CB1 receptors (K$_i$ = 3.4 μM) (Melck et al. 1999). Capsaicin (**Fig. 7**), its analogue pseudocapsaicin (**Fig. 7**) and the saturated C16 vanilloid derivative palvanil (**Fig. 7**) have been reported to reduce the uptake of [^{14}C]AEA in intact RBL-2H3 cells, but only at high μM concentrations (IC$_{50}$ values of 46 μM, 60 μM and > 50 μM respectively) (Di Marzo et al. 1998; Melck et al. 1999).

Olvanil
IC₅₀ = 9.0 µM

Arvanil
IC₅₀ = 3 6 µM

Linvanil
IC₅₀ = 8.0 µM

Palvanil
IC₅₀ > 50 µM

Capsaicin
IC₅₀ = 46.9 µM

Pseudocapsaicin
IC₅₀ = 60 µM
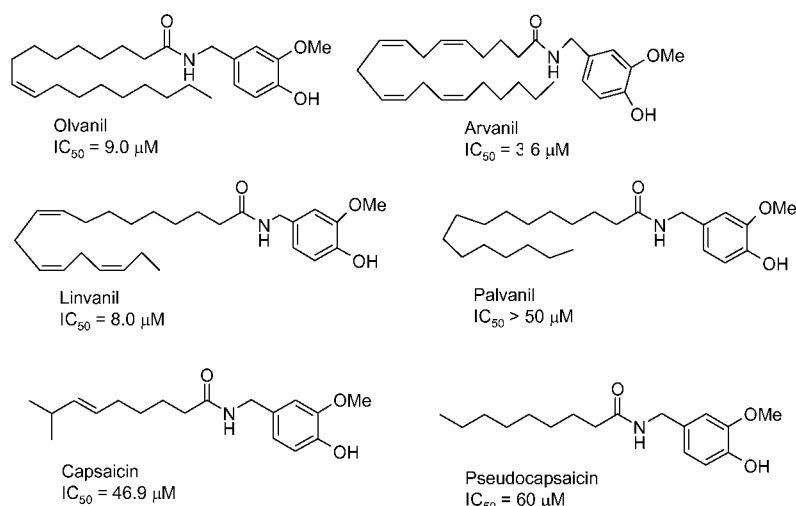
Fig. 7. Chemical structures and half-maximal concentrations for AEA uptake inhibition (IC_{50}) of vanilloid analogs. The IC_{50} values were measured in RBL-2H3 cells (Di Marzo et al. 1998; De Petrocellis et al. 2000).

2.2.1.3 Novel more selective uptake inhibitors

Recently, De Petrocellis et al. (2000) attempted to develop selective AEA uptake inhibitors that were devoid of VR1 agonist activity. Three arachidonyl-derivates were synthesised; VDM11 (N-(1-methyl-4-hydroxyphenyl)-arachidonylamide), VDM12 (N-(1-hydroxy-4-hydroxyphenyl)-arachidonylamide) and VDM13 (arachidonoyl-5-methoxy-tryptamine) (**Fig. 8**), of which VDM12 was a low-potency full agonist for VR1, and both VDM11 and VDM13 exhibited almost negligible agonist activity for VR1. These compounds inhibited the uptake of [^{14}C]AEA as potently as AM404, with IC_{50} values of 10 µM for VDM11, 25 µM for VDM12 and 12 µM for VDM13 in rat C6 glioma cells (De Petrocellis et al. 2000). These compounds were weak inhibitors of AEA hydrolysis in N18TG2 cell membranes (IC_{50} > 50 µM for VDM11, IC_{50} = 25 µM for VDM12 and IC_{50} = 27 µM for VDM13) and poor CB1 receptor ligands (K_i >5 – 10 µM) (De Petrocellis et al. 2000).

Lopez-Rodriguez et al. (2001; 2003) reported the synthesis of novel AEA transport inhibitors, where the ethanolamine moiety of AEA was replaced with a molecular fragment containing a five-membered ring with one heteroatom. Most compounds acted as potent inhibitors of the AEA transport in a human lymphoma cell line (IC_{50} = 0.8 – 24 µM) with low affinities for FAAH, the cannabinoid receptors CB1 and CB2 and the vanilloid VR1 receptor. The most potent AEA transport inhibitor reported to date, N-(3-

furylmethyl)-arachidonylamide (UCM707, **Fig. 8**) was 5-fold more potent than AM404 in inhibiting AEA re-uptake in human lymphoma cells (IC_{50} = 0.8 µM vs. IC_{50} = 4 µM for AM404, respectively), and displayed some binding affinity towards both cannabinoid (K_i > 1 µM and = 0.067 µM for CB1 and CB2 receptors, respectively) and vanilloid receptors (K_i > 5 µM for VR1). In addition, UCM707 inhibited FAAH with IC_{50} value of 30 µM in rat brain homogenates. *In vivo*, UCM707 has been reported to potentiate both the antinociceptive and hypokinetic effects of exogenous AEA, but has a negligible effect when administered alone (de Lago et al. 2002).

Recently, Ortar et al. (2003) developed a series of (R)- and (S)-1'-(4-hydroxybenzyl) derivatives of both *N*-oleylethanolamide and AEA, in order to study their ability to inhibit the cellular accumulation of AEA. Their most potent compounds, OMDM-1 and OMDM-2 (**Fig. 8**) inhibited [^{14}C]AEA accumulation in intact RBL-2H3 cells at a similar potency as arvanil (K_i = 2.4 µM, 3.0 µM and 2.9 µM for OMDM-1, OMDM-2 and arvanil, respectively). Neither of the compounds exhibited significant FAAH inhibition activity in N18TG2 cell membranes (K_i > 50 µM) nor agonist activity towards the vanilloid VR1 receptor ($EC_{50} \geq 10$ µM). OMDM-1 displaced the CB1 receptor antagonist [^{3}H]SR141716A from its specific CB1 receptor binding site in rat brain membranes, but only at high concentrations (K_i = 12.1 µM), whereas OMDM-2 was slightly more potent (K_i = 5.1 µM). Both OMDM-1 and OMDM-2 were also considerably more stable towards enzymatic hydrolysis than either AM404 or VDM-12 when incubated in rat brain homogenates.

VDM11
IC_{50} = 11.2 µM^A

VDM12
IC_{50} = 13.2 µM^A

VDM13
IC_{50} = 12 µM^A

UCM707
IC_{50} = 0.8 µM^B

OMDM-1
IC_{50} = 2.4 µM^A

OMDM-2
IC_{50} = 3 µM^A
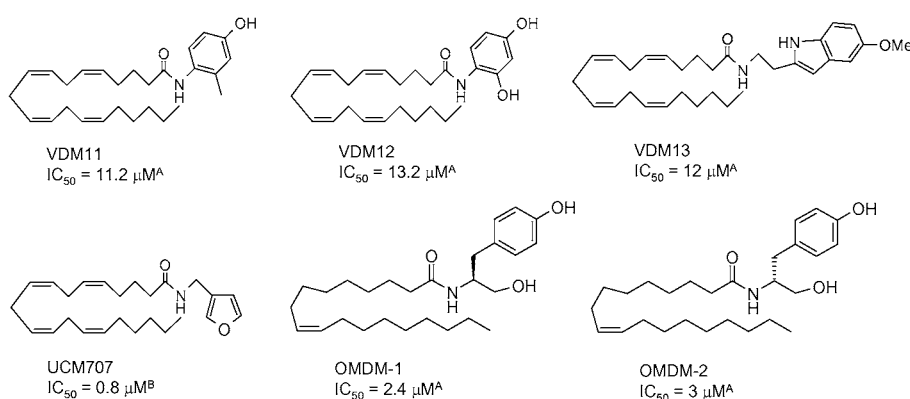
Fig. 8. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) of novel, more selective, AEA transport inhibitors. The IC_{50} values are measured in either RBL-2H3 cells (^{A}) or in human lymphoma U937 cells (^{B})(De Petrocellis et al. 2000; Lopez-Rodriguez et al. 2003; Ortar et al. 2003).

2.2.2 FAAH enzyme inhibitors

Initial studies revealed that the non-selective serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) potently inhibited the AEA hydrolysing activity in rat neuroblastoma and glioma cells (Deutsch and Chin 1993). Other non-selective compounds, including diisofluorophosphate, thiomerosal, iodoacetic acid, p-bromophenacylbromide and p-hydroxymercuribenzoate, were also reported to abolishing FAAH enzymatic activity (Hillard et al. 1995; Bisogno et al 1997a; Maccarrone et al. 1998). Later, several classes of more selective FAAH inhibitors were identified, including trifluoromethylketones, fluorophosphonates, diazomethyl- and chloromethyl-ketones and non-steroidal anti-inflammatory drugs, and recently the more target-selective FAAH inhibitors such as fatty acid sulfonyl fluorides, α-keto-oxazolopyridines and aryl-substituted carbamate derivatives.

2.2.2.1 Trifluoromethyl ketones, α-keto esters and amides

Koutek and colleagues (1994) first described the three classes of putative transition state FAAH inhibitors; α-keto amides, α-keto esters and fatty acid trifluoromethyl ketones (**Fig. 9**), which were developed on the hypothesis that the polarized carbonyl groups in trifluoromethyl ketones and α-keto carboxylate derivatives may form enzyme adducts that mimic the tetrahedral intermediates formed during the enzymatic reaction between a nucleophilic residue and the carbonyl group of AEA. The most effective FAAH inhibitors of these groups were arachidonyl trifluoromethyl ketone (ATFMK, **Fig. 9; 3a**) and ethyl-2-oxostearate (**Fig. 9; 2b**), which resulted in almost 100 % inhibition of FAAH in rat brain homogenates, whereas the α-keto amides were least active (Koutek et al. 1994).

Boger et al. (1999) developed a series of novel trifluoromethyl ketones in order to clarify the structural features necessary for FAAH inhibition in rat liver membrane preparations. In the simple, straight chain aliphatic trifluoromethyl ketones (**Fig. 9**), the inclusion of an oleamide $\Delta^{9,10}$ *cis* double bond (**Fig. 9; 4**) increased the inhibitor potency by 3-fold compared to the corresponding saturated trifluoromethyl ketone (**Fig. 9; 3c**), whereas its replacement with *trans* double bond in the same position (**Fig. 9; 5**) slightly reduced the activity. In saturated aliphatic trifluoromethyl ketones, the most potent inhibitors were C12 (**Fig. 9; 3f**) and C10 (**Fig. 9; 3g**) compounds, and shortening of the aliphatic chain below C10 led to the progressive loss of inhibitor activity (Boger et al. 1999). In the trifluoromethyl ketones having a ring structure (**Fig. 10**), the most
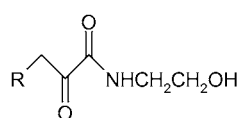
1a: R= $(CH_2)_{16}CH_3$ = 2-oxoeicosanoyl ethanolamide   **2a:** R= $(CH_2)_{16}CH_3$ = ethyl-2-oxoeicosanoate
1b: R= $(CH_2)_{14}CH_3$ = 2-oxostearyl ethanolamide   **2b:** R= $(CH_2)_{14}CH_3$ = ethyl-2-oxostearate
1c: R= $(CH_2)_{12}CH_3$ = 2-oxopalmitoyl ethanolamide   **2c:** R= $(CH_2)_{12}CH_3$ = ethyl-2-oxopalmitate
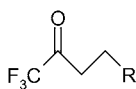
3a: R= $CH_2(CH=CHCH_2)_4(CH_2)_3CH_3$ = arachidonyl trifluoromethyl ketone (IC_{50} = 0.9 μM[A])
3b: R= $(CH_2)_2(CH=CHCH_2)_3(CH_2)_3CH_3$ = γ–linolenoyl trifluoromethyl ketone
3c: R= $(CH_2)_{14}CH_3$ = stearyl trifluoromethyl ketone (IC_{50} = 1.2 μM)
3d: R= $(CH_2)_{12}CH_3$ = palmitoyl trifluoromethyl ketone
3e: R= $(CH_2)_{10}CH_3$ = myristyl trifluoromethyl ketone (IC_{50} = 2.3 μM)
3f: R= $(CH_2)_8CH_3$ = lauryl trifluoromethyl ketone (IC_{50} = 0.72 μM)
3g: R= $(CH_2)_6CH_3$ = capryl trifluoromethyl ketone (IC_{50} = 0.60 μM)
3h: R= $(CH_2)_4CH_3$ = capryloyl trifluoromethyl ketone (IC_{50} = 6.6 μM)
3i: R= $(CH_2)_3CH_3$ = enantoyl trifluoromethyl ketone (IC_{50} = 52.8 μM)
3j: R= $(CH_2)_2CH_3$ = caproyl trifluoromethyl ketone (IC_{50} = 185.2 μM)
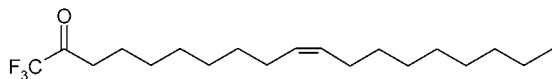
4: (IC_{50} = 0.46 μM)
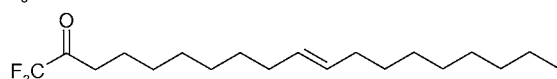
5: (IC_{50} = 0.78 μM)

Fig. 9. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) in rat liver homogenate of some α-keto ethanolamides (1), α-keto ethyl esters (2) and fatty acid trifluoromethyl ketones (3-5) (Koutek et al. 1994; Boger et al. 1999) ([A] = measured in rat brain homogenate, ref. Deutsch et al. 1997a).

effective FAAH inhibitors in rat liver membrane preparation were found to be compounds **8, 16** and **17**, with IC_{50} values of 0.59, 0.12 and 0.45 μM, respectively, implying that the FAAH inhibition preferred extended versus restricted conformation (Boger et al. 1999).

2.2.2.2 Sulfonyl fluorides

Phenylmethylsulfonyl fluoride (PMSF) (**Fig. 11**) is the first compound reported to be a potent inhibitor of AEA breakdown activity *in vitro* (Deutsch and Chin 1993). Originally, PMSF was reported to act as a non-specific inhibitor of various proteases
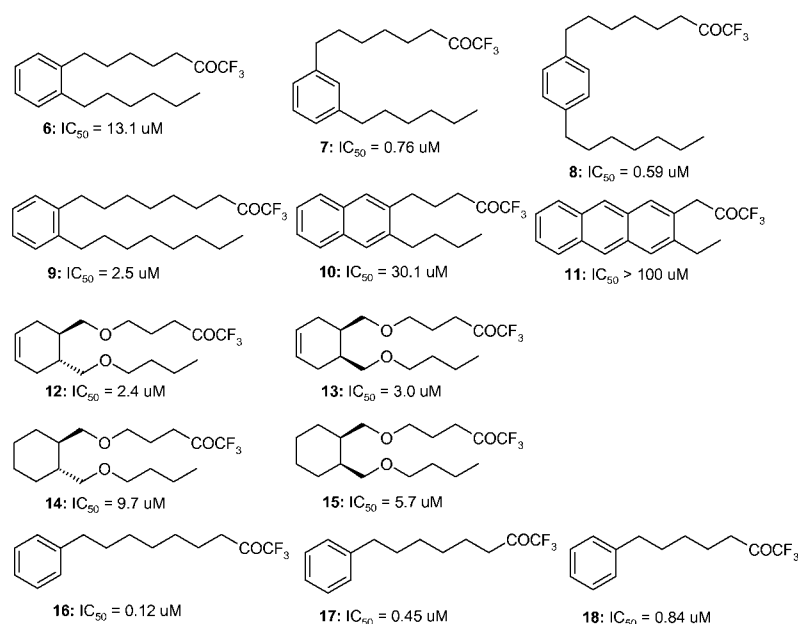
Fig. 10. Trifluoromethyl ketone inhibitors of FAAH with ring structure (Boger et al. 1999).

and other enzymes, including trypsin, chymotrypsin and acetylcholinesterase (Turini et al. 1969), and it was reported to inhibit the AEA hydrolysis with IC_{50} values of 0.3 - 3.7 μM in rat brain homogenates (Deutsch et al. 1997a; Deutsch et al. 1997b; Fowler et al. 1997). Generally, PMSF is assumed to inhibit FAAH by sulfonylating the hydroxyl groups of active site serine residues, which causes an irreversible inhibition (Gold 1965; Moss and Fahrney 1978). PMSF does not bind to CB1 receptors ($IC_{50} > 10 \ \mu M$) (Deutsch et al. 1997a) and, therefore, it has typically been included in various receptor binding and other *in vitro* assays to increase the apparent potency of AEA (Childers et al. 1994; Hillard et al. 1995).

PMSF (30 – 100 mg/kg, i.p.) has been shown to potentiate the effects of AEA on antinociception, spontaneous activity, catalepsy and mobility in mice, suggesting that besides *in vitro* metabolism, PMSF may also prevent the rapid *in vivo* metabolism of AEA (Compton and Martin 1997; Watanabe et al. 1999). Indeed, the administration of PMSF (30 mg/kg, i.p.) significantly increased brain levels of AEA when followed by an intravenous injection of [^{3}H]AEA (10 mg/kg) in mice (Wiley et al. 2000). The 10 mg/kg dose of AEA with PMSF resulted in similar brain levels as seen after the administration of a 50 mg/kg dose of AEA alone (1.94 vs. 2.04 $\mu g/g$, respectively). The PMSF pre-treatment also reduced the formation of the primary FAAH metabolite of

AEA, as the brain and plasma levels of arachidonic acid remained lower than those seen after administration of 10 mg/kg AEA alone (0.11 vs. 0.25 µg/g in brain and 0.05 vs. 0.49 µg/g in plasma) (Wiley et al. 2000).

