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Effects of Antiestrogens on Retinal Cells *in Vitro* -Glutamate transporter as a novel target?

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

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I Mäenpää H, Mannerström M, Toimela T, Salminen L, Saransaari P and Tähti H (2002): Glutamate uptake is inhibited by tamoxifen and toremifene in cultured retinal pigment epithelial cells. Pharmacol Toxicol 91: 116-122.

II Mäenpää H, Gegelashvili G and Tähti H (2003): Expression of glutamate transporter subtypes in cultured retinal pigment epithelial and retinoblastoma cells. Current Eye Research, in press.

III Mäenpää H, Saransaari P and Tähti H (2003): Kinetics of inhibition of glutamate uptake by antioestrogens. Pharmacol Toxicol 93: 174-179.

IV Mäenpää H, Toimela T, Saransaari P, Salminen L and Tähti H (1997): Mechanism of tamoxifen's retinal toxicity studied in pig pigment epithelial cell cultures. ATLA 25: 297-302.

V Mäenpää H, Toimela T, Mannerström M, Saransaari P and Tähti H (2004): Toxicity of selected cationic drugs in retinoblastomal cultures and in co-cultures of retinoblastomal and retinal pigment epithelial cell lines. Neurochem Res 29: 305-311.

ABBREVIATIONS

ab/am	antibiotic/antimycotic solution
AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
L-AP4	L-2-amino-4-phosphonobutyrate
AP-5	D-2-amino-5-phosphonopentanoate
ARPE-19	human retinal pigment epithelial cell line ARPE-19
ATP	adenosine triphosphate
BCA	bicinchoninic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CRALBP	cellular retinaldehyde-binding protein
D407	human retinal pigment epithelial cell line D407
DMEM	Dulbecco's Modified Eagle Medium
EAAC1	excitatory amino acid carrier 1
EAAT4	excitatory amino acid transporter 4
EAAT5	excitatory amino acid transporter 5
EDTA	ethylenediaminetetra-acetate
EC_{50}	effective concentration for half maximal effect
ER	estrogen receptor
FBS	fetal bovine serum
GABA	γ-aminobutyrate
GFAP	glial fibrillary acidic protein
GLAST	glutamate/aspartate transporter
GLT	glutamate transporter1
GTRAP	glutamate transport-associated protein
GTRAP3-18	glutamate transport-associated protein 3-18
GTRAP41	glutamate transport-associated protein 41
GTRAP48	glutamate transport-associated protein 48
β-HA	β-hydroxyaspartate
HeLa	Henrietta Lack's cervix carcinoma cell line
IC_{50}	inhibitory concentration for half maximal effect
MDCK	Madin-Darby canine kidney cell line
NMDA	N-methyl-D-aspartate
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PVR	proliferative vitreoretinopathy
RA	retinoic acid
RPE	retinal pigment epithelium
RPE65	retinal pigment epithelial specific protein 65
SDS-PAGE	sodium dodecylsulfate–polyacrylamide gel electrophoresis
SEM	standard error of mean
SERM	selective estrogen receptor modulator
TBS	Tris-buffered saline
WST-1	tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate)
Y79	human retinoblastoma cell line Y79

ABSTRACT

Glutamate is the main excitatory neurotransmitter in the retina and brain, but it may also exert excitotoxic harmful effects on cells, a phenomenon related to retinal diseases such as glaucoma and diabetic retinopathy. An excess of extracellular glutamate may activate an excitotoxic reaction cascade via its synaptic receptors. Efficient glutamate transport is crucial in keeping extracellular glutamate below toxic levels in the retina. Müller glial cells and the retinal pigment epithelium (RPE) are believed to regulate the glutamate concentration in the subretinal space, but little is known of the specific features of the glutamate transporter expressed in the RPE. The purpose of the present study was to investigate the functional characteristics and the expression of glutamate transporter in RPE cells *in vitro*. The main hypothesis was that selected drugs (tamoxifen and chloroquine) affect the function of the glutamate transporter in the RPE. Tamoxifen and chloroquine have caused retinal defects as an adverse effect of the treatment of breast cancer and rheumatoid arthritis, respectively. In the present study, the effects of these drugs on glutamate uptake in RPE cells and the cytotoxic effects on retinoblastoma Y79 cells were evaluated *in vitro*.

Pig RPE cultures, two human RPE cell lines ARPE-19 and D407 and a human retinoblastoma cell line Y79 were used in the experiments. As a more complex *in vitro* model, a retinal co-culture of ARPE-19 and Y79 cell lines was also established. Glutamate transport was characterized by glutamate uptake assays and the expression of glutamate transporter subtypes detected by immunoblotting and immunocytochemistry. Changes in cell viability were estimated by measuring mitochondrial enzyme activity and cellular ATP levels.

The RPE cells expressed active, Na^+-dependent high-affinity glutamate transporters. Their activity was affected by tamoxifen and its structural analogue toremifene, whereas chloroquine was ineffective. In fact, tamoxifen and toremifene competitively inhibited glutamate transport in both the pig RPE cultures and the RPE cell line, suggesting that this function might constitute a new cellular target for these drugs. The glutamate transporter subtypes expressed in the retinal cells were EAAC1 and EAAT4, which were now detected in the RPE cells and in the retinoblastoma cells for the first time. The results corroborate atypical, strong expression of EAAT4 in the cell lines. The drugs effected a dose-dependent reduction in the viability of Y79 cells, this being discernible with antiestrogens at concentrations attainable in tissues at clinically relevant drug doses. Evaluation of cell viability in the Y79 cell line implies that robust cytotoxicity can be studied with cell lines, while retinal co-cultures and more specific cellular mechanisms such as glutamate uptake may be used for more detailed evaluations.

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the retina and the brain. Besides being pivotal in neurotransmission, it may also act as a powerful neurotoxic substance (Choi 1992). The phenomenon termed excitotoxicity is apparently implicated in neurological and retinal diseases, including epilepsy, glaucoma and diabetic retinopathy (Doble 1999, Naskar et al. 2000). Glutamate transporters have key roles in excitotoxicity. Their dysfunction leads to extracellular accumulation of glutamate, which in turn may trigger a toxic reaction cascade via glutamate receptors (Danbolt 2001). In the retina, Müller glial cells are considered essential in regulation of the retinal glutamate homeostasis (Rauen et al. 1998). However, the retinal pigment epithelium (RPE) has also been thought to contribute to regulation of the glutamate concentration in the subretinal space (Miyamoto and Del Monte 1994, Pow 2001). The RPE is the outermost layer in the retina and participates in various important functions, e.g. partial formation of the blood-retinal barrier and phagocytosis of photoreceptor outer segments.

Tamoxifen and chloroquine, widely used drugs, are known to cause retinal defects as an unfortunate adverse effect (Bernstein 1983, Pavlidis et al. 1992). Tamoxifen is an antiestrogen used mainly in the therapy of breast cancer, while chloroquine is an antirheumatic and antimalarial drug. The RPE is thought to be an important target of chloroquine, but little is known of the specific mechanisms related to retinal defects induced by these drugs. *In vitro* studies have especially proved their power when single cell types and solitary mechanisms are investigated. Furthermore, there is an increasing need to reduce the use of laboratory animals in the safety testing of drugs and other chemicals. The present study focuses on the toxic effects of tamoxifen and its structural analogue toremifene, and chloroquine in retinal cells *in vitro*. A non-continuous RPE culture, human RPE cell lines and a human retinoblastoma cell line were used as target cells. Drug-induced changes in cell viability were tested, but the experiments also probed further into alterations in glutamate transport as a specific mechanism.

REVIEW OF THE LITERATURE

1 Retinal pigment epithelium (RPE)

1.1 General features of RPE in vivo and in vitro

The retinal pigment epithelium (RPE) is a monolayer of highly differentiated cells between the photoreceptors and choriocapillars (Fig. 1). Under normal conditions RPE cells are believed to be mitotically inactive (Stroeva and Mitashov 1983). They contain melanin pigment which absorbs light but also binds many toxins, including certain drugs. The RPE serves several specific functions, e.g. phagocytosis of the photoreceptor outer segments and supply of retinoids to photoreceptors. Polarized distribution of cell organelles and asymmetric membrane domains is characteristic of RPE cells; the apical side (photoreceptor side) with long microvilli and the basal side with smaller infoldings.

RPE cells, joined by tight junctions, form with the capillary endothelium the blood-retinal barrier. This barrier is important in regulating the passage of water, ions and foreign compounds to the subretinal space and further to the retinal microenvironment (Marmor 1998). The subretinal space is bordered by the apical microvilli of RPE cells and by the outer segments of photoreceptors. The disadvantage of tight junctions is that they may impede removal of subretinal fluid. There is thus a need for powerful transport mechanisms to control dehydration and homeostasis in the subretinal space.

RPE cells derived from human and animal eyes have for years been used in studies on the RPE structure and function. These studies have produced much detailed information on the RPE, but the assumption that the RPE cells *in vitro* mimics the RPE *in vivo* should not be made too hastily. An essential consideration here is the enhanced proliferation and cell death occurring *in vitro,* whereas *in vivo* individual cells may persist for many years (Uebersax et al. 2000). A further aspect is transdifferentiation by further passages or culture manipulations (Campochiaro et al. 1991, Grisanti and Guidry 1995, Chen et al. 2003). Several studies have shown the effect of culture components, e.g. growth factors and coating, on cell viability and differentiation (Janssen et al. 2000, Uebersax et al. 2000). Incomplete epithelialization is likely to occur in RPE cultures, since several weeks of culturing in confluence are needed to develop high epithelial organization and molecular polarity (Matsumoto et al. 1990, Bok et al. 1992, Dunn et al. 1996). After long periods in confluent culturing, changes also appear which reveal that the RPE cells are aging (Burke and Skumatz 1998), though the senescence of RPE is thought to manifest itself *in vivo* as well (Hjelmeland et al. 1999).