Deutsch and colleagues developed and tested a series of saturated alkyl sulfonyl fluorides with chain lengths varying from C12 to C20 as inhibitors of FAAH activity (Deutsch et al. 1997a). The fatty acid sulfonyl fluorides with chain lenghts from C18 to C12 inhibited rat brain FAAH at similar potency, with IC_{50} values around 5 µM, whereas the analog with a C20 chain was the least potent inhibitor (IC_{50} > 48 nM). Of the alkyl sulfonyl fluorides, palmitylsulfonyl fluoride (AM374, **Fig. 11**) was observed to be the most selective inhibitor of FAAH with an IC_{50} value of 7 nM. This compound was found to be approximately 40-fold more potent than PMSF (IC_{50} = 290 nM) and approximately 130-fold more potent than ATFMK (IC_{50} = 900 nM) in preventing AEA breakdown in rat brain homogenate. However, AM374 may produce direct effects on cannabinoid receptors when employed at high concentrations, as AM374 is reported to be capable of displacing [^{3}H]CP55,940 binding from the CB1 binding site of rat forebrain synaptosomal preparations with an IC_{50} value of 520 nM (Deutsch et al. 1997a).

Recently, Segall et al. (2003) described the synthesis and evaluation of a novel arachidonoyl derivative, arachidonylsulfonyl fluoride (**Fig. 11**), which was found to inhibit mouse brain FAAH with an IC_{50} value of 0.1 nM. Arachidonylsulfonyl fluoride possessed some inhibitory effect towards a mouse brain neuropathy target esterase (IC_{50} = 780 nM), but did not significantly affect mouse brain acetylcholinesterase (AchE) or butyrylcholinesterase (IC_{50}'s > 100 µM). However, arachidonylsulfonyl fluoride was noticed to be capable of displacing the specific [^{3}H]CP55,940 agonist binding to the CB1 receptor in mouse brain with an IC_{50} value of 304 nM (Segall et al. 2003). Like PMSF, the alkylsulfonyl fluorides presumably inhibit FAAH in an irreversible fashion via covalent modification of the active site serine (Deutsch et al. 1997a).

PMSF
IC_{50} = 290 nM (rat brain)

Palmitylsulfonyl fluoride (AM374)
IC_{50} = 7 nM (rat brain)

Arachidonylsulfonyl fluoride
IC_{50} = 0.1 nM (mouse brain)
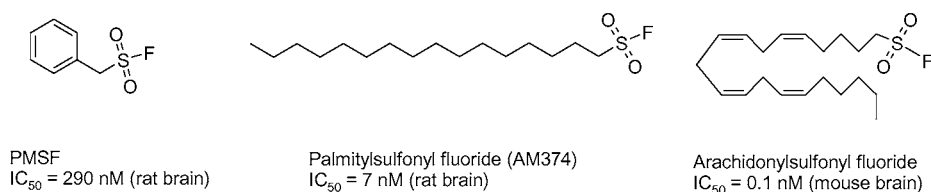
Fig. 11. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) of the most common sulfonylfluoride FAAH inhibitors (Deutsch et al. 1997a; Segall et al. 2003).

2.2.2.3 Methylfluorophosphonates

Methyl arachidonyl fluorophosphonate (MAFP, **Fig. 12**) was originally designed as an irreversible, active site-directed inhibitor of calcium- and arachidonyl sensitive cytosolic phospholipase A_2 ($cPLA_2$) (Huang et al. 1996). In rat brain homogenates, MAFP has been found to prevent the hydrolysis of AEA at low nanomolar concentrations (IC_{50} = 2.5 nM), indicating that it is approximately 800-fold more potent than ATFMK (IC_{50} = 1.9 μM) and 400-fold more potent than PMSF (IC_{50} = 0.9 μM), respectively, in inhibiting FAAH activity (Deutsch et al. 1997b). MAFP is reported to be selective towards nucleophiles within the arachidonyl binding region of this enzyme, and irreversibly inhibits the catalytic activity of FAAH. MAFP has been shown to act as a poor inhibitor of trypsin and chymotrypsin, although better than PMSF. Furthermore, MAFP has been reported to irreversible interact with the cannabinoid receptors in rat brain membrane preparations and prevent the subsequent binding of the cannabinoid agonist CP55,940. It exhibits a greater binding affinity towards CB1 receptors than AEA in the presence of PMSF (IC_{50} values of 40 nM and 20 nM for AEA and MAFP, respectively). According to IC_{50} values, it is assumed that approximately 10 % of CB1 receptors would be occupied at the concentration expected to inhibit 90 % of the FAAH activity (Deutsch et al. 1997b). Besides the enzymes mentioned above, MAPF has been reported to inhibit the catalytic activity of the calcium-independent arachidonic acid selective phospholipase A_2 ($iPLA_2$) and cyclooxygenases (Deutsch et al. 1997b).

Martin et al. (2000) investigated the ability of various methylfluorophosphonate analogs to inhibit FAAH, interact with CB1 receptors and produce pharmacological effects in mice. In that study, novel methylfluorophosphonate compounds were synthesised with saturated alkyl chains, ranging from C8 to C20 along with C20 unsaturated derivatives.
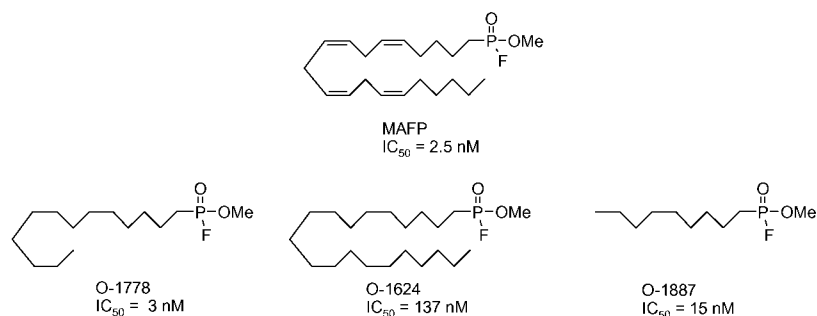
Fig. 12. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) in rat brain of some methylfluorophosphonate FAAH inhibitors (Martin et al. 2000).

All methylfluorophosponates were found to potently inhibit FAAH. The saturated short-chain C12 derivative O-1778 (**Fig. 12**) was observed to be the most potent FAAH inhibitor, yielding a 50 % FAAH inhibition at 3 nM, whereas the least active compound was the saturated C20 derivative O-1624 (**Fig. 12**), with an IC_{50} value of 137 nM. All of the analogs, except the saturated C20 (O-1624) and C8 (O-1887) (**Fig. 12**) derivatives effectively bound to the cannabinoid CB1 receptor at low nanomolar concentrations. However, none of the compounds completely displaced [^{3}H]CP55,940 binding even at high concentrations. In mice, the saturated short chain C12 (0-1778) and C8 (O-1887) analogs were very potent and produce Δ^9-THC –like effects *in vivo*, whereas the other analogs either acted as partial agonists or were inactive. Only the C20 analog (O-1624) was able to increase the levels of AEA in mice striatum, suggesting that, in addition to FAAH inhibition, these compounds may exert effects through their own activity towards CB1 or non-CB1 receptors (Martin et al. 2000).

2.2.2.4 Diazomethyl- and chloromethylketones

Arachidonoyl-diazo-methylketone (ADMK), arachidonoyl-chloro-methylketone (ACMK) and *O*-acetyl-arachidonoyl-hydroxamate (AcAHA) (**Fig. 13**) were designed on the basis of the possibility that inclusion of arachidonic acid and chemical moieties, which are known to covalently bind to serine/cysteine protease active sites, may result in more potent and selective FAAH inhibitors (De Petrocellis et al. 1997). The capability of compounds to inhibit AEA hydrolysis was investigated in porcine brain microsomes, mouse N18TG2 neuroblastoma and RBL-1 cells. ADMK was found to act as the most potent FAAH inhibitor in all studied enzyme sources with IC_{50} values of 6, 3 and 2 μM in porcine brain, N18TG2 and RBL-1 cell lines, respectively, whereas AcAHA was the less active compound with IC_{50} values of 25, 34 and 15 μM. ACMK

ADMK
IC_{50} = 3 μM

ACMK
IC_{50} = 10 μM

AcAHA
IC_{50} = 34 μM
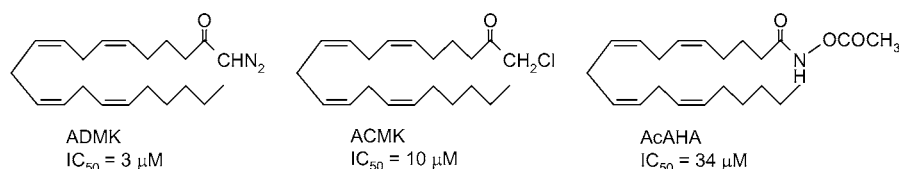
Fig. 13. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) in N18TG2 cell membranes of diazomethyl- and chloromethylketone FAAH inhibitors (Bisogno et al. 1998).

inhibited FAAH with IC_{50} values of 23, 10 and 3 µM in porcine brain, N18TG2 and RBL-1 cells, respectively. Unlike sulfonylfluorides and methylfluorophosphonates, ADMK, ACMK and AcAHA acted as reversible FAAH inhibitors with competitive inhibitory action, suggesting that they may also act as putative transition state inhibitors (De Petrocellis et al. 1997).

2.2.2.5 Arachidonyl serotonin and -ethylene glycol

Bisogno et al. (1998) screened for possible FAAH inhibitory action with 37 molecules that were extracted from different algae, or derived from the incorporation of arachidonic acid with bioactive amines or with ethylene glycol. Of the 32 algae-derived natural compounds, only malhamensilipin A and grenadadiene were observed to inhibit the hydrolysis of [^{14}C]AEA in mouse neuroblastoma N18TG2 cell homogenates with estimated IC_{50} values of 21 and 22.5 µM, respectively, whereas the others exhibited less than 40 % inhibition of [^{14}C]AEA hydrolysis at 100 µM concentration. Of the arachidonic acid derivatives, the most potent inhibitors of [^{14}C]AEA metabolism in N18TG2 cell homogenates were arachidonylserotonin (AA-5-HT) (**Fig. 14**) and arachidonyl ethylene glycol (AEG) (**Fig. 14**), with estimated IC_{50} values of 12 and 23.5 µM, respectively, while arachidonylhistamine, arachidonyldopamine and eicosapentaenoyl-serotonine were less active (IC_{50} > 30 µM). Unlike AA-5-HT, AEG behaved as a cannabimetic agent in the mouse model of cannabinergic activity, indicating that it could represent a novel class of CB1 receptor agonists (Bisogno et al. 1998).

AA-5-HT
IC_{50} = 12 µM

AEG
IC_{50} = 23.5 µM
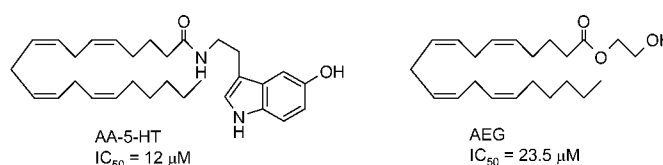
Fig. 14. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) in N18TG2 cell homogenates of arachidonylserotonin and –ethylene glycol (De Petrocellis et al. 1997).

2.2.2.6 α-Keto heterocycle inhibitors

Boger et al. (2000) developed novel α-keto heterocycle compounds with exceptionally high FAAH inhibitor potency. They synthesised compounds with an oleoyl chain possessing a 9-*cis*-double bond and a carbonyl at the site of the carboxylamide of oleamide, adjacent to an electron-deficient five- or six-membered monocyclic heterocycles or the bicyclic heterocycles, benzthiazole, benzimidatzole and benzoxazole. Only two of their compounds, **19** and **20** (**Fig. 15**), inhibited rat liver FAAH at a potency comparable to the trifluoromethyl ketone ATFMK (K_i-value of 82 nM). Further introduction of an additional nitrogen into the benzoxazole structure increased the inhibitor potency 50-200 –fold, providing compounds that were 10-50 - fold more potent than ATFMK, of which, the most effective inhibitor was observed to be the N4-substituted benzoxazole derivative (**21, Fig. 15**, K_i = 2.3 nM) (Boger et al. 2000).

Modification of the fatty acid chain revealed that the α-keto heterocyclic inhibitors based on the arachidonyl skeleton inhibited rat liver FAAH 4-5 times more potently than oleoyl-based derivatives, but degraded rapidly under normal test conditions. Incorporating unsaturation into the fatty acid chain enhanced efficacy for FAAH inhibition, and inclusion of a benzene ring into the fatty acid chain provided FAAH inhibitors at subnanomolar concentrations, the most potent inhibitor being compound **22**
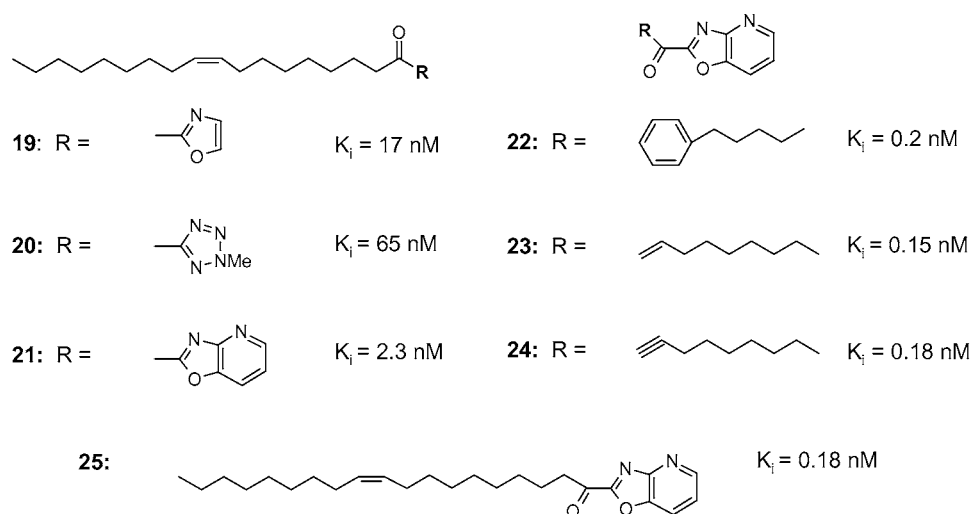
Fig. 15. Chemical structures and K_i-values for FAAH inhibition in rat liver homogenates of some α-keto heterocycle FAAH inhibitors (Boger et al. 2000).

(Fig. 15), which blocked the hydrolysis of AEA with a K_i-value of 200 pM. Furthermore, the incorporation of a double or triple bond at the oleamide $\Delta^{9,10}$ position produced even more effective FAAH inhibitors; compounds 23 and 24 (Fig. 15), which inhibited AEA hydrolysis with K_i-values of 150 and 180 pM, respectively. The most potent inhibitor of rat liver FAAH, with a K_i-value of 140 pM, was the oleoyl-based compound 25 (Fig. 15), which had triple bond at the $\Delta^{9,10}$ position (Boger et al. 2000).

Recently Boger et al. (2001) investigated the effects of carbonyl replacement and substitution at the 2-position on the inhibitory potency of α-keto heterocycle inhibitors. Their results suggested that an electrophilic carbonyl is essential for efficient FAAH inhibition and substitution at the 2-position may limit effective binding to the FAAH active site (Boger et al. 2001).

2.2.2.7 Alkylcarbamic acid aryl esters

Recently, Tarzia and co-workers reported on the synthesis of various alkylcarbamic aryl esters designed as FAAH inhibitors by modification of a known acetylcholinesterase (AchE) inhibitor (Tarzia et al. 2003) (Fig. 16). The ability of compounds to inhibit FAAH was measured in rat brain membranes using [^{3}H]AEA as a substrate. Their most effective FAAH inhibitior was observed to be m-biphenyl derivative URB524 (32), which inhibited [^{3}H]AEA hydrolysis with IC_{50} value of 63
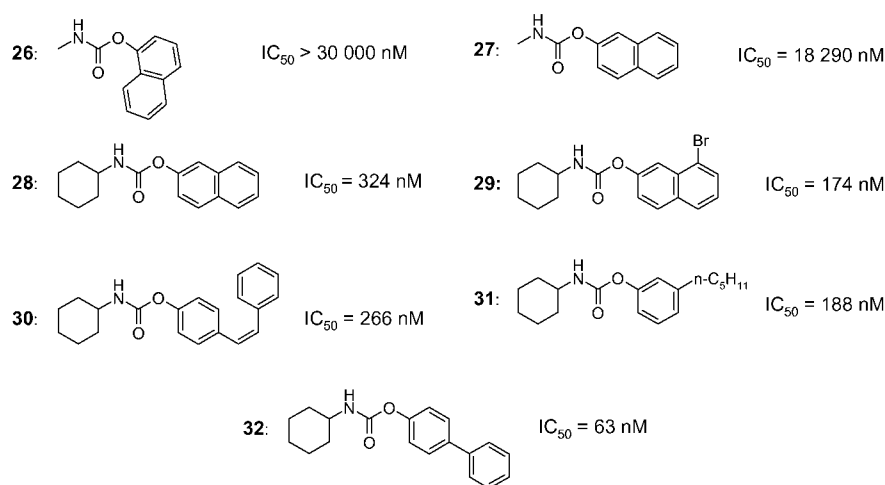
Fig. 16. Chemical structures and half-maximal concentrations for inhibition (IC_{50}) in rat brain membranes of some alkylcarbamic aryl ester FAAH inhibitors (Tarzia et al. 2003).

nM. None of these novel compounds (1-100 µM) displaced the specific binding of [^{3}H]WIN55,212-2 in the rat cerebellar CB1 receptor or in the human recombinant CB2 receptor, nor affected the transport of [^{3}H]AEA in human astrocytoma cells. Except the carbaryl derivative **26** (**Fig. 16**), these novel inhibitors had no significant effect on the activities of AchE or butyrylcholinesterase, indicating that these alkylcarbamic aryl esters possessed relatively good specificity towards FAAH (Tarzia et al. 2003).