Recently, RPE cell lines have become important tools in studies on the RPE *in vitro*. They have been created by transformation or have arisen spontaneously, as for example the human RPE cell lines D407 and ARPE-19 (Davis et al. 1995, Dunn et al. 1996). The advantage of cell lines is maintenance of their characteristics after several passages and long survival time compared to primary cultures. While cell lines may be to a great extent homologous, primary cultures exhibit heterogeneity and donor-to-donor variability. However, the RPE *in situ* has also been found to form a mosaic of similar but not identical cells (Burke et al. 1996). It is important to bear such differences and similarities to RPE cells *in vivo* in mind when using cell lines (Dunn et al. 1996).

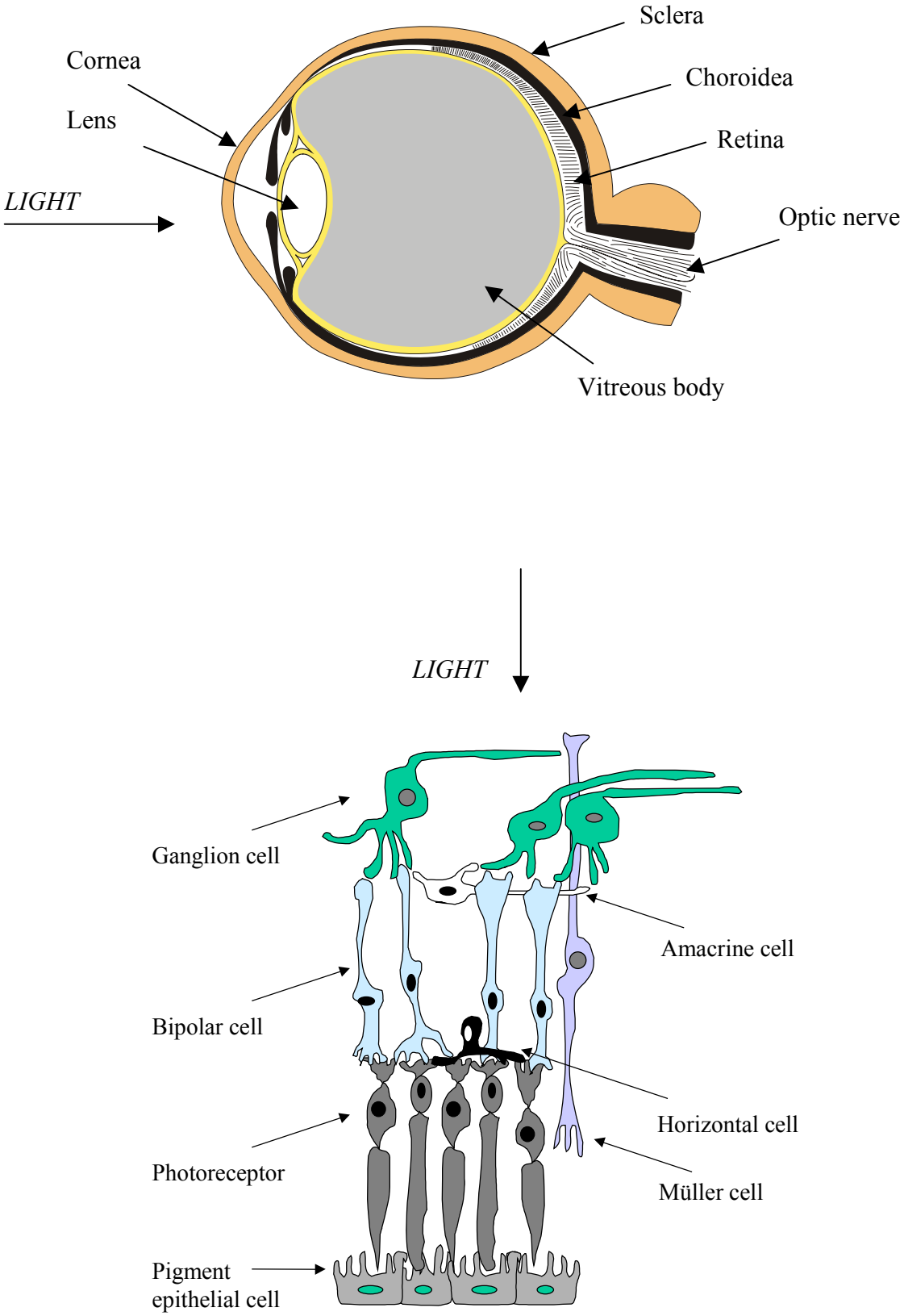

Fig 1. Schematic section of the eye (above) and simplified illustration of cellular organization in the retina (below).

1.2 RPE and proliferative vitreoretinopathy

Proliferative vitreoretinopathy (PVR) is the end point of a number of intraocular diseases. Proliferation, migration and transdifferentiation are salient features seen in RPE cells in this disorder (Hiscott and Sheridan 1998). In PVR, RPE cells take the shape of fibroblasts (Lee et al. 2001). Culturing of RPE cells may somehow mimic changes occurring in PVR, since RPE cells have been found to convert their epithelial characteristics to mesenchymal type with repeated passages *in vitro* (Grisanti and Guidry 1995). However, differences have been reported e.g. in gene expression between cultured RPE cells and RPE cells in PVR (Abe et al. 1996). The protein kinase C (PKC)-mediated pathway is thought to have a crucial role in triggering RPE migration or proliferation (Murphy et al. 1995). Furthermore, hepatocyte growth factor, connective tissue growth factor and glutamate have also affected the pathogenesis of PVR and proliferation of RPE cells (Uchida et al. 1998, Hinton et al. 2002).

2 Glutamate in the retina

2.1 Neurotransmitter and toxic agent

As noted, glutamate is the major excitatory neurotransmitter in the brain and retina. It acts as a neurotransmitter in more than 90 % of retinal synapses. Glutamate is released from photoreceptors and bipolar cells during the complex process of visual signal transmission and it interacts with different types of glutamate receptors at retinal synapses (Massey 1990, Wu and Maple 1998). In darkness, glutamate is continuously released from photoreceptors and the release is regulated by light (Dowling and Ripps 1972, Cervetto and MacNichol 1972, Copenhagen and Jahr 1989). The responses to glutamate in the two distinct types of bipolar cells are different. Glutamate hyperpolarizes one and depolarizes the other (Wu and Maple 1998). Furthermore, on-bipolar cells release glutamate in light, whereas off-bipolar cells release it in dark. The postsynaptic glutamate receptors in the off-bipolar cells are ionotropic (kainate or 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors), in the on-bipolar metabotropic (L-2-amino-4-phosphonobutyrate (L-AP4) receptor) and in ganglion cells, again, ionotropic (N-methyl-D-aspartate (NMDA), kainate or AMPA receptors) (Wu and Maple 1998).

When the extracellular concentrations of glutamate are excessively elevated, glutamate acts as a powerful neurotoxin, inducing e.g. Na^+ and Ca^{2+} influxes and cell swelling (Olney 1982, Choi 1992). As far back as 1957, glutamate-induced necrotic lesions in the retina were demonstrated (Lucas and Newhouse 1957) and by now the phenomenon has been extensively studied. Glutamate excitotoxicity entails neuronal death due to excessive or prolonged activation of glutamate receptors (Choi 1992). Important repercussions of excitotoxic processes are the generation and progression of several neuropathological conditions and retinal defects (Doble 1999, Sherry and Townes 2000, Ientile et al. 2001). Precise control of extracellular glutamate concentrations is thus crucial, and this is effected by glutamate transporters.

Excitotoxic cell death is the end point of a reaction cascade which involves prolonged depolarization of neurons, changes in the intracellular calcium concentration and activation of enzymes, including nucleases (Doble 1999, Arundine and Tymianski 2003). Activation of

NMDA and AMPA receptors is considered to be a principal step in triggering excitotoxic events. Receptor activation is followed by a sodium influx which perturbs the osmotic balance and may cause cell swelling and lead to cell lysis. Depolarization also elevates the intracellular calcium concentration, which in turn leads to activation of various enzymes and enhances calcium-dependent protein interactions. Moreover, enzyme activation may enhance the production of free radicals, which are detrimental to intracellular organelles. Amplification of neuronal damage occurs via an increase in the extracellular glutamate concentration. The main causes of elevated glutamate are 1) decrease in glutamate uptake or reversal of glutamate transport, 2) cell lysis and 3) exocytosis of synaptic vesicles.

2.2 Some other roles of glutamate in the retina

Regulation of the phagocytotic function of RPE cells, a fundamental process for visual acuity, also involves glutamate, (Besharse and Defoe 1998). In phagocytosis, apical processes of RPE cells engulf extracted discs. The phagosomes formed in the cells are broken down by lysis. Glutamate markedly increases the phagocytotic activity of RPE (Greenberger and Besharse 1985), which indicates its role in the basic tasks of the RPE. Exogenous glutamate may enter the retina via the RPE, whereas endogenous glutamate may be transported to RPE cells from the subretinal space. All cells have basal requirements for amino acids such as glutamate by reason of processes such as protein synthesis. In retinal cells, which do not undergo cell division, these demands are relatively limited (Pow 2001). However, glutamine synthetase, which takes part in glutamate recycling, is present in the RPE (Derouiche and Rauen 1995) and may affect glutamate degradation. Furthermore, the formation of γ-aminobutyrate (GABA) is also linked to glutamine/glutamate metabolism (Wiessner et al. 2002).