2.2.2.8 Other inhibitors

The mechanism-based inhibitor of Ca^{2+} -independent PLA_2 ($iPLA_2$), bromoenol lactone [(E)-6-(bromometylene) tetrahydro-3-(1-naphthalenyl), 2H-pyran-2-one] (BEL) has been reported to inhibit FAAH with an IC_{50} value of 0.8 µM in rat brain microsomes (Beltramo et al. 1997b). The inhibition potency of BEL was observed to be approximately 5 times higher than that of ATFMK, which inhibited rat brain FAAH with an IC_{50} value of 4 µM under similar test conditions. Additionally, BEL inhibited the hydrolysis of AEA in rat liver microsomes with an IC_{50} value of 79 µM. The observation that the inhibitory potential of BEL was about 100-fold lower in liver than in rat brain suggests this the difference might result from the degradation of bromoenol lactone by liver enzymes. Kinetic and dialysis experiments indicated that the inhibitory effect of BEL towards FAAH was non-competitive and irreversible (Beltramo et al. 1997b).

Various non-steroidal anti-inflammatory drugs (NSAID's) have been shown to affect the metabolism of AEA (Fowler et al. 1997). A series of NSAID's inhibited the hydrolysis of AEA in rat brain minus cerebellum with estimated IC_{50} values of 170, 270, 480, 550 and 650 µM for suprofen, ibuprofen, fenoprofen, naproxen and ketoprofen, respectively. Diclofenac and sulindac were markedly less potent than suprofen or ibuprofen but contained some FAAH inhibitory activity. The enantiomers of flurbiprofen and ketorolac inhibited FAAH even more potently, with IC_{50} values for R- and S- ketorolac of 50 and 440 µM, respectively, and for R- and S-flurbiprofen 60 and 50 µM, respectively, in rat brain homogenates (Fowler et al. 1999).

Recently, the general anesthetic propofol (100 mg/kg, i.v.) has been reported to increase whole-brain concentrations of AEA in mice (Patel et al. 2003). Propofol was found to act as a competitive inhibitor of FAAH with IC_{50} values of 14 and 52 µM when delivered in intralipid or DMSO vehicles, respectively, whereas the other common general anesthetics, etomidare, midazolam, ketamine and thiopental were inactive in sedative-relevant concentrations (Patel et al. 2003).

Furthermore, cannabidiol, cannabinol and Δ^9-THC have been reported to inhibit FAAH activity at high micromolar concentrations in mouse brain microsomes (Watanabe et. al. 1996). The endogenous compound 2-octyl-γ-bromoacetoacetate, which was originally isolated from human cerebrospinal fluid, and its most potent chloro analog, 2-octyl-γ-chloroacetoacetate, have been shown to competitively inhibit FAAH activity in solubilized liver plasma membranes with IC_{50} values of 3 and 0.7 μM, respectively, as measured by the reduction of [^{14}C]-oleamide hydrolysis at physiological pH (Patricelli et al. 1998).

2.3 THERAPEUTIC APPLICATIONS OF CANNABINOIDS

The medicinal properties of natural cannabinoids, extracted from the plant *Cannabis sativa*, have been known for thousands of years. *Cannabis sativa* is known to produce over 400 chemical entities, of which at least 60 belong to the cannabinoid class. The most abundant and widely studied natural plant cannabinoids include Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabidiol (CBD) and cannabinol (CBN). This plant, plant extracts and isolated Δ^9-THC have traditionally been used to alleviate symptoms of several medical conditions, including pain, anxiety, glaucoma, nausea, emesis, muscle spasms and wasting diseases. Discovery of the specific molecular targets for cannabinoid action has revealed a possibility to develop cannabinoid analogues with various therapeutic actions (**Table 2**). Only a few examples of the major potential therapeutic indications for cannabinoids are included in following chapters.

2.3.1 Cancer chemotherapy

Cannabinoids are reported to suppress human breast cancer cell proliferation *in vitro* (De Petrocellis et al. 1998) and induce apoptotic death of cultured glioma (Sanchez et al. 1998b; Jacobsson et al. 2000), astrocytoma (Sanchez et al. 1998b), neuroblastoma (Sanchez et al. 1998b), and pheochromocytoma (Sarker et al. 2000) cells. Intratumoral Δ^9-THC or WIN55212-2 administrations have induced considerable regression of malignant glioma growth in rats and immunodeficient mice (Galve-Roperh et al. 2000). Recently, malignant glioma growth was inhibited *in vivo* by local administration of the selective CB2 receptor agonist JWH-133 (Sanchez et al. 2001). JWH-133 -induced tumor regression was prevented by a selective CB2 receptor antagonist, but not by a CB1 receptor antagonist, suggesting that antitumoral actions of cannabinoids on

Table 2. Suggested therapeutic indications for compounds acting via the endogenous cannabinoid system.

INDICATION / DISEASE	SUGGESTED MECHANISM OF ACTION			REFERENCES
	VIA CB1	VIA CB2	OTHER	
Neurodegenerative disorders				
-Parkinson disease	X			Brotchie et al. 2003
-Tourette's syndrome	X			Müller-Wahl et al. 1998
-Huntington's disease	X		VR1	Lastres-Becker et al. 2002; 2003
Other neurological disorders				
-Brain ischemia	X		NMDA	Hampson et al. 1998; Nagayama et al. 1999
-Epilepsy	X			Wallace et al. 2001; 2003
-Migraine	X(?)			Russo 1998
Cardiovascular disorders				
-Shock	X			Wagner et al. 1997; Varga et al. 1998
Arthritis		X(?)	X(?)	Malfait et al. 2000
Cancer chemotherapy		X		Galve-Roperh et al. 2000
Multiple sclerosis	X			Baker et al. 2000; 2001
Pain	X	X	X	Hochmann et al. 2002
Glaucoma	X			Green 1979; Pate et al. 1998; Song and Slowey 2000
Emesis	X			Darmani 2000; 2001
Control of appetite	X			Cota et al. 2003

(?) No complete evidence

gliomas may be exerted via CB2 receptor activation. Local cannabinoid receptor activation by CB2 receptor agonist JWH-133 was reported to induce regression of malignant non-melanoma skin tumor growth by an apoptotic mechanism in mice (Casanova et al. 2003). Under certain circumstances, however, Δ^9-THC may inhibit host antitumor immune reactivity, and therefore accelerate tumor growth because of its immunosuppressive activity (Zhu et al. 2000).

2.3.2 MS-disease

The immunomodulatory properties of cannabinoids might have therapeutical value for the treatment of autoimmune diseases such as multiple sclerosis (MS), which is the most common human primary demyelinating disease of the CNS. Synthetic and endogenous cannabinoid agonists have been found to ameliorate the tremor and spasticity in an experimental MS-disease mouse model with chronic relapsing experimental allergic encephalomyelitis (CREAE) (Baker et al. 2000; 2001). Additionally, the AEA uptake inhibitors AM404 and VDM11 (10 mg/kg, i.v) and the FAAH inhibitor AM374 (10 mg/kg, i.v.) significantly reduced the spasticity of CREAE mice (Baker et al. 2001). The antispastic effects of cannabinoid agonists, AEA uptake inhibitors and FAAH inhibitor were reversed with both CB1 and CB2 receptor antagonist SR141716A and SR144528 (5 mg/kg i.v.), respectively, and both antagonists

exacerbated signs of spasticity in spastic mice, suggesting that both cannabinoid receptors may control spasticity (Baker et al. 2000; 2001). Furthermore, a marked increase in the levels of AEA, 2-AG and PEA in the spinal cord was observed in spastic mice, but not in normal or nonspastic remission CREAE mice (Baker et al. 2001). Recently, the cannabinoid agonists WIN55,212-2, arachidonyl-2'-chloroethylamide (ACEA) and JWH-133 were shown to improve motor coordination, reduce inflammation and promote remyelination in Theiler's murine encephalomyelitis virus (TMEV) infected mice (Arevalo-Martin et al. 2003). In the same MS-disease model, WIN55,212-2 treatment markedly ameliorated the progression of TMEV-induced demyelinating disease, possibly by inhibiting the Th1 cell differentiation and by inhibiting the production of proinflammatory cytokines (Croxford and Miller 2003). In contrast, in patients with MS, orally administered cannabinoid extract have been observed to increase the proinflammatory cytokine production at physiologically relevant doses (Killestein et al. 2003). However, in recent phase III clinical trials, treatment with a Δ^9-THC/cannabidiol inhalation significantly reduced neuropathic pain and improved other symptoms of MS in patients (GW Pharmaceuticals 2002).

2.3.3 Neurodegenerative diseases

Cannabinoids have been suggested to be beneficial in neurodegenerative diseases such as Huntington's disease (HD), Parkinson's disease and Tourette's syndrome. In HD, a significant loss of cannabinoid agonist binding and CB1 receptor density was observed in the substantia nigra of *post mortem* human brain (Glass et al. 1993; 2000). Denovan-Wright and Robertson (2000) reported a decreased CB1 receptor mRNA levels in the lateral striatum and cortical regions of transgenic HD mice prior to development of the HD phenotype or neuronal degeneration. Autoradiographic binding studies were also indicated a significant reduction in the density of CB1 receptors in gaudate-putamen and striatal projection areas of transgenic HD mice, and the loss of CB1 receptors reflected a loss of receptor activation efficacy, suggesting that the endocannabinoid system becomes hypofunctional in HD (Lastres-Becker et al. 2002). It has been suggested that compounds increasing endocannabinoid transmission may be useful in alleviating symptoms of HD (see review by Romero et al. 2002). In contrast, elevated striatal levels of AEA and lowered activities of both FAAH and AEA transporter protein have been shown to be accompanied with dopamine loss in 6-hydroxydopamine-lesioned rats that represents an experimental model of Parkinson's disease (PD) (Gubellini et al. 2002). The levels of 2-AG have been shown to be elevated by 7-fold in the globus pallidus of reserpine-treated rats, with reduced locomotor

activity (Di Marzo et al. 2000). Furthermore, the administration of D1 and D2 dopamine receptor agonists have shown to reduce levels of 2-AG and AEA in the globus pallidus of reserpine-treated rats, suggesting that overactivation of the endocannabinoid system may participate in the generation of PD (Di Marzo et al. 2000). It has been suggested that CB1 receptor antagonists may be beneficial in the treatment of parkinsonian symptoms and levodopa-induced dyskinesia, whereas CB1 receptor agonists could be useful in reducing levodopa-induced dyskinesia (see review by Brotchie 2003). Inhaled marijuana has been reported to improve both motor and vocal tics in patients with Tourette's syndrome (Müller-Vahl et al. 1998).

2.3.4 Regulation of appetite

Administration of the exogenous AEA (0.5 – 10 mg/kg, s.c.) has provoked overeating in pre-satiated rats (Williams and Kirkham 1999). This hyperphagic effect of AEA was dose-dependently attenuated with the CB1 receptor antagonist SR141716A. It has been recently reported that CB1 receptor knockout mice eat normally less than their wild-type littermates and the food intake of wild-type mice, but not CB1 knockout mice, was reduced by treatment with this CB1 receptor antagonist (Di Marzo et al. 2001). Leptin is a hormone secreted by adipose tissue that regulates normal food intake by increasing levels of appetite-reducing and reducing the levels of appetite-stimulating factors (Friedman and Halaas 1998). Administration of recombinant mouse leptin (125 or 250 μg) by intravenous injection has been shown to reduce endogenous levels of AEA and 2-AG by approximately 40-50 % in normal rat hypothalamus (Di Marzo et al. 2001). Moreover, the hypothalamic levels of the AEA precursor NArPE were markedly increased after leptin treatment in rats, whereas the hypothalamic levels of FAAH activity remained unchanged. In contrast, the hypothalamic levels of 2-AG precursors, sn-2-arachidonate-containing DAGs, were reduced by leptin treatment, suggesting a role for the endocannabinoid system as a modulator of food intake (Di Marzo et al. 2001). The systemic administration of oleylethanolamide (OEA), an endogenous lipid that does not bind to cannabinoid receptors, has been shown to suppress food intake and reduce body weight in food-deprived rats (Rodriguez de Fonseca et al. 2001). Recently, OEA has been found to produce its anorectic effects by activating the nuclear peroxisome-proliferator-activated receptor-α (PPAR-α) (Fu et al. 2003). Cannabinoid agonists that have appetite enhancing effects might be beneficial in the treatment of diseases that are associated in appetite and weight loss, such as AIDS or cancer chemotherapy -induced anorexia, whereas cannabinoid antagonists that reduce food intake might be useful in the treatment of obesity (see review by Cota et al. 2003).

2.3.5 Pain

Cannabinoids have been shown to suppress behavioural responses for acute and chronic noxious thermal, mechanical and chemical stimuli, suggesting that pain reduction may be the one of the physiological functions of endocannabinoids (see reviews: Hohmann 2002; Walker et al. 2002). Indeed, AEA has been shown to attenuate formalin-evoked pain behaviour in rats and this antinociceptive effect of AEA has been blocked by systemic administration of the CB1 receptor antagonist SR141716A, but not by CB2 receptor or opioid antagonists (Calignano et al. 1998). The CB1 receptor antagonist SR141716A has prevented the antinociceptive and cataleptic effects of intracerebroventricularly administered CP55,940 and WIN55,212-2 in rats (Lichtman and Martin 1997). In contrast, the administration of SR141716A alone has produced hyperalgesia in rats (Calignano et al. 1998). Recently, the CB2 receptor agonist AM1241 has been found to reduce the thermal nociception in rats, suggesting a role for CB2 receptors by inhibiting the local peripheral responses to noxious acute stimuli (Malan et al. 2001). It has been reported that the thalamic levels of CB1 receptors are upregulated in a rat model of chronic neuropathic pain (Siegling et al. 2001). The nociceptive primary sensory neurons have been shown to co-express CB1 and VR1 receptors at very high levels, suggesting an anatomical basis for VR1 mediated excitation and CB1 mediated inhibition of noxious stimuli at central and peripheral terminals of nociceptive primary neurons (Ahluwalia et al. 2000). The antinociceptive activity of cannabinoids has been reported to be comparable to opiates both in potency and efficacy (Bloom et al. 1977; Jacob et al. 1981).

2.3.6 Cardiovascular effects

AEA has been shown to cause a brief pressor response, followed by hypotension and mild bradycardia in anesthesized normotensive and hypertensive rats (Varga et al. 1995; Lake et al. 1997). The prolonged hypotension and bradycardia were inhibited by the CB1 receptor antagonist SR141716A, which suggests the involvement of CB1 receptors in those effects (Varga et al. 1995). A bolus injection of 2-AG has been shown to cause dose-dependent CB1 receptor mediated hypotension and non-CB1 receptor mediated tachycardia in anesthetized rats (Varga et al. 1998). In contrast, results with CB1 receptor deficient mice give evidence that the cardiovascular effects of 2-AG may also be mediated via non-CB1 receptors (Jarai et al. 2000). The CB1 receptor antagonist SR141716A has prevented endotoxic shock -induced hypotension in rats (Varga et al. 1998). Additionally, AEA and 2-AG are synthesised by rat lipopolysaccharide-

stimulated circulating macrophages and platelets, respectively, suggesting that some endocannabinoids may act via vascular CB1 receptor activation as mediators of endotoxin-induced hypotension (Varga et al. 1998). Moreover, the CB1 receptor antagonist SR141716A has been reported to increase blood pressure in rats subjected to haemorrhagic shock (Wagner et al. 1997), suggesting that treatment with a CB1 receptor antagonist may be beneficial in the prolonged hypotension that occurs during shock. There is also recent evidence that CB1 receptor antagonists might be useful in cardiac remodeling therapy after myocardial infarction (Wagner et al. 2003).

2.4 CANNABINOIDS AND INTRAOCULAR PRESSURE
2.4.1 Glaucoma

Glaucoma is one of the leading causes of irreversible blindness worldwide. Recent estimations suggest that 66.8 million persons suffer from glaucoma, 6.7 million of which are bilaterally blind (Quigley 1996). Glaucoma can occur at of any age, including the infancy, but it is generally considered to be a disease of the ageing eye. The prevalence of glaucoma has been shown to be considerably increased with increasing age and is reported to be around 1.5 % in patients over 50 years of age (Klein et al. 1992, Dielemans et al. 1994, Mitchell et al. 1996).

Glaucoma is characterized by a progressive degeneration of the retinal ganglion cell axons that comprise the optic nerve and a loss of peripheral vision early in disease, which may ultimately lead to a loss of central vision. The glaucomatous changes in the eye include increased cupping or excavation of optic nerve head, notching or thinning of the neuroretinal rim and loss of the retinal nerve fibre layer. Often, but not always glaucoma is accompanied by elevated intraocular pressure (IOP) (Coleman 1999). Glaucoma patients with average intraocular pressures greater than 17.5 mm Hg are predicted to have increased progression of visual field deterioration than patients with an average intraocular pressure of less than 14 mm Hg (AGIS Investigators 2000).

Glaucoma is a genetically heterogenous disease that results from the interaction of multiple genes and environmental influences (Sarfarazi 1997). Several risk factors are known to be associated with the development and progression of this disease, of which the most common risk factor is an elevated IOP. Elevated IOP alone is sufficient to result in glaucomatous optic neuropathy (Flammer et al. 2002). As IOP increases, there is a corresponding increase in the risk of glaucomatous optic-nerve damage and subsequent visual field loss (Tielsch et al. 1991). Other known glaucoma risk factors include older age (Klein et al. 1992, Dielemans et al. 1994, Mitchell et al. 1996; Gordon

et al. 2002), sex (Gordon et al. 2002), race (Tielsch et al. 1991; Gordon et al. 2002), myopia (Mitchell et al. 1999a), pseudoexfoliation (Mitchell et al. 1999b) and family history of glaucoma (Wolfs et al. 1998). In addition, there have been suggestions that other diseases such as diabetes mellitus (Mitchell et al. 1997) and systemic hypertension (Dielemans et al. 1995) may influence the risk of primary open angle glaucoma but these associations have been controversial.

Several glaucoma variants are identified. Glaucoma can be divided in open-angle glaucoma (POAG) and angle-closure glaucoma (PACG), which differ in whether the trabecular meshwork is obstructed by the iris. In POAG, the angle of the eye seems to be normally open, but the trabecular meshwork cannot filter out the aqueous humour properly. Conversely, in PACG, the periphery of the iris obstructs the trabecular meshwork in the angle of eye, therefore causing inadequate drainage of aqueous humour from the anterior chamber (Coleman 1999). Normal tension glaucoma (NTG) can be classified as a distinct type of POAG, where the typical optic disc damage with glaucomatous cupping and loss of neuroretinal rim is present without raised IOP (Kamal and Hitchings 1998). In contrast, patients with increased IOP, but no evidence of glaucomatous ocular changes, are described as ocular hypertensives (Coleman 1999).

The goal of glaucoma treatment is to stop the progression of irreversible optic nerve damage, and further loss of functional vision during the remainder of the patient's life. For glaucoma patients, drug therapy, laser surgery and conventional surgical therapy are generally used for lowering the IOP to a level that prevents progressive loss of vision (Aung and Chew 2002; Hoyng and Kitazava 2002). Reduction of IOP significantly delays, or even prevents, the progression of glaucomatous damage in both old and young glaucoma patients, high- or normal tension glaucoma and in eyes with less or greater visual field loss. Every 1 mm Hg reduction in the IOP has been shown to reduce the risk of glaucomatous damage progression by 10 % (Heijl et al. 2002).

The mainstay in IOP-lowering drug therapy includes topical prostaglandin analogs, beta-adrenergic antagonists, topical carbonic anhydrase inhibitors and selective alpha-adrenergic agonists (**Table 3**). The basic mechanisms of IOP lowering drugs is to either decrease aqueous humor secretion from the ciliary body of the eye or increase its outflow via the trabecular meshwork or via the uveoscleral route of the eye. However, since glaucoma patients must, in numerous cases, use more than one single active ingredient to control their disease, and since currently available glaucoma drugs are in many cases administered more frequently than once a day, patient compliance appears to be the major problem. In addition, current glaucoma medications tend to provoke several local and systemic side effects, particularly in elderly patients with history of

Table 3: Currently approved antiglaucoma agents (Lääketietokeskus Oy 2003).

Class / Generic Name	Dosage	Mechanism of Action	Common Side Effects
1. Beta-adrenergic receptor antagonists (non-selective): -Timolol maleate -Timolol hemihydrate (*) -Carteolol (*) -Levobunolol (*) -Metipranolol (*)	(1-)2 times / day	Reduced aqueous humor production	Fatigue, faintness, headache, bradycardia and asthma. Blurred vision, ocular aching, redness and foreign-body sensation within eye. Carteolol has an intrinsic symphatomimetic activity.
2. Beta-adrenergic receptor antagonists (selective): -Betaxolol	2 times / day	Reduced aqueous humor production	Ocular stinging and blurred vision. Bradycardia. Erythema, insomnia and depression.
3. Cholinergic agents: -Carbachol -Pilocarpine HCl -Pilocarpine nitrate	3 times / day 2-6 times / day 4-6 times / day	Increased aqueous humor outflow	Headache, nausea, dizziness, and asthma. Blurred vision, ocular aching, redness and foreign-body sensation within the eye
4. Carbonic anhydrase inhibitors: -Acetatzolamide (oral) -Brinzolamide -Dorzolamide	(1-)2 times / day 2 times / day 2-3 times / day	Reduced aqueous humor production	Lassitude, altered taste, loss of appetite and dizziness. Blurred vision, ocular aching, stinging and redness with topical products.
5. Adrenergic-receptor agonists (non-selective): -Adrenalin borate (*) -Adrenalin HCl (*) -Dipivefrin HCl	 2 times / day	Increased aqueous humor outflow	Conjunctival irritation, ocular burning, stinging, headache, photophobia, macular edema and allergic plepharoconjuctivitis. Tachycardia, extrasystoles and raised blood pressure.
6. Adrenergic-receptor agonists (α_2-receptor selective): -Apraclonidine HCl -Brimonidine tartrate	 3 times / day 2 times / day	Reduced aqueous humor production. Increased uveoscleral outflow (Brimonidine).	Ocular burning, stinging, foreign-body sensation, blurred vision and allergic reactions. Dry mouth, headache and fatigue.
7. Prostaglandin analogues: -Bimatoprost -Latanoprost -Travoprost	Once daily Once daily Once daily	Increased uveoscleral outflow.	Hyperpigmentation of iris and eyelashes, conjuctival hyperemia, cystoid macular edema, and anterior uveitis. Hypertension.
8. Combination products: -Dorzolamide / timolol maleate -Latanoprost / timolol maleate -Timolol maleate / pilocarpine HCl - Adrenalin HCl / pilocarpine (*)	2 times / day Once daily 2 times / day	See groups separately	See groups separately

(*) Not available in Finland. Approved in United States (Schuman 2000).

multiple morbidities, indicating that there is a serious need for novel therapeutic agents or treatment strategies in the treatment of glaucoma (Schuman 2000), especially as the elderly population continues to increase.

2.4.2 Ocular endocannabinoid system
2.4.2.1 Ocular cannabinoid receptors

Indirect evidence for the possible existence of ocular cannabinoid receptors was first provided in 1996 when Schlicker et al. (1996) inhibited dopamine release in guinea pig retinal discs by the application of CB1 receptor agonists WIN55,212-2 and CP55,940. The effect was reversible with the CB1 receptor antagonist SR 141716A. Subsequently, CB1 receptor mRNA has been identified in the various ocular tissues of rat (Porcella et al. 1998), where the CB1 receptor mRNA was more abundant in the ciliary body area and iris than in retinal tissues and choroid (**Table 4**). In the human eye, most of the CB1 receptor mRNA appears to be located in the ciliary body (Porcella et al. 2000). In addition to cells of the ciliary processes, the presence of CB1 receptor mRNA has recently been reported in cells isolated from the trabecular meshwork tissues of bovine and human (Stamer et al. 2001).

By the use of subtype-specific affinity-purified polyclonal antibodies against CB1 receptor protein, a wide distribution of CB1 receptors has been identified within the human anterior eye and retina (Straiker et al. 1999a). CB1 receptors seem to be present in the human ciliary epithelium, corneal epithelium and endothelium, trabecular meshwork, Canal of Schlemm, ciliary muscle and in blood vessels of the ciliary body. Retinal tissues of humans (Straiker et al. 1999a) and several other animal species (Straiker et al. 1999b; Yazulla et al. 1999; Yazulla et al. 2000) have been shown to contain CB1 receptor protein. Recently, the presence of functional G-protein coupled CB1 receptors has been demonstrated in the ciliary process, and in the trabecular meshwork tissues of bovine and human by immunofluorescence microscopy (Stamer et al. 2001). Evidence for the expression of CB2 receptor mRNA has been obtained from rat retinal tissues by the use of *in situ* hybridization histochemistry and reverse transcription PCR (Lu et al. 2000). The reported tissue distribution of ocular cannabinoid receptors is summarized in **Table 4**.

Table 4. The regional distribution of cannabinoid receptors in various ocular tissues.

Ocular tissue	CB1	CB2	Species	References
Retina; Ganglion cell layer IPL, INL, OPL, Photoreceptors	X,Y	Y	Human, rat, mouse, monkey, goldfish, chick	Straiker et al. 1999a, 1999b; Yazulla et al. 1999, 2000; Lu et al. 2000; Porcella et al. 2000
Cornea; Epithelium, Endothelium	X		Human	Straiker et al. 1999a
Iris	X,Y		Human, rat	Porcella et al. 1998, 2000; Straiker et al. 1999a
Trabecular Meshwork	X, Y		Human, bovine	Straiker et al. 1999a; Stamer et al. 2001
Sclemm's Canal	X		Human	Straiker et al. 1999a
Ciliary Body; Non-pigmented Epithelium Ciliary muscle	X, Y		Human, rat, bovine	Porcella et al. 1998, 2000; Straiker et al. 1999a; Stamer et al. 2001
Choroid	Y		Rat	Porcella et al. 1998

X = expression of receptor protein; Y = expression of receptor mRNA; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer.

2.4.2.2 Ocular endocannabinoids

The biosynthesis and hydrolysis of AEA from arachidonic acid and ethanolamine has been reported in various porcine ocular tissue homogenates (Matsuda et al 1997), including retina, iris, choroid and optic nerve. A single enzyme seemed to be responsible for AEA hydrolase and synthase activities in eye. Both AEA synthase activity (1.9 - 4.2 nmol/min^{-1}/mg^{-1} protein at 37 °C) and hydrolase activity (1.2 - 3.5 nmol/min^{-1}/mg^{-1} protein at 37 °C) in the ocular tissues were comparable to those of brain homogenate. The highest enzyme activity was detected in the retina. However, condensation of AEA from arachidonic acid and ethanolamine under physiological conditions is unlikely, as the required arachidonic acid and ethanolamine concentrations are high (for recombinant rat liver FAAH, Km values are 190 μM and 36 mM for arachidonic acid and ethanolamine, respectively) (Ueda et al. 1995a; Kurahashi et al. 1997).

The lipid extract of bovine retina has been shown to contain AEA and N-docosa-hexaenoylethanolamine (DHEA), their putative biosynthetic precursors NArPE (N-

arachidonylphosphatidylethanolamine) and NDHPE (N-docosahexaenoylphospatidyl-ethanolamine) (Bisogno et al. 1999b), respectively, suggesting that the biosynthesis of AEA may occur through phosphodiesterase-mediate cleavage of NArPE in the eye. In addition, a FAAH-like enzymatic activity that can be inhibited by the FAAH-inhibitors phenylmethylsulfonyl fluoride and arachidonyltrifluoromethylketone has also been identified in bovine retina (Bisogno et al. 1999b), and an FAAH enzyme protein has been detected and localized in rat retina using immunocytochemical methods (Yazulla et al. 1999).

Both endogenous cannabinoids AEA and 2-AG have been identified in lipid extracts of bovine and rat retinas (Bisogno et al. 1999b; Straiker et al. 1999b). The amounts of AEA and 2-AG in the bovine retina, measured by GC/MS, were 64.0 ± 9.6 pmol/g and 1.63 ± 0.31 nmol/g of retinal tissue, respectively (Bisogno et al. 1999b). In rat retina, 2-AG (2.97 ± 0.066 nmol/g) was found at levels comparable to that of rat brain (Straiker et al. 1999b), but AEA was not detected. Tissue levels of AEA measured from human post mortem eyes using liquid chromatography-mass spectrometry, were found to be 10-fold higher in ciliary process tissues (49.42 pmol/g tissue) than in either trabecular meshwork (3.08 pmol/g tissue) or neurosensory retinal tissues (4.48 pmol/g tissue) (Stamer et al. 2001). Other endogenous cannabinoid-like lipids, such as N-palmitoylethanolamide (PEA) (a CB2-receptor agonist) and N-oleylethanolamide (OEA) were also identified in rat retina (Straiker et al. 1999b). The existence of cannabinoid receptors and their endogenous ligands in various ocular tissues suggests a possible physiological role for the endocannabinoid system in several ocular functions (Porcella et al. 1998; Straiker et al. 1999a; Lu et al. 2000; Stamer et al. 2001).

2.4.3 Intraocular pressure effects of cannabinoids

The current medical interest in cannabinoids as anti-glaucoma agents began in the 1970's, when Hepler and Frank (1971) first reported the IOP lowering effects of smoked marijuana in humans. Since this original report, various studies have followed using isolated, synthesised or endogenous cannabinoids administered by different routes or vehicles to demonstrate their activity on IOP reduction. According to their distinct molecular structures, the cannabinoids are generally classified into the four main groups that include the classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids (see review by Pertwee 1999). In following chapters, the reported IOP lowering effects of the cannabinoids are summarized.

2.4.3.1 Marijuana

Following the report by Hepler and Frank (1971), the IOP lowering properties of inhaled marijuana have been confirmed by various other studies (as reviewed by Green 1998). For example, one smoked marijuana cigarette, containing 2 % Δ^9-THC, has been shown to decrease IOP to approximately 6.6 mm Hg in a heterogeneous glaucoma population (Merritt et al. 1980a). However, the IOP reduction induced by marijuana inhalation was accompanied by decreased blood pressure and other side effects such as tachycardia, anxiety, orthostatic hypotension and conjunctival hyperemia (Merritt et al. 1980a). The fact that long-term marijuana use may lead to emphysema-like changes in the lungs from the production of the tars, carcinogens and other volatiles (Tashkin et al. 1987), indicates that smoked marijuana may not be the first-choice treatment for glaucoma patients.

2.4.3.2 Classical cannabinoids

Classical cannabinoids are the natural phytochemicals and their synthetic derivatives that have a dibenzopyrene structure. The most widely known classical phytocannabinoid is Δ^9-THC, which is the main psychoactive ingredient in marijuana. Other typical compounds belonging to this group include Δ^8-THC, cannabinol and cannabidiol (Pertwee et al. 1999) (Fig. 17).

Δ^9-THC has been shown to reduce IOP when given intravenously or orally. Intravenous injections of Δ^9-THC (1.0 mg/kg) have been reported to reduce IOP around 25 % in normotensive albino rabbits (ElSohly et al. 1984). At doses lower than 2 mg/kg (i.v.), the IOP lowering activity of Δ^9-THC is dose-dependent but this effect is lost at higher doses, suggesting that the effects of Δ^9-THC are bi-phasic (ElSohly et al. 1981; 1984). Orally administered Δ^9-THC decreased IOP in normotensive conscious monkeys by approximately 22 % at a dosage of 3 mg/kg (Waller et al. 1984). In addition, Δ^9-THC administered in soft gelatine capsules (20 and 25 mg) lowered IOP by 7.8 mm Hg in glaucoma patients who were naive to marijuana (Merritt et al. 1980b). However, the higher Δ^9-THC doses (> 20 mg) tended to provoke adverse CNS effects such as depersonalisation, acute panic reaction and paranoid feelings in these patients (Merritt et al. 1980b).

Intravenous and oral administration of other classical cannabinoids, including Δ^8-THC, cannabinol and nabilone, has been shown to reduce IOP in laboratory animals as well as in humans (Merritt et al. 1981; ElSohly et al. 1981; 1984). Δ^8-THC induced significant IOP reduction when injected in rabbits, but has slightly less activity than Δ^9-
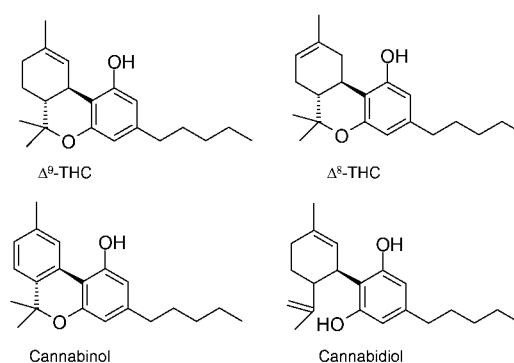
Fig. 17. Chemical structures of the four major cannabinoids in marijuana, Δ^9-THC, Δ^8-THC, cannabinol and cannabidiol.

THC within the same dosage range (ElSohly et al. 1981; 1984). At 10 mg/kg (i.v), cannabinol has been reported to lower IOP in normotensive albino rabbits at almost similar potency to 1.5 mg/kg Δ^9-THC, whereas cannabidiol has been reported to be inactive (ElSohly et al. 1981). Nabilone (0.25 – 0.5 mg/kg), a synthetic derivative of Δ^9-THC, was efficient at reducing IOP, but unfortunately, it was accompanied with adverse CNS effects, such as increased motor activity and vocalization in rabbits (ElSohly et al. 1981).

Variable results have been obtained from the IOP reducing properties of classical cannabinoids after their topical administration. For example, topically administered Δ^9-THC (1 %) failed to decrease IOP of normotensive rabbits when given in a light mineral oil, sesame seed oil or DMSO vehicles (ElSohly et al. 1981; 1984). Furthermore, the ocular administration of 0.05 - 0.1 % Δ^9-THC dissolved in light mineral oil has been ineffective in lowering the IOP of glaucoma patients (Merritt et al. 1981). In contrast, Δ^8-THC (0.4 %) induced an intense and long-lasting (> 8 hr) IOP reduction when administered topically as a submicron aqueous emulsion in hypertensive rabbits, but this effect has been less evident in normotensive rabbits (Muchtar et al. 1992). Δ^8-THC has been ineffective in reducing IOP when given topically in a light mineral oil vehicle in normotensive rabbits (ElSohly et al. 1984), suggesting that the choice of ocular vehicle may be crucial for the ocular activity of these very lipophilic cannabinoids.

2.4.3.3 Non-classical cannabinoids

Non-classical cannabinoids are typically bicyclic and tricyclic analogs of Δ^9-THC that lack a pyran ring. A particularly important member of this cannabinoid group is CP55,940 (**Fig. 18**) that is widely used as a radiolabelled probe in cannabinoid receptor-binding assays (Pertwee 1999).