3 Glutamate transporters

3.1 General features and subtypes of glutamate transporters

Glutamate transporters are essential for normal glutamatergic neurotransmission in the central nervous system (CNS) and the retina. They rapidly remove glutamate from the extracellular space to maintain physiologic glutamate concentrations. After glutamate has been released in synapses, it diffuses out of the synaptic cleft and is removed by uptake. In glial cells or nerve terminals this uptake is effected by glutamate transporters (Gegelashvili et al. 2001). The fact that the concentration of intracellular glutamate is approximately 10 000-fold higher (10 mM) than that in extracellular fluid bespeaks the efficacy of active uptake mechanisms. Characteristic of glutamate transporters is that they are driven by electrochemical gradients: when Na^+ and glutamate are translocated into the cell, K^+ is extruded (Fig. 2). The transport cycle is interrupted in the absence of potassium, this leading to incomplete transport cycles and exchange (Danbolt 2001). The stoichiometry of uptake involves three Na^+, one H^+ and one K^+ (Zerangue and Kavanaugh 1996). It is also to be emphasized that chloride currents coupled to the glutamate transporters EAAT4 and EAAT5 are glutamate-activated and Na^+-dependent, but are not coupled with glutamate translocation (Fairman et al. 1995, Arriza et al. 1997). Since the uptake systems are specific for glutamate, other neurotransmitters are not transported (Danbolt 2001). However, glutamate transporters may take up the amino acids

aspartate and cysteate (Danbolt 2001). The prevailing K_m values (ranging from 1 to 100 μM, usually 5-30 μM) characterize the affinity of glutamate uptake systems. They vary depending on different preparations and experimental conditions (Bridges et al. 1999).

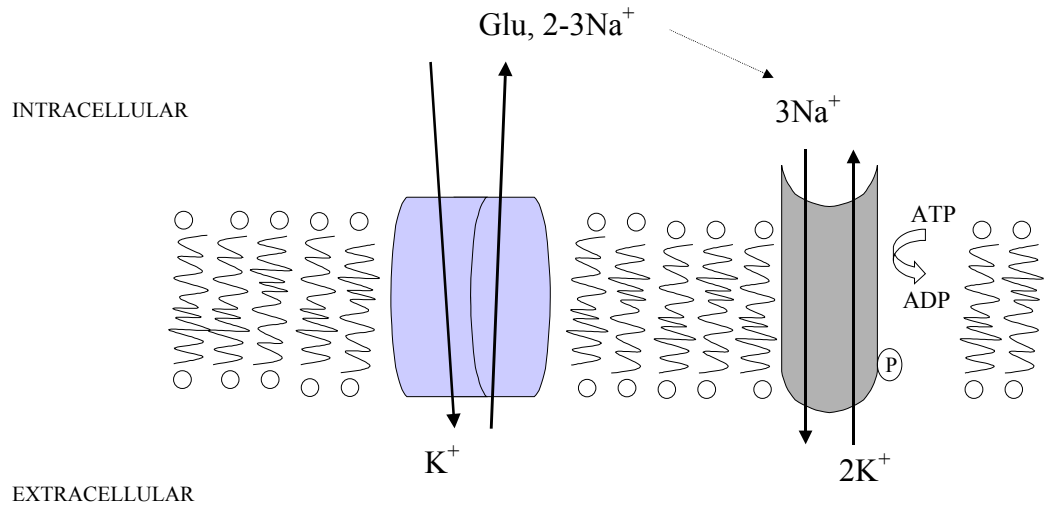

Fig. 2. Glutamate uptake. Glutamate is removed from the extracellular space by the sodium-coupled glutamate transporter, which also translocates a potassium ion in the opposite direction. The main driving force is the electrochemical gradient of sodium ions, which is maintained by the sodium pump (Na/K ATPase).

Since 1992, five subtypes of glutamate transporters have been identified: GLAST, GLT, EAAC1, EAAT4 and EAAT5 (for reviews, see (Gegelashvili and Schousboe 1998, Danbolt 2001, Gegelashvili et al. 2001). The amino acid sequences of the glutamate transporter subtypes share 50-60 % identity and the same transporter homologues represent 90 % identity in mammals (Danbolt 2001). GLT and GLAST are the most abundant glutamate transporters in the brain and are enriched in glial cells. EAAC1 is considered to be a neuron-specific protein in the brain, but it is also discernible outside the CNS. In the brain, EAAC1 is thought to have a minor role in glutamate uptake when compared to GLT and GLAST (Danbolt 2001). EAAT4 is believed to be mainly expressed in cerebellar Purkinje cells and EAAT5 has been detected in the retina. Both EAAT4 and EAAT5 exhibit features of the glutamate-gated chloride channel (Fairman et al. 1995, Arriza et al. 1997).

3.2 Glutamate transporter subtypes in the retina

Strict regulation of extracellular glutamate concentration is pivotal for retinal function, especially for glutamate neurotransmission. All subtypes of glutamate transporters are found in the retina with the exception of EAAT4 (Rauen et al. 1996, Rauen 2000). Müller glial cells and their glutamate transporter GLAST have been suggested to play the major role in retinal glutamate transport (Lehre et al. 1997, Rauen et al. 1998, Rauen and Wiessner 2000). Besides forestalling excitotoxicity, glial cells have a further role: they provide glutamate for metabolic processes, including the glutamate-glutamine cycle (Hertz et al. 1999). The glutamate transporter GLT is expressed in retinal neurons but not in glial cells of the retina, whereas EAAC1 is located only in neurons (Gegelashvili and Schousboe 1998, Rauen and Wiessner 2000, Gegelashvili et al. 2001). Expression of EAAT5 has been detected in Müller cells and retinal neurons (Pow et al. 2000, Rauen 2000). The findings on the expression of glutamate transporter subtypes in the RPE are controversial. GLAST was first detected (Derouiche and Rauen 1995) but a later study produced a negative result (Lehre et al. 1997). Kinetic studies support the presence of glutamate transporters in the RPE, although the subtype has not been determined (Salceda and Saldana 1993, Miyamoto and Del Monte 1994).

3.3 Regulation of glutamate transporters

A number of neurological disorders (e.g. amyotrophic lateral sclerosis and epilepsy) are related to a decrease in the expression or activity of glutamate transporters. Unfortunately, not many of the available substances are known to significantly increase glutamate transporter activity without problematic side effects. On the other hand, various conditions and compounds affect glutamate transporters (Gegelashvili and Schousboe 1997). This research field is crucial for the development of new strategies in the fight against neurological diseases.

Glutamate itself regulates its transporter. The mechanism underlying this may involve changes in the expression of GLAST via activation of metabotropic glutamate receptors (Gegelashvili et al. 2000). Furthermore, transporter substrates may cause rapid redistribution of the transporters (GLAST or EAAT4) from the intracellular compartment and translocation to the cell surface (Duan et al. 1999, Gegelashvili et al. 2000). Molecules which regulate glutamate transporters are often growth factors or modulators of the cellular signalling pathways. They may induce changes in both the activity and the expression of transporters, but the effects are not necessarily direct and may involve different cascades. One essential candidate as a regulator of glutamate transporters is PKC. It has been held to increase glutamate transporter activity via direct phosphorylation (Casado et al. 1993). However, the present data are more or less inconclusive in respect of the direct inhibiting and activating effects on transporter activity (Gegelashvili et al. 2001). Moreover, the PKC activator phorbol 12-myristate 13-acetate (PMA) has been found to increase the cell surface expression of EAAC1, but not to affect the total amount of EAAC1 (Davis et al. 1998). This intracellular trafficking can rapidly regulate functional activity.

A novel discovery related to the regulation of glutamate transporters is the implication here of glutamate transport- associated proteins (GTRAPs). GTRAP 3-18 interacts with EAAC1, whereas GTRAP41 and GTRAP48 interact specifically with EAAT4 (Lin et al. 2001, Jackson et al. 2001). They apparently modulate glutamate transport by anchoring EAAT4 to the actin

cytoskeleton (GTRAP41, GTRAP48) and coupling it to the G-protein signalling cascades (GTRAP48) (Jackson et al. 2001). The substrate affinity of EAAC1 and thus the EAAC1-mediated glutamate transport are reduced by up-regulated expression of GTRAP3-18 (Lin et al. 2001, Butchbach et al. 2002).

3.4 Role of glutamate transporters in retinal diseases

As in neurological diseases, excessive levels of glutamate have also been linked to the pathogenesis of ophthalmic diseases such as glaucoma and diabetic retinopathy (Naskar et al. 2000, Li and Puro 2002). The risk of glutamate toxicity increases especially when the uptake system is impaired, whereas abundant release is believed to have a minor role (Izumi et al. 2002). Müller cells, the principal glial cells in the retina, regulate the extracellular concentration of glutamate by means of efficient uptake (Rauen et al. 1998). In diabetes, viable glutamate transport is of paramount importance, since the compromised blood-retinal barrier may cause glutamate to leak into the retina. Unfortunately, hyperglycemia itself causes GLAST dysfunction in Müller cells (Li and Puro 2002). In glaucoma, attention is focused on GLAST, since it is reduced in the glaucomatous retina, whereas the level of GLT is unaffected (Naskar et al. 2000). The dysfunction of glutamate transport has been observed to have drastic consequences, e.g. resulting in diminished viability of ganglion cells and a notable increase in the vitreal glutamate concentration (Vorwerk et al. 2000). Indeed, retinal ganglion cells seem to be the retinal neurons most vulnerable to glutamate toxicity (Vorwerk et al. 1996, Luo et al. 2001).

4 Investigated drugs

The drugs investigated were tamoxifen, toremifene and chloroquine. Chemically, these drugs are cationic amphiphilic compounds having a hydrophobic moiety and a positively charged hydrophilic side chain (Wolfensberger 1998). Based on this property, they may form polar lipid complexes which are accumulated in lysosomes (Wolfensberger 1998). The molecular structures of the drugs are presented in Fig. 3.