The IOP reducing capacity of CP55,940 has been investigated by Pate and co-workers (1998) using normotensive pigmented rabbits as an animal model. After the topical administration of CP55,940 (dose = 25 μg) in a hydroxypropyl-β-cyclodextrin (HP-β-CD) vehicle, a clear IOP reduction (approximately − 4 mm Hg) was observed in the treated, but not in the untreated, eyes of rabbits. This hypotensive effect of CP55,940 was reversed by pre-treatment with subcutaneously injected CB1 receptor antagonist SR141716A (dose = 6 mg), suggesting that the ocular CB1 cannabinoid receptors may be involved in the IOP lowering action of CP55,940 (Pate et al. 1998).
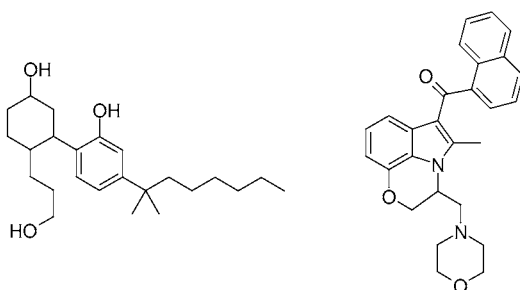
Fig. 18. Chemical structures of CP55,940 (left) and WIN55,212-2 (right). These prototype compounds belong to the groups of non-classical cannabinoids and aminoalkylindoles, respectively.

2.4.3.4 Aminoalkylindoles

Aminoalkylindoles are a class of cannabimimetic agents with a chemical structure that is completely different from those of natural cannabinoids. A prototypical compound of this class is WIN55,212-2 (**Fig. 18**), which has been shown to bind to both CB1 and CB2 receptors and exhibit cannabinoid-like activity *in vivo*.

Hodges et al. (1997) reported that the systemic administration of WIN55,212-2 (3 mg/kg, i.v.) gave only a marginal IOP reduction compared to the vehicle treatment in normotensive rabbits. In contrast, Song and Slowey (2000) investigated the IOP effects

of topically administered WIN55,212-2 in normotensive albino rabbits and observed a significant time- and dose-dependent IOP reduction in the treated eyes of rabbits after topical administration of WIN55,212-2 (doses = 20 – 100 µg) compared to the hydroxypropyl-β-cyclodextrin (HP-β-CD) vehicle. Pre-treatment with topical CB1 receptor antagonist SR141716A (25 µg) significantly attenuated the IOP reductive effects of WIN55,212-2, suggesting the involvement of CB1 receptors in the IOP lowering effects of WIN 55,212-2 (Song and Slowey 2000). Furthermore, topically administered WIN55,212-2 in aqueous 45 % HP-β-CD formulation has been shown to decrease IOP in patients with bilateral glaucoma and a history of multiple topical medical therapy (Porcella et al. 2001). The maximal IOP reductions observed after 60 min from WIN55,212-2 administration were 20 % and 31 % below the baseline values at doses of 25 and 50 µg, respectively (Porcella et al. 2001).

Recently, the IOP lowering activity of WIN55,212-2 has been confirmed in normal and glaucomatous monkeys (Chien et al. 2003). Single doses of WIN55,212-2, dissolved in 45 % HP-β-CD, reduced IOP in a time- and dose-dependent manner. The lowered IOP was observed for 4 - 6 hr after WIN55,212-2 administration and the maximal IOP reductions were 1.4, 2.9 and 3.4 mm Hg at the WIN55,212-2 concentrations of 0.02 %, 0.2 % and 0.5 %, respectively, in treated normal monkey eyes. In glaucomatous monkeys, the twice-daily administration of 0.5 % WIN55,212-2 that was continued for 5 days reduced IOP up to 3.5 mm Hg on day 1, 5.9 mm Hg on day 3 and 8.3 mm Hg on day 5, respectively, compared to baseline values. The topical administration of WIN55,212-2 was observed to reduce aqueous humor flow in normal monkey eyes suggesting, that its IOP lowering activity may be mediated at least partly through this mechanism (Chien et al. 2003). Topically administered aminoalkylindole WIN55,212-3, the enantiomer of WIN55,212-2, has been shown to be inactive in reducing IOP in rabbits (Song and Slowey 2000).

2.4.3.5 Eicosanoids

Eicosanoids include derivatives of arachidonic acid and typical examples of this class are the endogenous AEA and 2-AG. Thus far, all naturally occurring cannabimimetic fatty acid amide derivatives belong to this class of cannabinoids (Pertwee 1999).

Pate et al. (1995) first observed that the topical administration of AEA in hydroxypropyl-β-cyclodextrin (HP-β-CD) vehicle induced a dose-dependent bi-phasic IOP profile; i.e., an initial increase followed by IOP reduction in the treated eyes of normotensive rabbits. The IOP lowering activity of AEA was subsequently confirmed

R-methyl-AEA   R-isopropyl-AEA
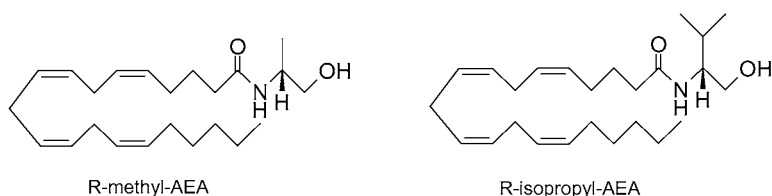
Fig. 19. Structures of R-α-methyl-anandamide and R-α-isopropyl-anandamide.

by Mikawa et al. (1997) in rabbits using light mineral oil as an ocular vehicle. Topical administration of other unsubstituted AEA analogs (Pate et al. 1996) and 2-AG (Pate et al. 1997) in the hydroxypropyl-β-cyclodextrin (HP-β-CD) vehicle induced similar bi-phasic IOP profiles as AEA in normotensive rabbits. In contrast, the metabolically more stabile α-substituted AEA analogs, such as R-α-isopropylanandamide and R-α-methanandamide (**Fig. 19**), immediately reduced IOP without the initial IOP elevation (Pate et al. 1997). Pre-treatment with the cyclooxygenase inhibitor indomethacin eliminated the IOP responses of arachidonic acid, AEA and 2-AG, but had no effect on the IOP effects of the more stabile R-α-methanandamide, indicating that these endocannabinoids act via a separate mechanisms in normotensive rabbits (Pate et al. 1996; Pate et al. 1997). The CB1 receptor antagonist SR141716A antagonized the IOP lowering effects of metabolically more stabile R-α-isopropylanandamide, but the IOP profile of AEA was unaffected by SR141716A pre-treatment (Pate et al. 1998).

2.4.3.6 Related compounds

The CB1 receptor antagonist SR141716A (**Fig. 20**) has been shown to increase IOP when given as a subcutaneous injection (dose = 6 mg) (Pate et al. 1998) or topically in hydroxypropyl-β-cyclodextrin (HP-β-CD) vehicle (dose = 25 μg) (Song and Slowey 2000) in normotensive rabbits, suggesting that SR141716A may either act as a inverse agonist at ocular CB1 receptors or block the tonic regulative effects of endogenous cannabinoids on ocular tension by preventing the endogenous agonist action.

An intravenous injection of the non-psychoactive synthetic cannabinoid-like compound HU-211 (dexanabinol, doses = 0.025-0.5 mg/kg) (**Fig. 20**) has been shown to induce a significant dose-related reduction of IOP in normotensive rabbits (Beilin et al. 2000). An adrenergic system was suggested to be associated with the ocular activity of HU-211 after its systemic administration (Beilin et al. 2000). Topical administration of HU-211 (0.12 % w/w) in a submicron emulsion resulted in a 6 hr IOP reduction in the treated eyes of normotensive rabbits (Naveh et al. 2000). A minor but still statistically

significant elevation of IOP was also observed in contralateral eyes (Naveh et al. 2000). HU-211 has practically no cannabimetic activity, but exhibits a neuroprotective NMDA -antagonist activity (Feigenbaum et al. 1989; Howlett et al. 1990).

Buchwald et al. (2000; 2002) attempted to develop a series of topical nitrogen-containing cannabinoid analogues that had local anti-glaucoma effect but no systemic effects by using a retrometabolic/soft-drug approach. One of their cannabinoid soft-drug analogues (**Fig. 20**) induced a short-lasting IOP reduction with a maximum decrease of 18 % after its systemic administration (1 mg/kg, i.v.) in rabbits. According to this soft-drug approach, its main metabolite demonstrated essentially no ocular activity after its i.v. administration. A minor but longer lasting IOP lowering effect was achieved in rabbits after a single-dose topical administration of some of these soft-drug derivatives (1 %) in emulphor. Their main metabolites were again found to be inactive after topical administration. Soft-drug technology may be one opportunity to reduce unwanted CNS side effects of cannabinoid antiglaucoma therapeutics (Buchwald et al. 2000; 2002).

Bimatoprost (Lumigan®) (**Fig. 20**) is a highly potent novel ocular hypotensive agent with long lasting IOP lowering activity (Brubaker 2001; Woodward et al. 2001). It is chemically related to prostamide $F_{2\alpha}$, which is a newly discovered fatty acid amide. Prostamide $F_{2\alpha}$ is suggested to be biosynthesised from AEA through a COX-2 catalyzed enzymatic conversion. Since bimatoprost has no demonstrated activity on cannabinoid CB1 and CB2 receptors, it is classified as prostaglandin analog. Bimatoprost is currently approved in clinical use in Finland (Lääketietokeskus Oy 2003).


SR141716A

Soft-drug analogue

HU-211

Bimatoprost
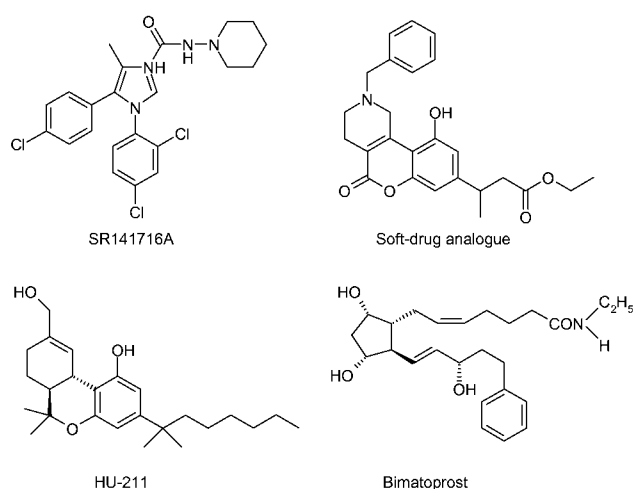
Fig. 20. Chemical structures of related compounds that have IOP reducing activity.

2.4.4 Cyclodextrins in topical delivery of drugs

Ophthalmic medications are administered either systematically or locally to the eye. In order to treat ocular disorders, the topical delivery of eye drops into the lower cul-de-sac of the eye is preferred, as the drug effects are localized and less drug gains entry into the systemic circulation. In addition, with systemic administration the adequate ocular drug concentrations may be difficult to achieve, as blood-aqueous and blood-retinal barriers effectively restrict access of drugs from systemic circulation to the intraocular targets. Indeed, lower drug doses that can be used with topical administration reduce the risk for systemic adverse effects. Furthermore, topical ocular administration is a relatively simple and painless method of drug administration.

Ophthalmic drugs are most commonly formulated as topically applied aqueous eye drop solutions. Other topical drug formulations, such as suspensions, ointments and solid ocular inserts have been used, but these may provoke blurred vision, ocular irritation and other side effects. In order to reduce IOP, antiglaucoma drugs must be absorbed from the pre-ocular region into the inner eye, where the target tissues are generally located. However, the ocular bioavailability of topically applied drugs is unfortunately very low; typically only 1 - 10 % of administered dose (Lee 1993). The main reasons for the low ocular bioavailability are poor drug penetration through corneal barrier and rapid loss of instilled solution from precorneal area (Lee and Robinson 1979).

The most important features affecting corneal penetration of ophthalmic drugs seem to be the lipophilicity and water solubility of a drug molecule. Indeed, the optimal partition coefficient (log P_{app} in pH 7.4) for drug penetration across the cornea has reported to be between 2 – 3 in logarithmic value, indicating that the absorption of moderately lipophilic compounds is favoured (Shoenwald and Huang 1983). Furthermore, the aqueous solubility of a potential ophthalmic drug has to be high enough to enable both the formulation of aqueous eye drop solutions and dissolution of the drug molecule into the tear film (Loftsson and Stefansson 1997). Due to the fact that only a few of the potential ophthalmic molecules fulfil this criteria of being simultaneously water and lipid soluble, various drug delivery techniques, including cyclodextrins, have been developed to increase the topical bioavailability of ophthalmic drugs (e.g. Järvinen and Järvinen 1996; Bourlais et al. 1998; Loftsson and Järvinen 1999; Sasaki et al. 1999; Davies 2000) (Table 5).

Table 5: Examples from the different strategies that are used to increase bioavailability of the ophthalmic drugs.

OPHTHALMIC DRUG DELIVERY STRATEGIES		
Chemical technologies	**Formulation strategies**	**Physical approach**
Prodrug technology	Penetration enhancers	Iontophoresis
Cyclodextrin technology	- Surfactants	Phonophoresis
	- Bile salts	
	- Fatty acids	
	- Chelating agents	
	Emulsifiers / liposomes	
	Suspensions	
	Bioadhesive hydrogels	
	Ocular inserts	

Cyclodextrins are a group of cyclic oligosaccharides that consist of α-D-glucopyranose subunits, joined together with α-1,4-bonds (Frank 1975). The cyclodextrin molecules are shaped as a truncated cone with a somewhat lipophilic central cavity and a hydrophilic outer surface (**Fig 22**). The most important feature of cyclodextrins is their ability to act as a "host" to form non-covalent inclusion complexes with hydrophobic "guest" molecules in an aqueous environment. These inclusion complexes lead to the altered physical, chemical and biological properties that differ from those of either the parent molecule or cyclodextrin, and can be used to increase the aqueous solubility and dissolution rate, decrease volatility, alter release rates, mask an unpleasant odour or taste, decrease local irritation and increase the stability of drugs (Uekama and Otagiri 1986; Rajewski and Stella 1996).

R = H => beta-cyclodextrin
R ≠ H => beta-cyclodextrin derivative

(A)   (B)
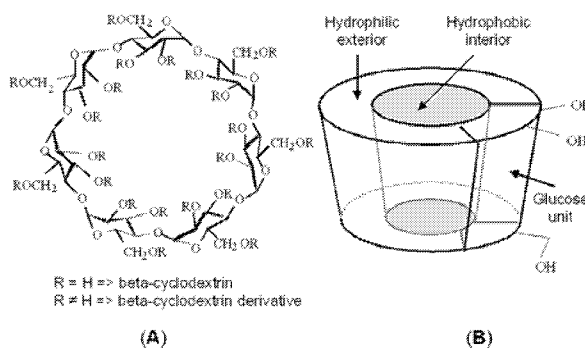
Fig. 22. Chemical structure (A) and conical shape (B) of β-cyclodextrin. All hydroxyl groups of the glucose units are located in the hydrophilic exterior of the cyclodextrin molecule.

The natural cyclodextrins (CD's) include α-CD, β-CD and γ-CD, which contain six, seven and eight glucose subunits, respectively. Each of these parent cyclodextrins exhibits various inclusion capabilities that result from differences in their central cavity dimensions (Uekama and Otagiri 1986; Loftsson and Brewster 1996). In order to increase the aqueous solubility and safety profile of natural cyclodextrins, several cyclodextrin derivatives have been developed, of which the randomly methylated derivatives, 2-hydroxypropyl derivatives and sulfobutylether derivatives of β-CD may have commercial pharmaceutical value (Thompson 1997; Szente and Szejtli 1999).

Cyclodextrins have been generally used to increase the aqueous solubility, aqueous stability and bioavailability of drugs in ophthalmic formulations (e.g. reviewed by Rajewski and Stella 1996; Loftsson and Stefansson 1997; Loftsson and Järvinen 1999; Järvinen et al. 2002). Cyclodextrins may act as a carrier for lipophilic drug molecules by maintaining them in solution and delivering the molecules to the surface of the cornea, where they are able to permeate across the membrane. However, the ophthalmic administration of cyclodextrins exhibits some essential differences to other drug delivery routes (Rajewski and Stella 1996). It is generally assumed that only the free drug, not the drug/cyclodextrin complex or free cyclodextrin may penetrate through biological membranes, including cornea. Thus, the drug has to be released from the complex before its absorption (Uekama and Otagiri 1986; Frijlink et al. 1990) (**Fig. 23**).
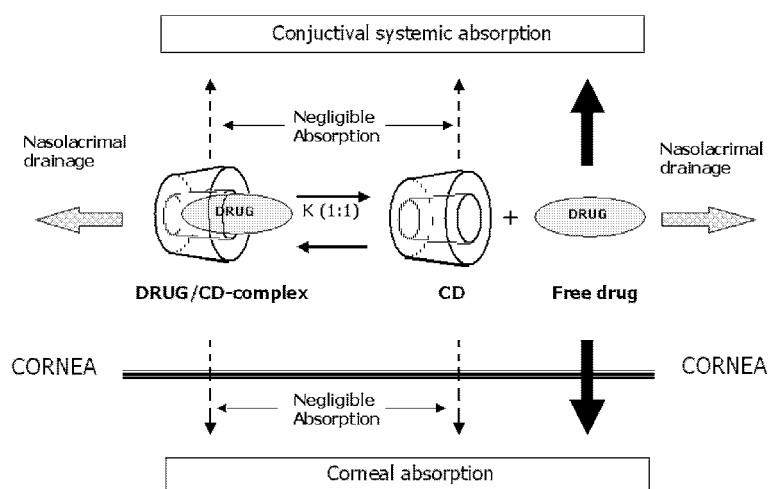
Fig. 23. The basic kinetics for ocular absorption with drug-cyclodextrin complexes (Modified from Järvinen et al. 2002).

In contrast to oral and parenteral routes, ophthalmic administration does not provide a significant increase in the fraction of free drug available after application of eye drops, as the small tear fluid volume (approximately 7 µl) hinders the drug/CD-complex dilution. Furthermore, the instilled eye drops are rapidly cleared from the precorneal area (Lee and Robinson 1979), and this limited time may be too short for the effective release of the drug dose from the drug/CD complex. Additionally, excessive complexation may decrease the ocular bioavailability of drugs by decreasing their free fraction, although this drawback may be circumvented by increasing the viscosity of an eye drop solution that prolongs the precorneal residence time of the drug (Jarho et al. 1996; Bary et al. 2000).

3 STUDY OBJECTIVES

The general objective of the present study was to evaluate the IOP effects of endogenous cannabinoids, their uptake and enzyme hydrolysis inhibitors in normotensive rabbits to obtain preliminary data for the possible utilization of the ocular endocannabinoid system in the development of novel IOP lowering agents for the treatment of IOP hypertension of glaucoma. The specific aims of this study were as follows.

1. To investigate whether the inhibition of AEA cellular uptake the eye affects IOP.

2. To investigate whether the inhibition of the FAAH, a main enzyme responsible for endocannabinoid AEA degradation, affects IOP.

3. To evaluate the *in vitro* stability of endocannabinoids in isolated bovine ocular tissue homogenates.

4. To investigate the IOP effects of topically administered noladin ether, a novel putative endocannabinoid.

5. To investigate the possible mechanisms of action by which endocannabinoids and their analogs reduce IOP.

6. To investigate whether the topically administered CB2 receptor specific agonist JWH-133 affects IOP.

4 MATERIALS AND METHODS
4.1 CHEMICALS (I-IV)

Endocannabinoid N-arachidoylethanolamide (AEA) (M.W. = 347.54 g/mol) was purchased either from Organix Inc. (Woburn, MA, USA) or from Deva Biotech Inc. (Hatboro, PA, USA), and 2-arachidonylglycerol (2-AG) (M.W = 378.6 g/mol) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Arachidonyl propionitrileamide (APN) (M.W. = 356.52 g/mol), the synthetic analogue of AEA, was purchased from Deva Biotech Inc. (Hatboro, PA, USA) and noladin ether (2-arachidonyl glycerol ether, HU-310) (M.W. = 364.57 g/mol) was synthesised by a method described earlier (Hanus et al. 2001). Arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI, USA).

The FAAH enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF) was obtained from Aldrich Chemie (Steinheim, Germany). Of the AEA uptake inhibitors, AM404 [N-(4-hydroxyphenyl)-arachidonylamide] (M.W. = 395.58 g/mol) was obtained either from Cayman Chemical (Ann Arbor, MI, USA) or from Deva Biotech Inc. (Hatboro, PA, USA), and olvanil [(N-vanillyl)-9-oleamide] (M.W. = 417.63 g/mol) was purchased from Tocris Cookson Ltd. (Bristol, UK). The cyclooxygenase inhibitor indomethacin was purchased from Sigma Chemical Co. (St. Louis. MO, USA).

The CB2 receptor agonist JWH-133 [3-(1',1'-dimethylbutyl)-1-deoxy-Δ^8-THC] (M.W. = 312.49 g/mol) was purchased from Tocris Cookson Ltd. (Bristol, UK), and the non-selective cannabinoid CP55,940 [(-)-5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)-cyclo-hexyl]-phenol] (M.W. = 376.58 g/mol) was kindly donated by Pfizer Inc. (Groton, CT, USA). Of the CB1 receptor antagonists, SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] was kindly provided by Sanofi Research (Montepellier, France) and AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (M.W. = 555.24 g/mol) was obtained from Tocris Cookson (Bristol, UK).

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, Encapsin®, average molecular weight = 1378.9 g/mol, average degree of substitution 0.6) was obtained from Janssen Biotech (Olen, Belgium), glycerol formal from Sigma Chemical Co. (St. Louis, MO, USA), poly(vinyl alcohol) (PVA; 98 - 99 % hydrolysed, molecular weight 124000 – 186000 g/mol) from Aldrich Chemical Company (Milwaukee, WIS, USA) and propylene glycol from Oriola Oy (Espoo, Finland). Isotonic 0.9 % sodium chloride solution and oxybuprocaine (Oftan Obucain 4 mg/ml) were purchased from Orion Pharma (Espoo,

Finland). A detailed description of the chemicals used for the $[^{35}S]GTP\gamma S$ autoradiography is listed in the original publication (**II**).

4.2 ANIMALS (I-IV)

Several animal models, such as chick, mouse, rat, rabbit, cat, dog and monkey with either normal or elevated IOP, have been developed for glaucoma drug research (Gelatt 1977). Rabbit is the most frequently used animal model in ocular drug reseach, as it is easy to maintain in a laboratory environment and is easy to handle (Gelatt 1977). Furthermore, the anatomical and physiological similarities and differences between human and rabbit eye have been extensively reported. The small size of rodent eyes has limited their usefulness in IOP studies (Gelatt 1977; Urtti and Salminen 1993).

Adult normotensive Dutch rabbits (range 2.3 – 4.0 kg) (**I-IV**) or adult New Zealand White albino rabbits (range 3.0 - 4.0 kg) (**III**) of either gender were selected as an animal model for the *in vivo* IOP experiments. The rabbits were housed singly in cages under controlled illumination (12-hour dark/12-hour light cycle) and environmental conditions (temperature $20.0 \pm 0.5\,°C$, 55 – 75 % relative humidity). Water and standard rabbit chow were available *ad libitum*, except during the tests. All rabbits were treated in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

4.3 EYE DROP SOLUTIONS (I-IV)

The topical eye drop solutions used in this study are summarized in **Table 6**. Because of the low aqueous solubility of test compounds, the eye drop solutions were formulated in either aqueous HP-β-CD solutions or in the more viscous propylene glycol. When preparing eye drop solutions containing HP-β-CD, an ethanol solution of the test compounds were evaporated under a nitrogen stream at room temperature and re-dissolved in an aqueous HP-β-CD solution, or the solid test compounds were dissolved directly into the HP-β-CD solution at room temperature. The concentration of HP-β-CD in eye drop solutions was kept as low as possible to ensure the required aqueous solubility for test compounds. The pH of eye drop solutions was adjusted to 7.4 with dilute sodium hydroxide or hydrochloric acid solutions, and the solutions were made isotonic with sodium chloride. The eye drop solutions were stored at fridge (+ 4 $°C$) and preserved from light until used. Drug concentrations in eye drop solutions were verified by high-performance liquid chromatography (HPLC) before experiments. Drug-free vehicles were used as ocular control treatments in all studies.

Table 6: Composition of prepared eye drop solutions.

DRUG	VEHICLE	DRUG CONCENTRATION	DRUG DOSE	DRUG:CD RATIO (mol:mol)
AEA (I, II)	10 % HP-β-CD (72.52 mM)	2.5 mg/ml (7.19 mM)	62.5 μg (179.84 nmol)	1 : 10
2-AG (III)	12.5% HP-β-CD (90.65 mM)	1.25 mg/ml (3.30 mM)	31.25 μg (82.48 nmol)	1 : 27
	25 % HP-β-CD (181.30 mM)	2.5 mg/ml (6.60 mM)	62.5 μg (165.08 nmol)	1 : 27
	Propylene glycol	0.3125 mg/ml (0.83 mM)	7.81 μg (20.64 nmol)	---
	Propylene glycol	1.25 mg/ml (3.30 mM)	31.25 μg (83.54 nmol)	---
	Propylene glycol	5 mg/ml (13.21 mM)	125 μg (330.16 nmol)	---
Noladin ether (III)	6.25% HP-β-CD (45.33 mM)	0.625 mg/ml (1.71 mM)	15.63 μg (42.86 nmol)	1 : 27
	25 % HP-β-CD (181.30 mM)	2.5 mg/ml (6.86 mM)	62.5 μg (171.43 nmol)	1 : 26
	Propylene glycol	5 mg/ml (13.71 mM)	125 μg (342.87 nmol)	---
APN (I)	15 % HP-β-CD (108.78 mM)	2.5 mg/ml (7.01 mM)	62.5 μg (175.31 nmol)	1 : 16
AM404 (II)	25 % HP-β-CD (181.30 mM)	2.5 mg/ml (6.32 mM)	62.5 μg (158.00 nmol)	1 : 29
	Propylene glycol	2.5 mg/ml (6.32 mM)	62.5 μg (158.00 nmol)	---
Olvanil (II)	Propylene glycol	2.5 mg/ml (5.99 mM)	62.5 μg (149.65 nmol)	---
	Propylene glycol	12.5 mg/ml (29.93 mM)	312.5 μg (748.27 nmol)	---
AM251 (III)	45 % HP-β-CD (326.35 mM)	0.5 mg/ml (0.90 mM)	12.5 μg (22.51 nmol)	1 : 363
CP55,940 (IV)	5 % HP-β-CD[A] (36.26 mM)	1.0 mg/ml (2.66 mM)	25 μg (66.44 nmol)	1 : 14
	10% HP-β-CD[A] (72.52 mM)	2.5 mg/ml (6.64 mM)	62.5 μg (165.97 nmol)	1 : 11
	Propylene glycol	2.5 mg/ml (6.64 mM)	62.5 μg (165.97 nmol)	---
JWH-133 (IV)	45 % HP-β-CD (326.35 mM)	0.4 mg/ml (1.28 mM)	10 μg (32.00 nmol)	1 : 255
	45 % HP-β-CD (326.35 mM)	1.0 mg/ml (3.20 mM)	25 μg (80.00 nmol)	1 : 102
	Propylene glycol	2.5 mg/ml (8.00 mM)	62.5 μg (200.03 nmol)	---

[A] The vehicle contained additionally 3 % PVA to increase viscosity of the eye drop solution in order to increase the pre-corneal residence time of the instilled eye drop. Increased residence time provides more time for the drug-CD complex to dissociate before clearance (Jarho et al. 1996).

4.4 INJECTABLE SOLUTIONS (I, II)

The FAAH inhibitor PMSF was dissolved in glycerol formal to concentrations of 3, 30 and 150 mg/ml and administered as a subcutaneous injection to rabbits (0.2 - 0.25 ml/rabbit), because of its low solubility in the ophthalmic vehicle (I, II).

The cyclooxygenase inhibitor indomethacin was dissolved in an aqueous 20 % HP-β-CD solution to a concentration of 5 mg/ml. The pH was adjusted at 7.4 and the solution was made isotonic with sodium chloride. Indomethacin (12.5 mg/rabbit) was administered to rabbits by subcutaneous injection (I).

A CB1 receptor antagonist SR141716A was prepared by dissolving the compound in a 45 % HP-β-CD solution of phosphate buffer at pH 4.2. Each rabbit in the CB1 antagonism study received 4.8 mg of SR141716A by subcutaneous injection (I). Subcutaneous pre-treatments of isotonic NaCl were used as negative injection controls. All subcutaneous pre-treatments were given approximately 30 min before topical ocular treatments.

4.5 MEASUREMENT OF IOP (I-IV)

To perform each experiment, the rabbits were placed in plastic restraining boxes located in a quiet room. A single drop (25 – 50 µl) of the test solution or eye drop vehicle was instilled unilaterally into the left eyes of rabbits. The contralateral eyes were left untreated. During the eye drop instillation, the upper eyelid was gently pulled away from the ocular globe. The IOPs were measured using a Digilab Modular One pneumatonometer (Bio-Rad, Cambridge, MA) (I-IV) or Mentor Tono-Pen XL tonometer (Norwell, MA) (III). One or two drops of oxybuprocaine (0.06 %) were applied to the cornea to reduce discomfort before each measurement. At least two readings were taken from treated (ipsilateral) and untreated (contralateral) eye for each determination, and the mean of these readings was used. IOP's of rabbits were measured 2 or 1 hours before, at 0 hour and at 0.5, 1, 2, 3, 4 and 5 hours after eye drop administration. IOP at the time of eye drop application (0 hour) was used as a baseline value. All studies were conducted at comparable times of day to eliminate possible circadian effects. The studies were set up using a non-blind randomized cross-over design. At least 72 hours of washout time was allowed for each rabbit between doses. The experiments were approved by the Ethics Committees of University of Kuopio (I-IV) and by Hebrew University (data with albino rabbits in original publication III), and were conducted in accordance with institutional regulations.

4.6 ENZYMATIC HYDROLYSIS EXPERIMENTS (III)
4.6.1 Eye homogenate preparation (III)

In order to determinate the rates for enzymatic degradation of endocannabinoids in ocular tissues, 20 % (w/v) bovine iris-ciliary body and cornea homogenates were prepared. To prepare homogenates, approximately twenty bovine eyes, obtained from the local slaughterhouse (Atria Oy, Kuopio, Finland), were immediately rinsed with ice-cold 0.9 % NaCl solution and the corneas were dissected within 3 h of slaughter. The iris-ciliary bodies were detached from the eye as a single tissue sample. The removed eye tissues were cut into smaller pieces, weighed into separate centrifuge tubes and homogenized with four volume of ice-cold isotonic 50 mM phosphate buffer, pH 7.4, using an X-1020 homogeniser (Ystral, Germany). The crude homogenates were centrifuged for 90 min at 9000 g at 4 °C with a Sorvall RC-26 Plus (DuPoint, Newtown, CT) centrifuge. Pellets were discharged and supernatants were transferred to a –80 °C freezer until use. The protein concentrations were determined using the Bradford

method (Bradford 1976), and were 7.1 mg/ml for cornea and 7.3 mg/ml for iris-ciliary body homogenates.

4.6.2 Degradation studies (III)

The degradation rates for AEA, 2-AG and noladin ether were determined in 10 % bovine cornea and iris-ciliary body homogenates. Endogenous cannabinoids were dissolved in one volume (e.g. 0.5 mL) of 50 mM isotonic phosphate buffer pH 7.4 (μ = 0.15) containing 0.1 % ethanol. Initial concentrations were 0.7 mM for AEA and 2-AG, and 0.1 mM for noladin ether, respectively. One volume (e.g. 0.5 mL) of preheated cornea or iris-ciliary body homogenate was added and the solution was incubated at +37 °C in a water path. At pre-determined time intervals, 200-μl aliquots were taken and mixed with 400 μl of ice-cold acetonitrile in order to terminate the enzymatic reaction. After 5 min of centrifugation at 16000 g, the clear supernatant was assayed for remaining amounts of endogenous cannabinoid by analytical HPLC.

4.7 DRUG ANALYSES (I-IV)

The high-performance liquid chromatography (HPLC) analyses were performed with a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-7100 pump, a L-7000 interface module, a L-7455 diode array detector (200 - 400 nm, set at 211 nm) and a L-7250 programmable autosampler. A Purospher RP-18 end-capped reversed-phase column (125 x 4 mm, 5 μm, Merck kGaA, Darmstadt, Germany), protected with a Purospher RP-18 end-capped reversed-phase guard column (4 x 4 mm, 5 μm, Merck kGaA, Darmstadt, Germany) was used for chromatographic separations. The chromatographic conditions were as follows:

Method 1 (I): The mobile phase consisted of acetonitrile and monobasic potassium phosphate buffer (20 mM KH_2PO_4, pH 7.0) at flow rate of 1.2 mL/min. The proportion of acetonitrile in the mobile phase was increased from 60 % to 95 % by a linear ramp over 15 min after injection, then maintained for a 5 min plateau and subsequently returned to the initial conditions over the course of 8 min. Method 1 was used for the determination of AM404, olvanil and AEA concentrations in eye drop solutions.

Method 2 (II, III): Similar to method 1, except the proportion of acetonitrile in the mobile phase was increased from 60 % to 90 % over 15 min, maintained for a 5 min plateau and returned to initial conditions over the course of 6 min. Method 2 was used for the evaluation of AEA, APN, 2-AG and noladin ether concentrations in eye drop solutions.

Method 3 (III): Isocratic elutions for the hydrolysis experiments were performed with a mobile phase mixture of 18 % phosphate buffer (20 mM KH_2PO_4, pH 3.0) in acetonitrile at a flow rate of 1.2 mL/min to detect the hydrolysis rates of AEA, 2-AG and noladin ether in bovine ocular tissues.

Method 4 (IV): Similar to method 3, except a mobile phase mixture of 25 % monobasic potassium phosphate buffer (20 mM KH_2PO_4, pH 2.5) in acetonitrile was used to analyse the concentration of CP55,940 in eye drop solutions.

Method 5 (IV): Similar to method 1, except the proportion of acetonitrile in the mobile phase was increased from 85 % to 95 % over 5 min, maintained for a 20 min plateau and returned to initial conditions over the course of 10 min. Method 4 was used for the determination of JWH-133 concentrations in eye drop solutions.

4.8 [^{35}S]GTPγS AUTORADIOGRAPHY (II)

[^{35}S]GTPγS Autoradiography was performed in the Department of Physiology, University of Kuopio, in order to confirm that the endogenous cannabinoid uptake inhibitors, AM404 and olvanil, do not activate the CB1 cannabinoid receptor. The [^{35}S]GTPγS autoradiography was conducted under optimised assay conditions, where the tonic adenosine A_1 receptor signal has been eliminated. The [^{35}S]GTPγS autoradiography used 20 μm-thick brain sections of 4-week-old male Wistar rats. Two coronal and two sagittal brain sections were used for one slide, each obtained from an individual animal. Briefly, the slides were pre-incubated for 20 min at 20 °C in buffer A, which contained 50 mM tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl and 5 M $MgCl_2$, followed by GDP loading for 1h at 20 °C in the buffer A additionally containing 2 mM GDP and 1 μM DPCPX. For [^{35}S]GTPγS binding, brain sections were incubated for 90 min at 20 °C in buffer A with added 0.3 - 0.5 nM [^{35}S]GTPγS, 2 mM GDP, 1 μM DPCPX, 1 mM DTT and cannabinoid ligands in concentrations of 10^{-4} M or 10^{-5} M, dissolved in 0.1 or 0.2 % (w/v) fatty-acid free BSA. Non-specific binding was measured in the presence of 10 μM [^{35}S]GTPγS. The brain sections were washed twice at 0 °C with washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) for 5 min each time, rinsed in ice-cold de-ionized water for 30 sec, air-dried and exposed to Hyperfilm^{TM}-βmax (Amersham) for 5 days, together with autoradiographic [^{14}C] microscale standards (Amersham). Films were developed in a Kodak D-19 developer for 4 min at 4°C.