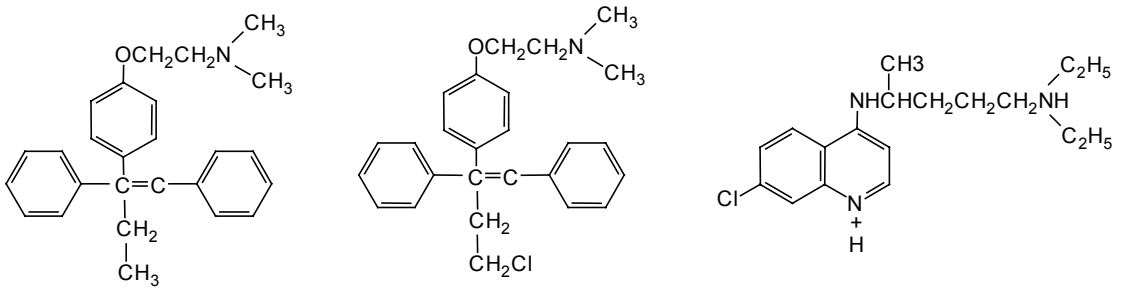

Fig. 3. Structures of the investigated drugs.

4.1 Antiestrogens tamoxifen and toremifene

Tamoxifen and toremifene are selective estrogen receptor modulators (SERM) in that they behave as estrogen antagonists in some tissues and as an agonists in others. In other words they are not completely antiestrogenic and may act as estrogen for example on the endometrium (Rang et al. 1999). Antiestrogens are used mainly in prevention and therapy of breast cancer (Benshushan and Brzezinski 2002), their antitumor activity being due primarily to their antiestrogenic action. Binding to estrogen receptor (ER) fails to activate the transcription machinery which would eventually lead to activation of cell proliferation (De Cupis et al. 1999). In Finland, tamoxifen has been available since 1980 for the treatment of breast cancer, while toremifene was approved for clinical use in 1988 (Mäenpää et al. 2000, Pukkala et al. 2002). These drugs resemble each other structurally, differing only by a single chloride atom (Fig. 3). The predominant metabolic pathways for tamoxifen and toremifene are demethylation and hydroxylation and they may be highly protein bound in plasma (Lien and Lønning 2000). Generally speaking, the clinical anticancer efficacy and the side-effect profiles of tamoxifen and toremifene are comparable (Holli et al. 2000, Holli 2002). Preclinical data point to some differences between the two drugs. For example, the growth of breast cancer MCF-7 cells is more effectively inhibited by tamoxifen (Tominaga et al. 1993) and it has been found to be hepatocarcinogenic and more genotoxic *in vivo* (Kärki et al. 2000, Hirsimäki et al. 2002). When comparing preclinical toxicity data and clinical findings, it should be borne in mind that there is a difference in the respective normal clinical doses used (tamoxifen 20 mg/day and toremifene 40-60 mg/day).

Tamoxifen retinopathy is a rare side effect of tamoxifen (incidence 3-6 %) when a low dose (20 mg/ day, duration 25 months) is used (Pavlidis et al. 1992, Lazzaroni et al. 1998). Retinal findings such as crystalline deposits, macular edema and RPE abnormalities belong to the clinical picture (Yanyali et al. 2001). Crystalline deposits are thought to represent areas of axonal degeneration (Kaiser et al. 1981, Alwitry and Gardner 2002). However, the pathogenic mechanism of tamoxifen-induced changes is largely unknown. It has been hypothesized that tamoxifen forms complexes which accumulate in lysosomes (Imperia et al 1989). Quite recently, a decrease in the activity of lysosomal enzymes and in the phagocytotic activity of RPE cells has been shown (Toimela et al. 1998, Mannerström et al. 2001). Considering the cellular targets of tamoxifen (Table 1), the mechanisms of its action may be assumed to be complex.

Table 1. Examples of the possible cellular targets for tamoxifen

Cellular target	Cell/ tissue studied
Membrane fluidity	Rabbit white muscle (Custodio et al. 1993), human RPE cell line (Engelke et al. 2002)
PKC	Rat brain (O'Brian et al. 1985), cultured rat chondrocytes (Schwartz et al. 2002)
Calmodulin	Bovine brain (Lopes et al. 1990)
Volume-activated chloride channels	Lens organ culture (bovine), human lung carcinoma cell line, mouse fibroblast cell line (Zhang et al. 1994), HeLa cell line (Sahebgharani et al. 2001)
Intracellular calcium concentration	Human neutrophils and various cell lines: canine kidney (MDCK), rat glioma and human prostate cancer (Jan et al. 2000)
NMDA receptor	Rat brain (cortical and hippocampal regions, Cyr et al. 2001)
Mitogen-activated protein kinases	Breast cancer cell line (Mandlekar and Kong 2001)

MDCK, Madin-Darby canine kidney cell line
HeLa, Henrietta Lacks cervix carcinoma cell line

4.2 Chloroquine

Chloroquine (4-aminoquinoline) is a drug used for the treatment and chemoprophylaxis of malaria. It is also used in rheumatoid arthritis and lupus erythematosus. The mechanism of its action is not fully understood, but chloroquine accumulates in lysosomes and inhibits the digestion of hemoglobin in parasites (Rang et al. 1999), which in turn reduces their viability. In rheumatoid diseases, the drug is also concentrated in lysosomes and interferes with hydrolase action. This may lead to the generation of toxic metabolites, decreased release of lysosomal enzymes or changes in lymphocyte proliferation (Rang et al. 1999). Chloroquine has been partially replaced by hydroxychloroquine in view of the fewer side effects of the latter. However, hydroxychloroquine has also been reported to possess lower efficacy and equal toxicity (Browning 2002, Warhurst et al. 2003).

The first description of chloroquine retinopathy was published by Goldman and Preston (1957). The incidence of this disorder ranges from 3 to 10 % of treated patients (Bernstein 1983). Clinically it can be classified as early, advanced and severe. First to appear are paracentral visual field defects and color vision deficiency, and with prolonged medication the defects progress, leading to pigment changes and generalized retinal degeneration (Kellner et al. 2000). The daily dosage is most crucial, cumulative doses and duration of therapy

having a minor role. The safe limit is considered to be 250 mg/day, but larger doses are commonly administered (Browning 2002). In the retina, RPE seems to be the most sensitive target of chloroquine (Bruinink et al. 1991). Pathological changes include degeneration of ganglion cells and photoreceptors (Rosenthal et al. 1978). RPE-linked chloroquine toxicity is believed to involve binding of chloroquine to melanin and lysosomal dysfunction (Rosenthal et al. 1978, Schraermeyer et al. 1999). The latter phenomenon in RPE cells is corroborated by reduced lysosomal enzyme activity in the RPE (Toimela et al. 1998). The blood-retinal barrier is apparently disturbed by chloroquine, though continued efficiency of the blood-brain barrier may partly explain why CNS neuropathy is not detected (Bruinink et al. 1991).

5 Primary cultures, cell lines and organotypic cultures

Tissue cultures were first used almost 100 years ago to elucidate questions of developmental biology (Mather and Roberts 1998). More than forty years later it was demonstrated that single cells could be grown in culture (Mather and Roberts 1998). Cell cultures can be roughly divided into primary cultures and established cell lines. In primary cultures, the availability of original tissue (e.g. human eye) and the developmental phase of cells used (neurons from the adult brain) may give rise to insuperable problems. The majority of established cell lines originate from tumors, which spontaneously give rise to a cell line (Drexler et al. 2000), but they can also be created by transformation *in vitro*. There are a variety of techniques available for the generation of immortalized cells, for example irradiation, chemical carcinogens, viruses and recombinant DNA vectors expressing oncogenes (Stacey and MacDonald 2001). Nowadays centralized cell banks provide uniform, contaminant-free cell lines whose history and characteristic features are well established.

An ideal cell line would exhibit good proliferative capacity, and maintain differentiated features and stability in the normal culture environment as well as in passaging. Cell lines usually provide homogenous populations of cells and grow rapidly. However, continuous cell lines may not express features typical of the original tissue, while primary cells furnish cultures with similar characteristics (Stacey and MacDonald 2001). In fact, the cells derived from an abnormal tumor may be significantly different from normal cells. Also primary cells, e.g. epithelial cells, may lose their characteristics during isolation procedures or in the first weeks *in vitro* (Sambruy et al. 2001). The features of non-continuous cell cultures may manifestly change, especially upon several passages (Grisanti and Guidry 1995). Furthermore, conspicuous disadvantages of primary cultures are the presence of contaminating cells and differences in the genetic background of the isolates (Unger et al. 2002). From the technical point of view, cell lines offer definite advantages: reproducibility, simplicity and reliability (Stacey and MacDonald 2001). As an example of continuous cell lines, two human RPE cell lines are described in Table 2.

The highest complexity *in vitro* is obtained with organotypic cultures. Such cultures may be derived e.g. from explants of embryonic brain or sensory organs (Stacey and Viviani 2001). Organotypic retinal cultures should retain their hierarchical organization, synapses and cellular differentiation as well as formation of outer segments (Pinzon-Duarte et al. 2000). The retinal organ culture is usually established by isolating the neural retina from the anterior segment of the eye as well as from the RPE (Mosinger Ogilvie et al. 1999, Winkler et al. 2002). This is advantageous when studying the degenerative changes induced by separation of the retina from the RPE, but the presence of this latter is known to influence e.g. the

alignment of photoreceptors (Pinzon-Duarte et al. 2000, Winkler et al. 2002). Regardless of technical difficulties, organotypic cultures with adherent RPE have also been established (Hoff et al. 1999, Pinzon-Duarte et al. 2000).