4.9 STATISTICAL ANALYSIS OF DATA (I-IV)

The IOP data are presented as a change in IOP (% of baseline) mean ± SE (standard error n = 5-6). A one-factor analysis of variance (ANOVA) for repeated measurements, followed by Fisher's protected least significant difference (PLSD) method (**I-II, IV**), or a 2-way ANOVA for repeated measures with Bonferroni correction (**III**) were used to compare treated and untreated groups. Significance in the differences between the two treatments was tested by a 2-tailed paired Student's *t*-test (**I-II**). A value of $p < 0.05$ was considered statistically significant.

5. RESULTS AND DISCUSSION
5.1 IOP EFFECTS OF DRUG-FREE VEHICLES (I-IV)

The highly lipophilic nature of cannabinoids and cannabinoid-like compounds has hindered their ocular delivery. Typically, different approaches, such as non-aqueous solvents, oils and emulsifiers, have been used to overcome their poor aqueous solubility and dissolution properties, and to provide eye drop formulations (ElSohly et al. 1981; Muchtar et al. 1992; Naveh et al. 2000). However, non-aqueous solvents and solvents with surfactant properties tend to be uncomfortable, irritable and exhibit corneal disrupting properties (Sasaki et al. 1999).

In this study, eye drop solutions were prepared with the use of aqueous HP-β-CD, in varying concentrations (5 - 45 %), and propylene glycol, which has a more lipophilic nature. Aqueous HP-β-CD was selected as a primary ocular vehicle; however, if the required drug solubility could not be achieved with HP-β-CD then propylene glycol was used (I-IV). Previously, cyclodextrins (particularly HP-β-CD) have been successfully employed as ocular excipients to obtain aqueous eye drop solutions from highly lipophilic drugs, including cannabinoids (Loftsson and Järvinen 1999; Järvinen et al. 2002). Complexation of AEA with HP-β-CD has been shown to markedly increase its solubility and stability in aqueous environment (Jarho et al. 1996). On the other hand, a high excess of cyclodextrin may decrease the corneal penetration of drugs due to increased complexation (Jarho et al. 1996; Bary et al. 2000). Thus, in order to maximize the ocular bioavailability of topically applied drugs, the amount of cyclodextrin should be optimised so that only the drug that is excessive to its own aqueous solubility is complexed (Bary et al. 2000).

The drug-free vehicles were always used as negative controls in the IOP studies of cannabinoid drugs, and were administered unilaterally to the rabbits. The results clearly show that HP-β-CD had no significant effect on the IOP of treated or untreated eyes in rabbits when administered alone, even at the high concentrations (up to 45 %) (I-IV). This observation is consistent with earlier reports (Pate et al. 1995; 1998; Song and Slowey 2000). In addition, no visible signs of irritation or discomfort were observed in rabbits after administration of drug-free HP-β-CD vehicles (I-IV). In fact, the ocular tolerability of HP-β-CD is reported to be apparently high, as it does not damage corneal tissues (Jansen et al. 1990). In contrast, propylene glycol tended to slightly increase the IOP of treated and untreated eyes after its topical administration in rabbits (I – IV). As an ocular vehicle, propylene glycol is less ideal. Like other viscous drug delivery systems, it is most probably uncomfortable, and leads to blurred vision and ocular irritation (Loftsson and Stefansson 1997).

5.2 IOP EFFECTS OF ENDOCANNABINOIDS IN TREATED EYES (I-III)
5.2.1 IOP profiles of endocannabinoids (I-III)

The IOP profiles and maximal IOP reductions induced by endocannabinoids AEA, 2-AG and noladin ether, and the other cannabinoid agonists, APN and CP55,940, that were investigated during this study, are summarized in **Table 7**. The IOP profiles of endocannabinoids AEA, 2-AG and their synthetic analog APN were found to be biphasic; i.e., an initial IOP increase followed by IOP decrease was observed, as reported earlier by Pate et al. (1995; 1997). In contrast, the novel putative endocannabinoid noladin ether (Hanus et al. 2001) and the non-classical cannabinoid CP55,940 lowered IOP directly without the initial IOP elevation, suggesting that they may act via a separate mechanism on IOP reduction.

The mean maximal IOP lowering effects (% of the baseline) of AEA, 2-AG and noladin ether, as well as the other cannabinoid agonists, at the dosage of 62.5 µg in HP-β-CD vehicle varied from −10 % to −18 % (**Table 7**). The IOP reducing activity of AEA has been reported to be comparable with commercial timolol solution (Oftan Timolol® 5 mg/ml, dose = 125 µg) at a dosage of 31.25 µg in normotensive rabbits (Pate et al. 1995).

Table 7. IOP profiles and maximal IOP reductions of endocannabinoids and relative compounds at a dosage of 62.5 µg in HP-β-CD vehicle in the treated eyes of normotensive rabbits (K_i-values adapted from Showalter et al. 1996; Ben-Shabat et al. 1998; Hanus et al. 2001 and Felder et al. 1995).

DRUG (dose)	VEHICLE	MAXIMAL EFFECT (%)	TIME OF MAX. EFFECT	IOP PROFILE [A]	Ki (CB1)	K_i (CB2)
AEA (180 nmol)	10 % HP-β-CD	-18.4 ± 5.3*	3 h	↑↓	89 nM[C]	371 nM[C]
2-AG (165 nmol)	25 % HP-β-CD	-10.3 ± 3.0	3 h	↑↓	58 nM	145 nM
APN (175 nmol)	15 % HP-β-CD	-12.6 ± 3.8	2 h	↑↓	---	---
Noladin ether (171 nmol)	25 % HP-β-CD	-11.4 ± 1.9*	2 h	↓	21 nM	>3 µM
CP55,940 [B] (166 nmol)	10 % HP-β-CD	-15.2 ± 2.6*	2 h	↓	2.6 nM	3.7 nM

Maximal effects are presented as % of baseline, mean ± SE, n = 5-6.
[A] Indicated as an increase (↑) and/or decrease (↓) in IOP.
[B] The vehicle contained additionally 3 % PVA.
[C] In the presence of 50 µM of phenylmethylsulfonyl fluoride (PMSF)
* Statistically significant compared to vehicle treatment (two-way ANOVA with Bonferroni correction or Fisher's PLSD)

5.2.2 Endocannabinoid hydrolysis in bovine ocular tissues (III)

The hydrolysis rates for endocannabinoids were investigated in bovine ocular tissues in order to investigate how susceptible they were to ocular enzymatic activities. The half-lives for endocannabinoid hydrolysis in bovine cornea and iris-ciliary body homogenates are presented in **Table 8**. Noladin ether degraded at a significantly slower rate than AEA or 2-AG in both 10 % (w/w) bovine cornea and iris-ciliary body homogenates. The hydrolysis of endocannabinoids followed pseudo first-order kinetics in both homogenates (**III; Fig. 2**). The main metabolite of AEA and 2-AG eluted at the same retention time as a synthetic arachidonic acid standard, whereas no arachidonic acid was formed from noladin ether in either 10 % (w/w) bovine cornea or iris-ciliary body homogenates (**III; Fig. 3**).

Various esterases (Nakamura et al. 1993) and FAAH-like enzyme activities (Matsuda et al. 1997; Bisogno et al. 1999b) have been reported to be present in ocular tissues, whereas the relevant ocular enzymes for cleaving ether groups have not been identified so far. FAAH has been reported to hydrolyze 2-AG at a rate four times faster than AEA (Goparaju et al. 1998) and this may correspond to the higher hydrolysis rate observed for 2-AG in the present study. In addition, the ester structure of 2-AG may be a good substrate for other esterase activities in biological tissues. Fezza et al. (2002) have recently suggested a shared cellular uptake with 2-AG and AEA, following slow incorporation into phospholipids as an elimination mechanism for noladin ether in intact C6 glioma cells. The oxidation of *cis*-double bonds may provide other possible routes of metabolism for noladin ether.

Table 8. Determined half-lives (T½) (mean ± SD) for endocannabinoid hydrolysis in 10 % (w/w) bovine cornea and iris-ciliary body homogenates (pH 7.4, 37 °C) (n = 2-3).

ENDOCANNABINOID	T½ (min)		MAIN METABOLITE
	CORNEA	IRIS-CILIARY BODY	
AEA	178.7 ± 21.2	8.7 ± 3.8	Arachidonic acid
2-AG	19.1 ± 2.6	3.1 ± 0.9	Arachidonic acid
Noladin ether	1128.7 ± 532.1	430 ± 0.0	Unknown

5.2.3 Mechanisms of action for IOP reduction (I-IV)

The bi-phasic IOP profiles and rapid hydrolysis rates of AEA and 2-AG suggest that these topically administered endocannabinoids may act via their metabolite(s) in the

eye. In fact, corresponding bi-phasic IOP profiles have been reported after topical doses of both prostaglandin $F_{2\alpha}$ (Lee et al. 1984) and arachidonic acid in rabbits (Pate et al. 1996). The enzyme COX-2 has been demonstrated to metabolize AEA and 2-AG to various oxygenated products (Yu et al. 1997; Kozak et al. 2000). Pretreatment with the non-selective cyclooxygenase inhibitor indomethacin (12.5 mg/rabbit, s.c.), which prevents the formation of prostanoid metabolites from arachidonic acid has, indeed, been shown to eliminate the IOP effects of arachidonic acid and AEA in rabbits (Pate et al. 1996), suggesting that their bi-phasic IOP profiles are most probably induced by these prostanoid metabolites. In addition, the CB1 receptor seems to not be involved in the IOP-lowering effects of AEA, as the CB1 receptor antagonist SR141716A (6 mg/rabbit, s.c.) had no influence on the bi-phasic IOP effects of AEA in normotensive rabbits (Pate et al. 1998).

In order to investigate the mechanisms by which 2-AG and noladin ether reduces IOP in normotensive rabbits (III), the CB1 receptor antagonist AM251 was employed. Topically administered AM251 (dose = 25 µg) pre-treatment had no influence on the bi-phasic IOP effects of 2-AG (dose = 62.5 µg) (III; Fig. 5A). In contrast, the same dose of AM251 almost totally antagonized the IOP reduction induced by noladin ether (dose = 62.5 µg) (III; Fig. 5B). Based on the differences in IOP profiles, different enzymatic hydrolysis rates and topical CB1 antagonist data, these results imply that the IOP effects of the primary endocannabinoids, AEA and 2-AG, are most probably mediated via their active prostanoids or other arachidonic acid metabolites, whereas noladin ether seems to decrease IOP via the CB1 receptor in normotensive rabbits. Direct CB1 agonism is suggested to decrease IOP without initial IOP elevation. In fact, synthetic cannabinoid agonists such as WIN55,212-2 and CP55,940 have earlier been shown to lower IOP without any initial IOP elevation (Pate et al. 1998; Song and Slowey 2000; Porcella et al. 2001).

5.3 IOP EFFECTS OF ENDOCANNABINOID METABOLISM INHIBITORS IN TREATED EYES (I-II)

AEA and 2-AG rapidly enter cells by a specific facilitated uptake mechanism, followed by enzymatic hydrolysis via FAAH or MGL. The general hypothesis for this part of the study was that prevention of the endocannabinoid metabolism might increase their extracellular concentrations, where the binding sites of cannabinoid receptors are located and, consequently, prolong and potentiate the action of endocannabinoids (Fig. 24).

A   B

Endogenous or exogenous cannabinoids (e.g. AEA)   Endogenous or exogenous cannabinoids (e.g. AEA)

Transport blockage through transporter inhibitor leads to increased extracellular levels of cannabinoids

CB1 receptor   transporter protein   CB1 receptor   transporter protein   FAAH / MGL

Arachidonic acid

COX

Hydrolysis inhibition by enzyme inhibitors leads to increased intracellular levels of cannabinoids, which decreases their facilitated cellular uptake and passive diffusion. Thus, their extracellular concentrations increase.   Prostanoids

Fig. 24. Theoretical approaches to increasing cannabinoid ligand concentrations in the extracellular space of cells, where the receptor binding sites are located, with transport (A) and enzyme (B) inhibitors.

5.3.1 FAAH inhibition without topical cannabinoid drug (I)

The effect of FAAH inhibition on the IOP of normotensive pigmented rabbits was tested by using the FAAH inhibitor phenylmethylsulfonyl fluoride (PMSF). PMSF is a non-selective serine protease inhibitor that has been reported to effectively prevent the enzymatic hydrolysis of various endocannabinoids and their derivatives (Deutsch and Chin 1993; Hillard et al. 1995; Deutsch et al. 1997a). In this study, PMSF failed to have any significant effect on rabbit IOP, compared to vehicle treatment when administered subcutaneously at the doses of 0.22 - 22 mg/kg, in the absence of topical cannabinoid drug (Table 9). Perhaps PMSF failed to affect IOP because this dosage was not enought to provide complete FAAH inhibition in the eye. In fact, Compton and Martin (1997) have reported that the pharmacological effects of PMSF in a mouse model of cannabimimetic activity were observed at the dosage of 100 mg/kg or higher, even though lower doses of PMSF were capable of potentiating the effects of exogenously administered AEA (Compton and Martin 1997). In addition, the subsequent endogenous levels of AEA may not have been high enough to produce a pharmacological action after FAAH inhibition by PMSF.

Table 9: IOP changes in the treated eyes of normotensive pigmented rabbits after the subcutaneous injection of FAAH inhibitor PMSF.

OCULAR TREATMENT	PMSF (mg/kg)	TIME AFTER EYE DROP ADMINISTRATION (hours)						
		0	0.5	1	2	3	4	5
10 % HP-β-CD	---[A]	0.0 ± 0.0	-4.1 ± 2.0	-5.2 ± 1.2	-8.3 ± 1.2	-2.1 ± 2.0	-0.4 ± 5.9	7.9 ± 5.6
10 % HP-β-CD	0.22	0.0 ± 0.0	-1.4 ± 2.4	-5.6 ± 6.1	-3.6 ± 3.1	-4.7 ± 3.8	-1.9 ± 5.8	3.9 ± 5.5
10 % HP-β-CD	2.2	0.0 ± 0.0	2.2 ± 5.0	3.4 ± 4.4	-4.5 ± 3.5	0.8 ± 4.7	0.6 ± 4.9	7.1 ± 5.5
10 % HP-β-CD	22	0.0 ± 0.0	-2.1 ± 4.1	-2.7 ± 2.3	-7.9 ± 3.3	-9.0 ± 4.6	-5.0 ± 4.1	1.9 ± 4.1

Data presented as % change from baseline values, mean ± SE, n = 5.
[A] = 0.9 % NaCl

5.3.2 FAAH inhibition with topical endocannabinoid (I)

To test whether the FAAH inhibitor PMSF might be effective in inhibiting enzymatic hydrolysis, and subsequently enhance the ocular effects of exogenous AEA, PMSF was administered subcutaneously at doses of 0.22–2.2 mg/kg prior to topical ocular unilateral cannabinoid agonist administration in rabbits. PMSF pre-treatment (2.2 mg/kg) was observed to be capable of altering the IOP profile of AEA (Table 10). Indeed, after PMSF administration, AEA exhibited an immediate IOP hypotensive effect, instead of the typical bi-phasic IOP profile that was observed after the administration of AEA alone (I; Fig. 2A). This indicates that PMSF may prevent the enzymatic hydrolysis of AEA to arachidonic acid, and subsequent formation of active arachidonic acid metabolites.

The observation that PMSF pre-treatment may inhibit the ocular degradation of AEA was confirmed with another cannabinoid agonist, arachidonyl propionitrileamide (APN), which is a synthetic derivative of AEA. APN has been previously reported to decrease IOP without initial IOP hypertension (Pate et al. 1996). In the present study, however, APN tended to slightly but not significantly increase IOP during the first 30 minutes of each experiment (mean initial IOP increase by 9.8 % over baseline values). As with AEA, PMSF pretreatment eliminated the initial IOP elevation of APN and induced an immediate but not significant IOP reduction, although a higher dose of PMSF was required (22 mg/kg) (Table 11). This small and variable initial IOP elevation suggests that APN is a poorer FAAH substrate and produces less arachidonic acid than AEA. However, APN may also be a weaker cannabinoid receptor agonist than AEA, as its IOP reducing efficacy seems to be slightly lower than that of AEA.

Table 10: IOP changes in the treated eyes of normotensive pigmented rabbits after subcutaneous injection of the FAAH inhibitor PMSF with topical administration of AEA.

OCULAR TREATMENT	PMSF (mg/kg)	TIME AFTER EYE DROP ADMINISTRATION (hours)						
		0	0.5	1	2	3	4	5
10 % HP-β-CD	---[A]	0.0	-4.1 ± 2.0	-5.2 ± 1.2	-8.3 ± 1.2	-2.1 ± 2.0	-0.4 ± 5.9	7.9 ± 5.6
AEA[B]	---[A]	0.0	31.3 ± 9.9*	17.8 ± 6.2*	-12.1 ± 3.7	-18.4 ± 5.3*	-10.7 ± 7.0	-7.1 ± 4.3
AEA[B]	0.22	0.0	30.6 ± 17.9*	5.6 ± 8.4*	-24.3 ± 2.6*	-20.3 ± 3.5*	-11.7 ± 3.7	-4.0 ± 3.2
AEA[B]	2.2	0.0	0.6 ± 2.9	-4.8 ± 3.2	-15.3 ± 5.8	-14.4 ± 2.9*	-7.3 ± 4.3	-0.4 ± 5.1

Data presented as % change from baseline values, mean ± SE, n = 5
[A] = 0.9 % NaCl
[B] = Topical dose of AEA was 62.5 μg (= 180 nmol)
* Statistically significant compared to vehicle treatment (two-way ANOVA with Fisher's PLSD test)

Table 11: IOP changes in the treated eyes of normotensive pigmented rabbits after subcutaneous injection of the FAAH inhibitor PMSF with topical administration APN.

OCULAR TREATMENT	PMSF (mg/kg)	TIME AFTER EYE DROP ADMINISTRATION (hours)						
		0	0.5	1	2	3	4	5
20 % HP-β-CD	---[A]	0.0	-1.4 ± 1.6	-1.4 ± 4.2	-1.2 ± 4.7	-1.2 ± 4.7	-4.8 ± 4.1	-2.8 ± 3.1
APN[B]	---[A]	0.0	9.8 ± 3.9	-3.4 ± 3.7	-12.6 ± 3.8	-9.2 ± 3.4	-6.3 ± 2.6	-6.0 ± 2.5
APN[B]	2.2	0.0	12.1 ± 8.2	-1.5 ± 6.8	-16.1 ± 2.1	-12.0 ± 2.1	-14.2 ± 3.2	-14.0 ± 3.5
APN[B]	22.0	0.0	-9.9 ± 3.5	-13.9 ± 3.3	-11.4 ± 3.0	-14.3 ± 5.1	-7.6 ± 4.5	-8.2 ± 3.0

Data presented as % change from baseline values, mean ± SE, n = 6
[A] = 0.9 % NaCl
[B] = Topical dose of APN was 62.5 μg (= 175 nmol)
* Statistically significant compared to vehicle treatment (two-way ANOVA with Fisher's PLSD test)

5.3.3 Mechanism of action for IOP effects of PMSF (I)

To investigate whether cyclooxygenase derived arachidonic acid metabolites are involved in the IOP lowering effects of AEA in the presence of PMSF, the nonselective

cyclooxygenase inhibitor indomethacin was used to prevent the cyclooxygenase metabolism. Indomethacin (12.5 mg/rabbit) was not able to alter the IOP profile of AEA in the presence of PMSF, suggesting that prostanoid synthesis through the cyclooxygenase pathway may not be involved (I; **Table 2**).

The CB1 receptor antagonist SR141716A was used to determine whether this cannabinoid receptor is involved in the IOP profile of AEA in the presence of PMSF. SR141716A had previously failed to antagonize the ocular effects of AEA (Pate et al. 1998). In the presence of PMSF, however, the IOP lowering effects of AEA were inhibited by this CB1 receptor antagonist (I; **Fig. 3**), and a long lasting IOP increase was observed in both treated and untreated eyes of rabbits, supporting the earlier hypothesis that the CB1 receptor may be involved in the physiological control of IOP (Pate et al. 1998; Song and Slowey 2000). SR141716A has also been reported to act as an inverse agonist (Landsman et al. 1997; Pan et al 1998). Taken together, these results indicated that PMSF inhibits the enzymatic hydrolysis of AEA and subsequent formation of arachidonic acid, and the AEA-induced immediate IOP reduction in the presence of PMSF is most probably mediated by intact AEA at the CB1 receptors, rather than its metabolites.

However, in addition to FAAH inhibitor activity, PMSF has been reported to be a potent inhibitor of other enzymes, such as cholinesterases and serine proteases (Turini et al. 1969). As PMSF rapidly crosses the blood-brain-barrier (Turini et al. 1969), its subcutaneous administration would be expected to inhibit FAAH and other enzymes in the brain, as well as in the periphery. Thus, it cannot be ruled out that the IOP effects of PMSF were caused by the inhibition of other enzymes. Recently the ability of PMSF to inhibit FAAH, neuropathy target esterase (NTE) or AchE was studied in mouse brain. At the dosage of 30 mg/kg (i.p.), PMSF inhibited 99.98 % of FAAH, 64 % of NTE and only 7 % of AChE activities, respectively (Quistad et al. 2002).

5.3.4 Transporter inhibition without topical cannabinoid drug (II)

The endocannabinoid uptake inhibitors AM404 and olvanil were administered topically to determinate whether these uptake inhibitors potentiate the activities of endogenously produced cannabinoids by blocking their cellular uptake in ocular tissues. The IOP effects of endocannabinoid uptake inhibitors, without any additional cannabinoid drug, are summarized in **Table 12**. The maximal IOP lowering effects (% decreases from baseline values) of olvanil and AM404 at the dosage of 62.5 μg in HP-β-CD or propylene glycol vehicle varied from 7 % to 14 %, respectively.

Table 12: The maximal IOP effects and IOP profiles induced by topical endocannabinoid uptake inhibitors, AM404 and olvanil, in treated eyes of normotensive rabbits.

INHIBITOR	DOSE	VEHICLE	MAXIMAL EFFECT (%)	TIME OF MAX. EFFECT	IOP PROFILE[A]
AM404	62.5µg (158 nmol)	25 % HP-β-CD	-13.8 ± 4.8*	4 h	↓
AM404	62.5µg (158 nmol)	Propylene glycol	-10.2 ± 2.1	3 h	↑↓
Olvanil	62.5µg (150 nmol)	Propylene glycol	-7.3 ± 2.7	2 h	↓
Olvanil	312.5 µg (748 nmol)	Propylene glycol	-15.0 ± 5.9*	3 h	↓

Maximal effects are presented as % of baseline, mean ± SE, n = 5-6
[A] Indicated as increase (↑) and/or decrease (↓) in IOP.
* Statistically significant compared to vehicle treatment (2-tailed paired Student's t-test or one-factor ANOVA with Fisher's PLSD test)

5.3.5 Transporter inhibition with topical endocannabinoid (II)

Previously AM404 has been found to potentiate the pharmacological action of AEA (Beltramo et al. 1997a; Calignano et al. 1997). In the present study, we investigated whether topically administered AM404 affected the IOP profile of topically administered exogenous AEA. Subcutaneous pre-treatment with the FAAH-inhibitor PMSF was used to prevent the enzymatic inactivation of AEA. Ocularly administered AEA (62.5 µg in 10 % HP-β-CD, 25 µl) decreased IOP in the presence of PMSF pre-treatment (24 mg/kg, 30 min prior to AEA), although no statistically significant differences were observed when compared to treatment with PMSF and topical vehicles (II; **Fig. 3**). The topically administered transporter inhibitor AM404 (125 µg in 25 % HP-β-CD, 50 µl) failed to either potentiate or prolong the ocular hypotensive effects of AEA in the presence of PMSF (II; **Fig. 3**).

AM404 (10 mg/kg i.v.) has been shown to significantly enhance and prolong the antinociceptive and hypotensive effects of AEA in mice and guinea pigs, but has no, or only a minor effect when administered alone without AEA (Beltramo et al. 1997a; Calignano et al. 1997). In contrast, results from the present work demonstrated that AM404 reduced IOP when administered topically without exogenous AEA. Furthermore, AM404 did not improve the ocular hypotensive effects of AEA. In this study, PMSF pre-treatment was used because AEA evokes a bi-phasic IOP profile (i.e., initial ocular hypertension followed by significant IOP hypotension) in the absence of a FAAH-inhibitor. As PMSF prevents AEA hydrolysis to arachidonic acid and other

cyclooxygenase products, only the hypotensive phase of IOP profile was observed. However, it has been reported that FAAH inhibitors may actually decrease AEA transport in the RBL-2H3 cell line (Rakhshan et al. 2000) probably by modifying the active site of the transporter protein and acting as a transporter inhibitor (Maccarone et al. 1998). FAAH inhibitors may also decrease the cellular uptake of AEA by enhancing its intracellular concentrations, therefore disturbing the concentration gradient that is needed to promote uptake.

5.3.6 Mechanism of action for IOP effects of AM404 and olvanil (II)

The binding affinities of AM404 (K_i = 1.8 μM) and olvanil (K_i = 1.6-7.1 μM) for the CB1 receptor are of the same magnitude as their respective affinities for the transporter protein (K_i = 2.1 and 14.1 μM for AM404 and olvanil, respectively) (Khanolkar et al. 1996; Di Marzo et al. 1998; Beltramo and Piomelli 1999). Therefore, it could be expected that the IOP actions of AM404 and olvanil originate from their respective agonist activities at the CB1 receptor. However, neither AM404 nor olvanil exhibited CB1 receptor -dependent G-protein activity at concentrations up to 10^{-4} M when monitored by [^{35}S]GTPγS autoradiography of rat brain sections (**II; Fig. 5**). This indicates that the IOP effects of transport inhibitors may not be mediated through direct agonism of the CB1 receptor. Additionally, AM404 and olvanil have been reported to activate the vanilloid receptor (VR1), which is a ligand-gated non-selective ion channel present in nociceptors that elicit a burning pain sensation (Caterina et al. 1997; Szallasi and Blumberg 1999). However, it is currently not known whether the VR1 receptor is expressed in intraocular tissues, or if its activation even affects IOP.

The ocular action of transport inhibitors is suggested to be due to the prevention of carrier-mediated uptake and subsequent intracellular metabolism of endogenous AEA or 2-AG those eventually act as a ligand for IOP reduction. However, the bi-phasic IOP profile of AM404, when administered in a propylene glycol vehicle, suggests that AM404 is degraded to arachidonic acid in ocular tissues, although AM404 is reported to be a poor substrate for FAAH (Lang et al. 1999). In addition, AM404 may serve as a substrate for the endocannabinoid transporter (Piomelli et al. 1999).

Recently, the existence of endocannabinoid transporter has been questioned. Glaser et al. (2003) have suggested that the cellular uptake of AEA is a simple diffusion, rather than a transporter-mediated process. However, they used bovine serum albumin (BSA), which itself possess some inhibitory effect on AEA accumulation, in their uptake studies. Recently, Ligresti et al. (2004) have reported that AEA accumulation in RBL-2H3 cells is saturable at low concentrations (μM), is time-dependent and decreased by

compounds that do not have an inhibitory action for FAAH, when BSA is excluded from the experiments, providing further evidence that the protein other than FAAH is involved in the cellular uptake of endocannabinoids.

5.4 IOP EFFECTS OF CB2 RECEPTOR AGONISM (IV)

A wide distribution of the cannabinoid CB1 receptor protein has already been detected in several human and bovine ocular tissues, including the trabecular meshwork, canal of Schlemm and ciliary epithelium (Straiker et al. 1999a; Straiker et al. 1999b), which are the major ocular structures that participate in maintaining IOP. While evidence for CB2 receptor mRNA has been obtained from rat retinal tissues (Lu et al. 2000), it is not yet clear if CB2 receptors participate in the IOP hypotensive actions of cannabinoids. As CB2 agonists seem to lack the psychoactive effects of CB1 agonists (Malan et al. 2001), they would offer an alternative approach to develop novel and possibly more tolerable glaucoma medications. Recent development of CB2 specific receptor ligands, such as JWH-133, has enabled the separation of pharmacological actions between CB1 and CB2 receptor subtypes.

The effect of direct CB2 agonism was investigated on IOP of normotensive rabbits in the present study by using the CB2 receptor agonist JWH-133, and its IOP effects were compared to those of non-selective non-classical cannabinoid CP55,940 (**Table 13**). The CB2 receptor binding properties of JWH-133 are approximately 200 times greater towards CB2 than CB1 receptors (K_i for CB1 = 677 nM and for CB2 = 3.4 nM, respectively) (Huffman et al. 1999). In contrast, CP55,940 binds to both CB1 and CB2 receptors at almost equal nanomolar concentrations (K_i for CB1 = 2.6 nM and for CB2 = 3.7 mM, respectively) (Felder et al. 1995). JWH-133 at doses of 10 and 20 µg in 45 % HP-β-CD vehicle did not generate IOP reductions in the treated eyes of normotensive rabbits. In contrast, at the doses of 25 µg (5 % HP-β-CD vehicle with 3 % PVA) and 62.5 µg (10 % HP-β-CD vehicle with 3 % PVA) CP55,940 produced statistically significant IOP reductions in treated eyes (**IV**; **Fig. 2A**). In the present study, JWH-133 (dose = 62.5 µg) did not decrease IOP of normotensive rabbits when formulated in propylene glycol, to provide higher drug concentrations in eye drop solutions, while CP55,940 at the same dose significantly reduced IOP (**IV**; **Fig. 2A**). These results suggest that topically administered CB2 receptor agonist JWH-133 does not lower IOP in normotensive rabbit animal model, at least in the dosage range studied. However, additional studies are required to determine the possible role of CB2 agonism in IOP regulation.

Table 13: Comparison of the effects of the CB2 receptor agonist JWH-133 and the non-selective cannabinoid agonist CP55,940 on the IOP of treated eyes in normotensive rabbits.

DRUG	DOSE	VEHICLE	MAXIMAL EFFECT (%)	TIME OF MAX. EFFECT	IOP PROFILE[A]
JWH-133	10 μg (32 nmol)	45 % HP-β-CD	4.5	2 h	No effect
JWH-133	25 μg (80 nmol)	45 % HP-β-CD	5.4	2 h	No effect
JWH-133	62.5 μg (200 nmol)	Propylene glycol	-2,5	2 h	No effect
CP55,940	25 μg (66 nmol)	5 % HP-β-CD, 3 % PVA[B]	-11.9*	2 h	↓
CP55,940	62.5 μg (166 nmol)	10 % HP-β-CD, 3 % PVA[B]	-15.2*	2 h	↓
CP55,940	62.5 μg (166 nmol)	Propylene glycol	-13.3*	1h	↓

Maximal effects are presented as % change in baseline values, n = 5-6
[A] Indicated as an increase (↑) and/or decrease (↓) in IOP.
[B] Additional 3 % of PVA was used to increase viscosity of the eye drop solution to increase residence time of the eye drop on the precorneal area.
* Statistically significant compared to vehicle treatment (two-way ANOVA with Bonferroni correction or Fisher's PLSD)

5.5 IOP EFFECTS OF TEST COMPOUNDS IN UNTREATED EYES (I-IV)

Overall, none of the topically applied endocannabinoids, their synthetic derivatives nor AEA transporter inhibitors that were investigated in this study significantly affected the IOP of the untreated eyes of rabbits when administered alone, suggesting that their IOP actions were local and mediated via ocular targets rather than via the CNS. These observations are consistent with earlier studies showing that, in the untreated eyes of rabbits, the IOP effects of various ocularly administered cannabinoids have only been minor and short-lasting (Pate et al. 1995; 1996; 1997; 1998; Song and Slowey 2000; Porcella et al. 2001). The observed marginal contralateral effects of topically administered cannabinoids might be caused by the systemic absorption of the applied dose (Urtti 1994; Pate et al. 1995; Naveh et al. 2000). In contrast, the CB1 receptor antagonist SR141716A induced a typical IOP hypertension in both treated and untreated eyes, which resulted from the subcutaneous route of administration.

6 SUMMARY AND CONCLUSIONS

The endogenous cannabinoid system involves two types of cannabinoid receptors, their endogenous ligands and mechanisms for both the formation and inactivation of these ligands. The endogenous cannabinoid system has recently been identified in various organs, including the eye. The present study investigated whether the ocular endogenous cannabinoid system might act as an appropriate target for the development of novel IOP-lowering agents for treating high intraocular pressure in glaucoma.

Endocannabinoids AEA and 2-AG are rapidly eliminated *in vivo* by their carrier-mediated transport into cells and subsequent enzymatic hydrolysis. In this study, the possibility of affecting IOP in normotensive rabbits by inhibiting the metabolism of endocannabinoids was investigated. The IOP effects and enzymatic stabilities of AEA, 2-AG and the novel putative endocannabinoid noladin ether were compared in order to investigate possible mechanisms by which endocannabinoids and their analogues reduce IOP in normotensive rabbits. In addition, the effect of direct CB2 receptor agonism on IOP of rabbits was also evaluated. On the basis of results from the present study it can be concluded that:

1. Topical administration of the endocannabinoid transport inhibitors AM404 and olvanil decrease IOP in treated eyes of normotensive pigmented rabbits without CB1 receptor activation.

2. The FAAH enzyme inhibitor PMSF (s.c.) eliminates the initial hypertensive phase associated with the bi-phasic IOP profiles of topically administered AEA and its synthetic analogue APN in normotensive rabbits. This bi-phasic IOP profile is probably caused by prostanoid metabolites. In contrast, the IOP reduction induced by AEA in the presence of PMSF is mediated via the CB1 receptor.

3. 2-AG and AEA are rapidly hydrolysed in bovine iris-ciliary body and cornea homogenates. The main hydrolysis product of AEA and 2-AG is arachidonic acid. Noladin ether is the first endogenous cannabinoid that is relatively stabile and does not degrade to arachidonic acid in bovine iris-ciliary body and cornea homogenates.

4. Noladin ether decreased IOP in normotensive rabbits. The IOP reductive effects of noladin ether are mediated via the CB1 receptor, whereas AEA and 2-AG affect IOP via bioactive prostanoid metabolites.

5. Direct CB1 receptor agonism is suggested to decrease IOP without initial IOP elevations.

6. Topical administation of a specific CB2 receptor agonist JWH-133 does not decrease IOP in normotensive rabbit model at the doses used in the present study.

Taken together, these results suggest that the IOP of normotensive rabbits can be lowered with topically administered CB1 receptor agonists. The inhibitors of FAAH and AEA transporter may potentiate the action of endogenous cannabinoids if their endogenous levels are high enough to produce a pharmacological action. These preliminary data indicate that it is possible to develop IOP lowering therapeutics that affect the endogenous cannabinoid system; however, more information is needed from safety and efficacy profiles.

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8 ORIGINAL PUBLICATIONS

I   Krista Laine, Kristiina Järvinen, David W. Pate, Arto Urtti, Tomi Järvinen:
Effect of the enzyme inhibitor, phenylmethylsulfonyl fluoride, on the IOP
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II   Krista Laine, Tomi Järvinen, Juha Savinainen, Jarmo T. Laitinen, David W.
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