Table 2. General features of the two human RPE cell lines

	ARPE-19[1]	D407[2]
Donor	Male, 19 years	Boy, 12 years
Establishment	Arose spontaneously	Arose spontaneously
Sub-culturing	Not defined	> 200 times
Chromosomes	46 (p.11)	70 (p.52)
Appearance	Cobblestone	Cobblestone
Polarization	Yes	No
Intercellular junctions	Yes	Yes
Barrier properties	Not very good	N.d.
Pigmentation	Variable	Divided, decreasing
Phagocytotic activity	N.d.	Yes
Expression of markers	CRALBP+, RPE65+,	CRALBP+, keratins 7, 8, 18 and 19+, vimentin+, GFAP-, spectrin+,

[1]Dunn et al. 1996 [2]Davis et al. 1995
p., passage of the subculture
N.d., not determined
CRALBP, cellular retinaldehyde-binding protein
RPE65, retinal pigment epithelial specific protein 65
GFAP, glial fibrillary acidic protein

6 Evaluation of toxicity *in vitro*: general considerations

During recent years, the need has increased to establish more acceptable alternatives to animal models. There is also a need for sensitive methods making it possible to investigate single mechanisms. At present, a wide spectrum of studies can be carried out with cultured cells: e.g. determinations of cytotoxicity and studies of single cellular targets such as receptors. The main advantage of an *in vitro* system is the possibility to control the chemical and physical environment of the cells studied. A battery of *in vitro* eye/skin irritation tests has been proposed to replace the classical toxicological *in vivo* method, the Draize test (Balls et al. 1995, Sina et al. 1995, Curren and Harbell 1998). However, attempts to develop, improve and validate *in vitro* tests have raised questions regarding their reproducibility and relevance to *in vivo* situation and acceptability for human safety evaluation (Ferro and Doyle 2001). In-depth studies of the mechanisms of toxicity constitute one of the most pivotal areas of *in vitro* toxicology (Louekari 1996).

Cytotoxicity is primarily understood as the potential of a compound to induce cell death. *In vitro* cytotoxicity tests are necessary in defining basal cytotoxicity and concentration ranges for further and more detailed studies (Eisenbrand et al. 2002). Two main approaches to cytotoxicity, put forward by Freshney (2001), include "negative" toxicity (e.g. quality assurance of pharmaceuticals) and "positive" (selective) toxicity, for example in the development of anticancer drugs. The end points in cellular toxicity are based on e.g. 1) breakdown of the cellular permeability barrier, 2) reduced mitochondrial function, 3) changes in cell morphology, 4) changes in cell replication, and 5) changes in energy metabolism (Stacey and Viviani 2001, Eisenbrand et al. 2002). The need is increasing for toxicity markers which would assist in early prediction of damage. This means detection of defects prior measurable cytotoxicity and at subtoxic levels. Early markers proposed for this purpose include reactive oxygen species, cellular calcium, changes in enzymes and perturbations of cell membranes (Eisenbrand et al. 2002).

In vitro assays are divided into three groups in respect of their duration: short-term assays of viability, long-term assays of cell survival, and the intermediate type (Freshney 2001). The intermediate assays are often based on the use of microtitration plates and a wide range of concentrations and are suitable for screening purposes. Comparisons of single compounds or combinations are possible by establishing the dose at which 50% of cells are affected (EC_{50}) (Eisenbrand et al. 2002). Some drawbacks are nevertheless encountered (Freshney 2001). It is in first place not possible to distinguish between cell loss and metabolic inhibition. Furthermore, differentiation between cytotoxicity and cytostasis and between reversible cell damage and growth of resistant cells is difficult.

AIMS OF THE STUDY

Glutamate transporters in the retina are important for retinal glutamate homeostasis. While the trafficking of glutamate between retinal neurons and glial cells has been widely studied, less is known about the glutamate transporters in RPE cells which form a part of the blood-retinal barrier. The RPE has many functions pivotal for normal visual acuity. Tamoxifen and chloroquine are widely used drugs which have been found to cause retinal side effects. The mechanisms of these harmful effects have remained for the most part obscure.

The aims of the present study were the following:

1. to characterize the properties and expression of glutamate transporters in RPE cells *in vitro* and to evaluate possible differences in glutamate transporters between pig RPE cell cultures with limited life span and human RPE cell lines,

2. to establish whether modulators of cell proliferation affect the expression of glutamate transporter EAAT4 in an RPE cell line,

3. to determine the effects of tamoxifen, toremifene and chloroquine on glutamate uptake in RPE cells and on the viability of human retinoblastoma cells, and

4. to establish a retinal co-culture for study of the protective role of RPE cells for retinal neurons.

MATERIALS AND METHODS

1 Cell cultures

1.1 Pig RPE culture (I-IV)

Fresh pig eyes from a slaughterhouse, delivered in ice-cold saline, were dissected and the retinas gently removed under a dissection microscope. Isolation of the RPE was accomplished by incubation with 0.25 % trypsin solution (Gibco, UK). The isolated cells were resuspended in growth medium (medium components for cell culturing from Gibco, UK): Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % fetal bovine serum (FBS) and 1 % antibiotic/antimycotic solution (ab/am); 4-6 x 10^4 cells/cm^2 were plated in culture flasks. After 11-14 days, the confluent cultures were sub-cultured and passages 1-3 (Fig. 4a) used for experiments. The content of FBS was reduced to 10 % in the medium for subcultures.

1.2 Human RPE cell lines ARPE-19 and D407 and retinoblastoma cell line Y79 (I-III, V)

The human RPE cell line ARPE-19 (Fig. 4c), characterized and introduced by Dunn and colleagues (1996), was obtained from American Type Culture Collection (ATCC, USA). The culture medium for ARPE-19 cells was DMEM/Ham´s F12 (1:1) supplemented with 10 % FBS and 1 % ab/am. Stock cultures were sub-cultured once a week (split ratio 1:6). The human RPE cell line D407 (Davis et al. 1995) was a generous gift from Dr. Hunt (University of South Carolina, USA). The D407 cell line (Fig. 4b) was cultured in DMEM supplemented with 3 % FBS and 1 % ab/am. D407 cells were trypsinized and sub-cultured twice a week (~ 4-6 x 10^4 cells/cm^2 or split ratio 1:4).

Human retinoblastoma cell line Y79 (Reid et al. 1974) was obtained from the European Collection of Cell Cultures (UK) and grown as a suspension of 5x10^5 - 10^6 cells/ml in RPMI 1640 medium supplemented with 10 % FBS and 1% ab/am (Fig. 4d). Sub-culturing of Y79 cells was done by removing one third or half of the cell suspension and replacing it with fresh medium or by centrifuging the cells and plating them in fresh medium (split ratio 1:2-1:4). Y79 cells formed small clusters and a part of the cells were faintly attached to the surface of tissue culture plastics. In general, Y79 cells were more sensitive to environmental and nutritional changes than RPE cells and their viability was more easily altered by simple culturing procedures.

1.3 Retinal co-culture (V)

Retinal co-cultures were established by culturing ARPE-19 cells on Millicell polytetrafluoroethylene filters (Millipore, Bedford, US) and Y79 cells in the lower chamber below them (Fig. 5 and V, Fig. 1). The experimental design of the co-culture is presented in V, Table I. In the co-culture, the cells were grown in serum-free RPMI medium containing 1% ab/am, except for the plating of ARPE-19 cells, which was performed in the normal medium for ARPE-19 cells. Briefly, on day 1, 25x10^4 or 4x10^5 ARPE-19 cells were seeded on coated filters and Y79 cells, 18x10^4 cells/cm^2, were plated on a 24-well plate. The ARPE-19 and Y79 cultures were grown separately for the first 24 h. On the second day, the culture

inserts with ARPE-19 cells were placed in the wells in which the Y79 cells were growing. For adaptation, the cells were allowed to grow in co-culture for 24 h, whereafter they were exposed to the drugs to be investigated (tamoxifen or chloroquine). At the time of exposure, the confluence of the ARPE-19 cell layer (100 %) and the Y79 cell suspension (~70 %) was assessed microscopically.

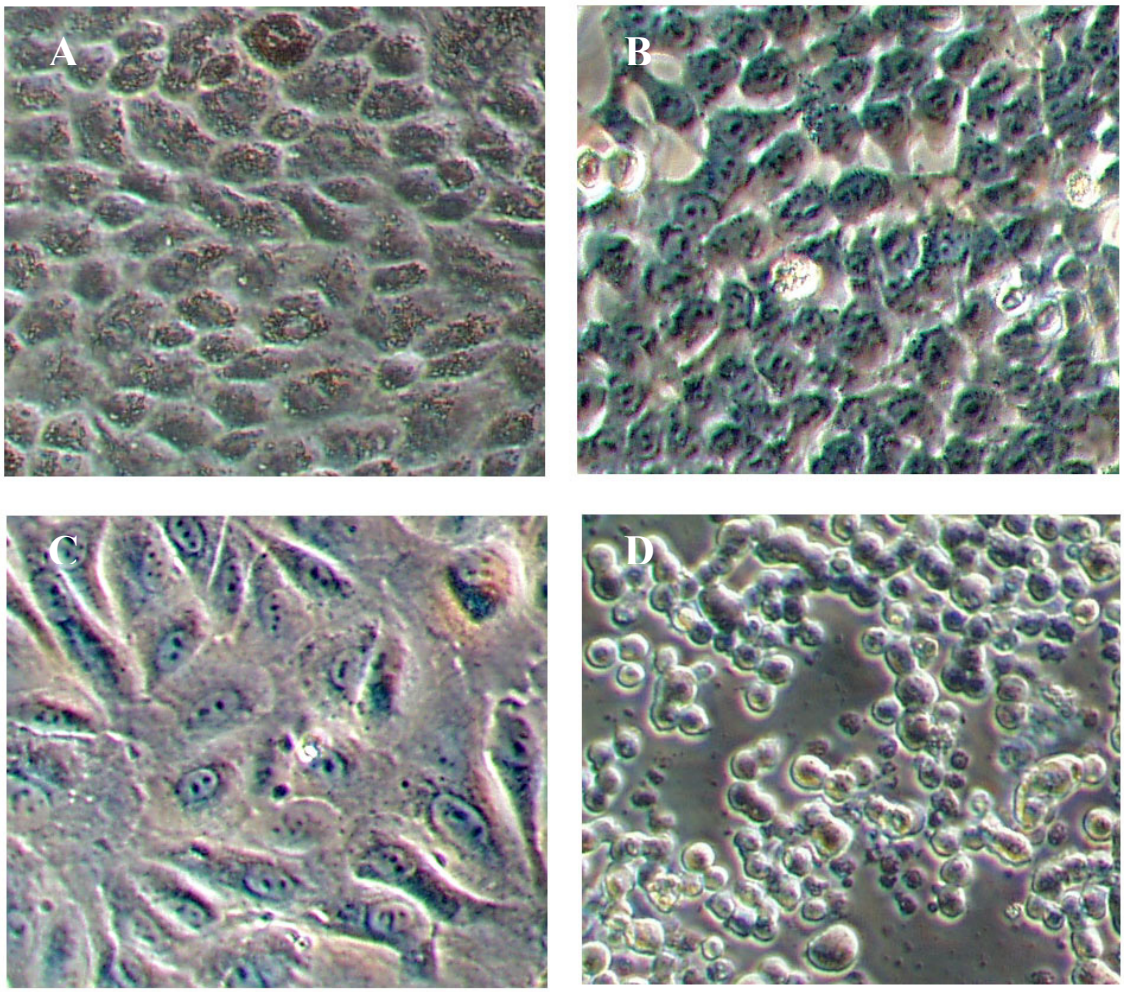

Fig. 4. Photographs of cell cultures. A) pig retinal pigment epithelial cells, B) human RPE cell line D407, C) human RPE cell line ARPE-19 and D) human retinoblastoma Y79 cell line.

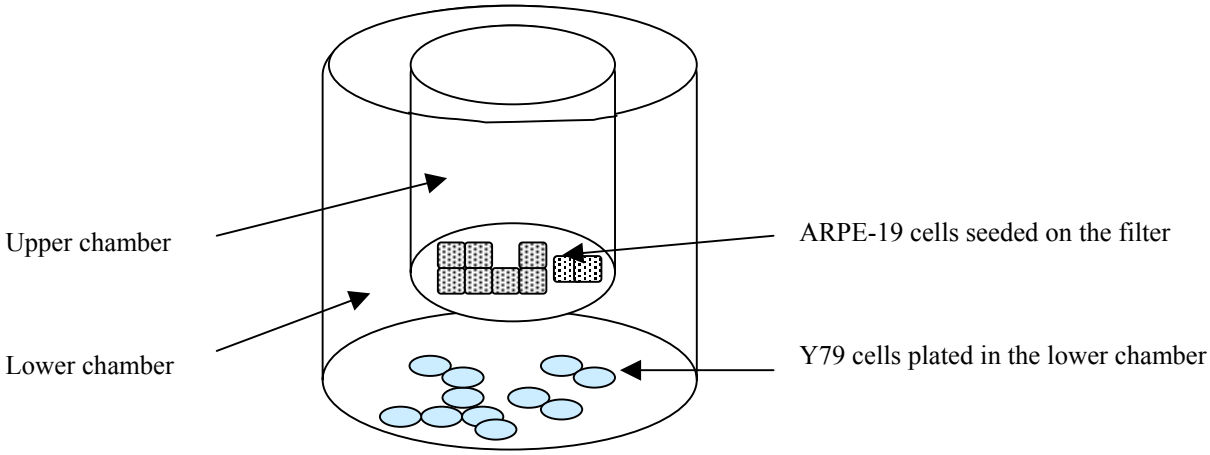

Fig. 5. Schematic illustration of retinal co-culture.

2 Glutamate uptake assays (I, III, IV)

For glutamate uptake assays, cells were cultured to ~80 % confluence in small culture dishes (Ø 35 mm). In the assay, L-[^{3}H]glutamate (1.55 PBq/mol, Amersham, UK) was used as tracer. Prior to pre-incubation the dishes were first washed with oxygenated uptake medium. The test compounds (drugs, selected antagonists and inhibitors) were added during the 10-min preincubation, except when D- and L- aspartate were tested as competitive substrates of glutamate transporters (added together with glutamate). After preincubation, glutamate (containing 23 MBq/l L-[^{3}H] glutamate) was added and the cell cultures incubated at 37°C for a further 10 min. The exception was the time-course experiment, where the incubation times varied from 1 to 60 min. The total glutamate concentration was usually 5 µM; the concentration dependence of uptake was however assessed with 1-1000 µM glutamate. Glutamate uptake was terminated by washing the cultures three times with cold medium. After drying the dishes, the radioactivity of the solubilized RPE was measured with an LKB Wallac 1219 Rackbeta liquid scintillation counter. The breakdown of L-[^{3}H]glutamate during the experiments was found to be negligible when analyzed by thin-layer chromatography.

3 Western blot analysis (II)

Samples for Western blots were prepared from 80-100% confluent cultures which were lysed in buffer (reagents from Sigma, USA, except where indicated) containing 150 mM NaCl, 100 mM Tris-HCl (pH 6.8), 1 % Triton X-100 (Bio-Rad Laboratories, USA) 0.2 % SDS (Bio-Rad Laboratories, USA), 0.5 % deoxycholate and a protease inhibitor cocktail (Complete Mini EDTA-free, Roche Diagnostics, Germany). The lysates were collected into Eppendorf tubes after a few minutes and incubated further at 5°C for 1 h, then triturated and centrifuged at 20 000 *g* for 20 min. The supernatant was used for Western blots (total cell fraction).

The protein samples were suspended in 5x sample buffer containing 60 mM Tris-HCl (pH 6.8), 25 % glycerol, 2 % SDS, 14.4 mM 2-mercaptoethanol and 0.1 % bromophenol blue. Separation of proteins was done by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After overnight protein transfer to a nitrocellulose membrane, the quality of transfer was examined by staining with Ponceau S (Sigma, USA). The membrane was blocked in Tris-buffered saline (TBS) containing 5 % non-fat dried milk and 0.1 % Tween 20 for 60 min. Antibodies against glutamate transporters EAAT4 (0.1 µg/ml, affinity purified by Dr. Gegelashvili and Dr. Rodriguez-Kern at BioSignal, USA,) and GLAST, GLT, EAAC1 and EAAT5 (2-7 µg/ml, Alpha Diagnostic International, USA) were used. The cross-reactivity of the antibodies with other glutamate transporter classes has previously been found to be insignificant, whereas a strong species cross-reactivity between e.g. rat, mouse and human has been detected. The membrane was probed with primary antibodies at 5ºC overnight. The comparable amount of protein in the different lanes was verified with monoclonal anti-β-actin (Sigma, USA), dilution 0.2 µg/ml. Immunoreactive bands were detected with goat peroxidase-conjugated secondary antibodies (Cappel, USA, dilution 1:50000) and by enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham, UK).

4 Immunocytochemistry (II)

The expression of glutamate transporters was investigated by immunocytochemistry in three different RPE cultures. Cells were plated on 8-well chamber slides ($5x10^4$ cells/cm^2) and grown to 80-100 % confluence for staining. The cultures were washed with TBS and fixed for 90 seconds in ice-cold acetone. Blocking of the background staining was effected by incubating the cultures with TBS containing 1.5 % goat serum for 30 min. The cultures were incubated with antibodies against GLAST, GLT, EAAC1, EAAT4 and EAAT5 (Alpha Diagnostic International, USA), diluted 10 µg/ml in TBS containing 0.1 % bovine serum albumin (BSA). Labelling was detected with a Vectastain Elite (peroxidase) staining kit (Vector, USA). After the 30-min incubation with the primary antibodies, the cultures were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:200), followed by incubation with the avidin-biotin-peroxidase complex. Immunoreactivity was visualized with a Vip substrate for peroxidase (acetonitrile, Vector, USA).

5 Cell viability tests: WST-1 and cellular ATP measurement (V)

WST-1 test (Roche, Penzberg, Germany) is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. For the test, $18x10^4$ human retinoblastoma Y79 cells /cm^2 were plated onto 96-well plates in 100 µl serum-free medium and grown for 24 h before drug exposure. The exposure (tamoxifen, toremifene or chloroquine added in 10-µl volume) lasted likewise 24 h. Thereafter 10 µl of the WST-1 reagent was added to each well. The cells were incubated for 45 min in 5% CO_2 at 37°, whereafter the absorbance at 450 nm was measured with a Multiscan MS reader. Cell viability in the test is expressed as a percentage of absorbance of the control wells.

ATP measurement is based on oxidation of the light emitting luciferin in the ATP-dependent reaction catalyzed by luciferase. The efficiency of the reaction reflects the cellular ATP levels as measured with luminescence. As an index of cell viability in the retinal co-cultures, the level of ATP was measured after the 24-h exposure (in both ARPE-19 and Y79 cells separately). Furthermore, for comparison with the co-cultures, ATP was also measured in Y79 cells grown in 24-well plates. For the ATP test, $18x10^4$ Y79 cells/cm^2 were plated on 24-well culture plates in 1 ml of serum-free medium and grown for 48 h before the 24-h drug exposure. Thereafter, 10 % trichloracetic acid (Merck, Germany) was added to the final concentration of 0.5 % and the samples frozen overnight at -80°C. Aliquots (25 µl) of defrosted samples were transferred to the 96-well plate and 100 µl of 1:5 combined ATP Monitoring Reagent and 0.1 M Tris-acetate buffer, pH 7.5, containing 2 mM EDTA (both reagents from Thermo Labsystems, Finland) were added to each well. After gentle agitation luminescence was measured with a Luminoscan Ascent luminometer. Changes in cellular ATP are expressed as percentage of luminescence of the control cells.

6 Protein measurement (I-IV)

The protein content of the samples was determined by a bicinchoninic acid (BCA) -based assay (BCA Protein Assay, Pierce, Rockford, USA). BSA was used as standard.

7 Data analysis and calculations (I, III-V)

Characterization of glutamate uptake involved kinetic analyses. The kinetic parameters for saturable uptake reflecting the affinity of the carrier for the substrate (K_m) and the maximal transport capacity (V_m) were estimated by non-linear regression analysis (Fig.P for Windows software, version 2.2a). The data were fitted with an equation consisting of two components, saturable conforming Michaelis kinetics and non-saturable: $v = V_m*s/(K_m+s) + NSB*s$, where v is the uptake velocity, V_m the maximal velocity of uptake, K_m the Michaelis constant, s the glutamate concentration, and NSB the proportionality constant for non-saturable uptake. The parameters were obtained from three independent determinations carried out in duplicate with different batches of cells. Statistical comparisons of the constants were made with Student's t-test (Graph Pad Prism software, version 3.0).

The estimates of inhibitor concentrations for 50 % inhibition (IC_{50} value) and the effective concentrations for 50 % reduction in cell viability (EC_{50} value) were obtained by fitting the

data to a four-parameter sigmoidal function (Fig.P for Windows). Statistical comparisons of the parameters were made by t-test as above. In general, the results from the independent experiments were pooled and the mean values ± standard error of the mean (SEM) calculated. Statistically significant differences from the corresponding controls were estimated by one-way ANOVA with an adequate post-test (GraphPad Prism) or by the above t-test.

RESULTS

1 Glutamate transport in pig RPE cells and human RPE cell lines

1.1 Kinetic properties of glutamate uptake (I, III)

The human RPE cell line D407 accumulated glutamate more effectively than pig RPE cells. Glutamate uptake consisted of two components, saturable and non-saturable. The transport constants K_m (mean ± SEM) for saturable uptake were 19.0 ± 4.0 μM in the human D407 cell line and 58.3 ± 17.5 μM in pig RPE cells. The maximal velocities (V_m) were 600 ± 22.2 and 195 ± 13.3 μmol kg^{-1} min^{-1}, respectively. In both RPE cells, glutamate uptake was strongly sodium-dependent, since the absence of sodium reduced it by 92 %. L- and D-aspartate significantly inhibited the uptake (84-72 % decrease), whereas the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphonopentanoate (AP-5) caused only minor changes. The transportable inhibitor of glutamate uptake β-hydroxyaspartate (β-HA) diminished glutamate uptake to 19 % in D407 cells and to 31% in pig RPE cells. β-HA also altered the kinetic parameters of saturable glutamate uptake in D407 cells. K_m was more than 10-fold over the control value in the presence of β-HA. In pig RPE cells, the saturable component of glutamate uptake was abolished by β-HA application and glutamate uptake was almost linear.

1.2 Expression of glutamate transporter subtypes (II)

Glutamate transporters EAAT4 and EAAC1 were detected in human RPE cell lines ARPE-19 and D407 according to Western blot analysis. In pig RPE cells, only EAAC1 immunoreactivity was observed. All the detected EAAT4 and EAAC1 bands corresponded to the size of ~65 kDa, as expected from cloned glutamate transporters. None of the other transporter subtypes GLT, GLAST or EAAT5 was found in the RPE cells. Immunocytochemical study of the expression of glutamate transporters in the RPE supported the results obtained by Western blots.

2 Effects of antiestrogens on glutamate uptake in RPE cells (I, III, IV)

The antiestrogens tamoxifen and toremifene reduced glutamate uptake in RPE cells dose-dependently. Concentrations of 5-50 μM tamoxifen and toremifene caused a significant ($p<0.01$) decrease in glutamate uptake in both human cell line D407 and pig RPE cells. In the RPE cell line D407, IC_{50} for tamoxifen was 7.4 ± 1.1 μM and for toremifene 7.6 ± 1.1 μM, and in pig RPE cells 4.6 ± 1.1 μM and 4.7 ± 1.0 μM, respectively. The differences between the IC_{50} values for cell line D407 and pig RPE cells were significant ($p<0.001$). Application of β-estradiol with tamoxifen slightly reduced its inhibitory effect in pig RPE cells, the reduction not however depending on the β-estradiol concentration. Furthermore, β-estradiol (0.1 nM – 1μM) did not affect glutamate uptake.

When glutamate uptake was measured over the concentration range of 5-1000 μM in the presence of 7.5 μM tamoxifen or toremifene, the uptake still comprised saturable and non-saturable components. Toremifene inhibited saturable glutamate uptake at all glutamate concentrations, whereas the inhibitory effect of tamoxifen was only seen at concentrations

below 300 μM (D407 cells) or 500 μM (pig RPE cells). The proportionality constants of non-saturable uptake in the presence of antiestrogens did not significantly differ from those in controls. K_m was increased by tamoxifen and toremifene in both RPE cells, although the effect was more pronounced in D407 cells. V_m was not changed by toremifene but was significantly increased by tamoxifen. When the RPE cultures were pre-exposed to 7.5 μM tamoxifen/toremifene, a slight inhibition of glutamate uptake by tamoxifen was seen only in D407 cells, whereas toremifene caused no significant changes. However, pre-exposure to a high concentration of tamoxifen (30 μM) was strongly inhibitory. Furthermore, when the tamoxifen/toremifene exposure time was lengthened to 24 h, detachment of cells and cell death were clearly discernible.

3 Effects of proliferation modulators on morphology and EAAT4 expression in ARPE-19 cells (II)

Insulin (5 mg/l) increased, whereas retinoic acid (RA, 5 μM) and tamoxifen (3 and 6 μM) inhibited the growth of ARPE-19 cells during the first three days of exposure. After three days there were changes in the shape of insulin- and RA-treated cells (II, Fig. 4). After insulin treatment, ARPE-19 cells formed clusters and some longer processes were microscopically detectable. The shape of RA-exposed cells became more epithelial and ARPE-19 cells started to resemble the primary pig RPE culture. With tamoxifen, there were no notable changes in morphology but cell density was lower when compared to the control culture. The expression of glutamate transporter EAAT4 was not affected by insulin, RA or tamoxifen exposure. The intensity and size of the immunoreactive bands were unchanged after all exposures (II, Fig. 3). There was no difference between the 4- and 7-day exposures or between two tamoxifen concentrations.

4 Expression of glutamate transporter in retinoblastoma cells (II)

Western blot analysis of Y79 cell suspension showed expression of EAAC1 and EAAT4 in Y79 cells. Glutamate transporter subtypes GLT, GLAST and EAAT5 were not detected.

5 Toxicity of drugs in retinoblastoma cultures (V)

After the 24-h exposure, tamoxifen, toremifene and chloroquine reduced Y79 cell viability dose-dependently, with EC_{50} values of 6.4 ± 0.7 μM, 9.6 ± 0.7 μM and 184 ± 22 μM, respectively. The EC_{50} value for tamoxifen was significantly ($p<0.05$) lower than for toremifene. Already 2 μM tamoxifen had detrimental effects on Y79 cells, whereas the lowest effective toremifene concentration was 10 μM. No additive effect in toxicity was seen with antiestrogen concentrations above 15 μM. The chloroquine concentrations 50-1000 μM were considered noxious and no additive reduction in cell viability was gained with concentrations above 400 μM.

6 Toxicity studies in retinal co-cultures (V)

Both ARPE-19 cell plating densities, $25x10^4$ and $4x10^5$, resulted in a microscopically confluent monolayer on the semipermeable filter in 48 h. No changes were detected microscopically in Y79 cells in the lower chamber in the co-culture prior to drug exposure. Tamoxifen (3-6 µM) had no effect on the viability of Y79 cells when dosed via coated or uncoated cell-free filters or via the filter with ARPE-19 cells. Changes in the solvent of tamoxifen (ethanol 0.4%) did not affect the results, but a DMSO content over 0.5 % detached the cells.

Chloroquine exposure via the empty coated filter caused changes in Y79 cell viability (Table 3). When dilution of the drug because of the volume difference between the upper and lower chamber is considered, the effect resembled changes induced in an open system (exposure without culture insert, V, Fig. 3.). The ARPE-19 cell layer on the filter reduced the toxic effect of 600 µM chloroquine (Table 3). The same effect was not seen with lower chloroquine concentrations of 200 and 400 µM. No chloroquine-induced changes in the viability of ARPE-19 cells were detected by ATP measurements in the co-culture.

Table 3. Chloroquine concentrations and changes in the viability of Y79 cells in the retinal co-culture

Chloroquine (µM)		**Chloroquine-induced change in Y79 cell viability (ATP measurement)**		
Upper chamber	Lower chamber (maximally)	Filter without ARPE-19 cells	$24x10^4$ ARPE-19 cells	$40x10^4$ ARPE-19 cells
200	80	+5 %	0 %	+2 %
400	160	-10 %	-8 %	-12 %
600	240	-27 %	0 %	-7 %

DISCUSSION

1 Glutamate transporter in RPE cells *in vitro*

Although the role of glutamate transporters in neural transmission in the brain and retina has been extensively studied (Gegelashvili and Schousboe 1998, Rauen 2000, Danbolt 2001, Gegelashvili et al. 2001), little is known of the glutamate transporter in RPE cells. The present study revealed a high-affinity transport system for glutamate in both the human RPE cell line D407 and in pig RPE cells (I). The capacity of glutamate uptake was higher in the cell line than in pig RPE cells. In both RPE cultures, glutamate transport was seen to be Na^+-dependent and inhibited by β-HA (I, III), the potent transportable glutamate uptake inhibitor (Arriza et al. 1994, Danbolt 2001). Aspartate is apparently one of the substrates of this transporter (I). These features, together with the estimated kinetic parameters for glutamate uptake, strongly support the hypothesis that the glutamate transporter in RPE cells exhibits the properties of the glutamate transporter in the CNS. However, only rough comparison between the kinetic parameters is possible, taking into account their dependency on experimental conditions. Previously, a few functional studies have focused on glutamate uptake in RPE (Salceda and Saldana 1993, Miyamoto and Del Monte 1994, Bridges et al. 2001). In general, our results are in accord with them, showing the presence of an active glutamate transporter in the RPE. However, in the human cell line 165, two saturable components have been detected (Miyamoto and Del Monte 1994), whereas our analysis showed only a single saturable component in both RPE cells studied. The above-mentioned study by Bridges and colleagues focused on glutamate transport in the absence of sodium. In our study only a minor part (less than 10 %) of glutamate uptake was Na^+-independent.

The expression of neuronal glutamate transporter subtypes EAAC1 and EAAT4 in RPE would further indicate the presence of an active glutamate transporter. While both EAAT4 and EAAC1 were detected in the RPE cell lines D407 and ARPE-19, EAAC1 was the only glutamate transporter detected in pig RPE cell cultures (II). Only a few other studies, focusing on the expression of GLAST, have elucidated the expression of glutamate transporter subtypes in the RPE. Derouiche and Rauen (1995) showed the presence of GLAST, while others (Lehre et al. 1997) failed to detect it. The present results strongly suggest, for the first time, the presence of EAAC1 in RPE cells *in vitro*. EAAC1 is a glutamate transporter found in various types of neurons in the retina and in the brain, but also in many types of cells outside the CNS (Gegelashvili and Schousboe 1998, Gegelashvili et al. 2001, King et al. 2001, Novak et al. 2002). EAAC1 is believed to have a quantitatively minor role in glutamate uptake in the brain, since GLT and GLAST are the glutamate transporter subtypes found in astroglia and are thus mainly responsible for the removal of extracellular glutamate (Danbolt 2001). Judging from our findings in the RPE, EAAC1 may have a significant role in the regulation of glutamate in the distal retina, since GLAST and GLT were not detected in the RPE. However, it is important also to bear in mind that e.g. soluble factors in culture medium may induce changes in the expression of transporters (Gegelashvili et al. 1997, Plachez et al. 2000).

Considering the characteristics of the glutamate transporter in the RPE and the location of the RPE cell layer, the transporter may have a vital role in normal retinal functions. The glutamate concentration in the subretinal space must be precisely controlled to obviate the neurotoxicity of glutamate. In this respect, the crucial role of Müller glial cells is noteworthy. They are believed to dominate in the total retinal glutamate transport (Rauen and Wiessner

2000). If the function of these cells is perturbed, the role of the glutamate transporter in RPE cells is particularly important. RPE cells also control the entry of exogenous glutamate from the choroidea analogously to the blood-brain barrier. In addition, the glutamate system is involved in photoreceptor disc shedding (Greenberger and Besharse 1985, Besharse and Defoe 1998).

2 Effects of tamoxifen and toremifene on the function of glutamate transporter in RPE cells

In view of the increased K_m of glutamate uptake in the presence of tamoxifen and toremifene it is to be concluded that they competitively inhibit glutamate uptake in RPE cells (III). When a competitive inhibitor is transportable (substrate inhibitor), it does not abolish the functions of the transport mechanism. It may thus foment glutamate release by hetero-exchange, while a non-transportable competitive inhibitor only attenuates it (Anderson et al. 2001). One is tempted to speculate that tamoxifen acts as a substrate for glutamate transport. The extracellular glutamate concentration is elevated by 1) replacement of glutamate as substrate and 2) increased glutamate release. This, in turn, would increase the risk of glutamate toxicity. Changes in glutamate transport may thus now be envisaged as mechanism related to the defects induced by tamoxifen in the retina. However, further studies on the intracellular tamoxifen concentration and the efflux of glutamate are needed to clarify the nature of competitive inhibition.

In addition to the direct effects of antiestrogens on the glutamate transporter, protein kinase C (PKC) -related and estrogen receptor (ER) -mediated effects are possible. PKC is an enzyme family which phosphorylates many cellular proteins, probably also glutamate transporters (Casado et al. 1993, Pan et al. 1995, Ganel and Crosson 1998, Frank 2002, Bull and Barnett 2002). However, there is a discrepancy in the reported consequences of PKC activation, which has both increased and reduced glutamate uptake in different studies (Dowd and Robinson 1996, Gonzalez et al. 1999, Bull and Barnett 2002). Moreover, PKC has also been shown to regulate the cell surface expression of glutamate transporter EAAC1 (Davis et al. 1998). The explanation for these different effects of PKC remains open. It may be cell type-specific or PKC-isoform-dependent or may vary with the glutamate transporter subtype (Dunlop et al. 1999, Gegelashvili et al. 2001). In any case, tamoxifen has been shown to inhibit PKC (O'Brian et al. 1985, Bignon et al. 1991), and may thus via PKC prevent the activation of glutamate transporter. In pig RPE cells, the PKC inhibitor chelerythrine chloride reduces glutamate uptake (Toimela and Tähti 2001), supporting the involvement of the PKC pathway.

Since tamoxifen and toremifene are partial agonists/antagonists of the ER, ER–related mechanisms should not be overlooked. However, the present results would indicate mechanisms unrelated to the ER, since estradiol did not change glutamate uptake in RPE cells and only slightly affected the reduction evoked by tamoxifen (IV). Furthermore, the exposure time in functional studies was short in view of the ER-mediated effects. Published data on estrogen-related neuroprotection and glutamate toxicity are abundant but inconclusive. For example, estrogen has enhanced the neurotoxicity of glutamate (Yang et al. 2003) while tamoxifen has partially blocked the protective effect of estrogen against excitotoxicity (Kajta and Lason 2000).

The IC_{50} values of glutamate uptake for tamoxifen and toremifene were almost identical. Tamoxifen has previously been found to inhibit glutamate uptake into synaptosomes with IC_{50} of 24 μM (Freund et al. 1995). This concentration is significantly higher than the cytotoxic concentration of tamoxifen and toremifene *in vitro* (Mannerström et al. 2002) and thus not comparable to the present values. Tamoxifen and toremifene are pharmacologically and structurally fairly similar, but retinal defects have been reported only with tamoxifen treatment (Pavlidis et al. 1992). An important reason for this may be the more limited long-term experience with toremifene compared to that with tamoxifen (Pukkala et al. 2002). The present *in vitro* results suggest that if the tamoxifen-induced changes in glutamate uptake are related to retinal defects, toremifene is equally capable of producing these changes. Comparing the present data to those from a previous cytotoxicity study (Mannerström et al. 2002), the changes in glutamate uptake were seen at lower antiestrogen concentrations than the reduction in cell viability. This implies that the measurement of glutamate uptake may be more sensitive to discern defects than the cell viability test.

3 Expression of EAAT4 in cell lines and effect of selected compounds on EAAT4 expression

In addition to what was observed with the RPE cell lines, the detection of EAAT4 in the retinoblastoma cell line Y79 supports the conception that EAAT4 is expressed in cells possessing a strong capacity to proliferate (II). Previously, this transporter has also been detected in a glioma cell line (Gegelashvili et al. 2000). EAAT4 evinces a dual action: it is both transporter and chloride channel (Fairman et al. 1995). Speculatively, EAAT4 may be thought to affect cell migration and proliferation via the following mechanisms: volume regulation by chloride channels affects cell invasiveness (Soroceanu et al. 1999) or EAAT4 binds to the glutamate transporter associated proteins (GTRAPs), these in turn binding to Rho-type GTPases, which affect tumorgenesis (Jackson et al. 2001, Pruitt and Der 2001, Boettner and Van 2002). The effects of drug manipulations on the expression of EAAT4 and on the proliferation of ARPE-19 cells were studied to ascertain whether proliferation changes in the cell lines are directly related to changes in EAAT4 expression. Tamoxifen and RA hindered proliferation of ARPE-19 cells, whereas insulin induced it. In spite of proliferation changes, the size and intensity of the EAAT4 band was not altered after these exposures (II). The constitutively intense expression of EAAT4 was apparently not sensitive to drug exposures. The results may be taken to imply post-translational regulation of EAAT4 influencing e.g. cellular distribution and the presence of this protein at the plasma membrane. It would appear that tamoxifen-induced changes in glutamate uptake are attributable to effects at the functional level as discussed above (section 2).

4 Toxicity of tamoxifen, toremifene and chloroquine *in vitro*

On the basis of the reduced viability of retinoblastoma cells due to antiestrogens, it is suggested that these drugs also have a potential in the therapy of tumors other than breast cancer. Such a conception is corroborated by various *in vitro* studies on different tumor cells (Treon et al. 1998, Simard et al. 2002, Picariello et al. 2003). Furthermore, the defects seen in the primitive neural Y79 cells may reflect the sensitivity of retinal neurons to the toxic effects of antiestrogens. There is no experimental evidence on the expression of ER in Y79 cells.

However, ER was detected in the retina (Ogueta et al. 1999, Munaut et al. 2001) and thus expression in the Y79 cells is also possible. The toxic effects of antiestrogens may involve both ER-dependent and -independent mechanisms. It has become clear that besides antagonizing ER, tamoxifen has multiple additional effects on tumor cells (Pollack et al. 1990, Kishino et al. 1997, Treon et al. 1998, Lehenkari et al. 2003). It has, moreover, induced apoptosis in both ER-positive and ER-negative cells (Chen et al. 1996, Treon et al. 1998, Franke et al. 2003). Since the present EC_{50} values for the antiestrogens were below 10 µM (V), they were within the range of plasma concentrations (2-7 µM) achieved with tamoxifen administration (Couldwell et al. 1996). Furthermore, the tissue levels of tamoxifen in drug therapy may be more than 10 times higher than the serum levels (Lien et al. 1991). The blood-brain barrier hinders tamoxifen penetration in the brain (Vertosick et al. 1992), but the barrier capacity of the RPE may be less efficient. This would help drug entry into the retina and hence increase the risk of toxicity. Such a surmise is corroborated by the finding that tamoxifen penetrates intraocular fluids (Flaxel et al. 2000).

In the co-culture of ARPE-19 and Y79 cells, the toxic effect of 600 µM chloroquine on the Y79 cells was reduced, suggesting a protective role of the RPE (V). It is not clear why there was no similar effect with the lower chloroquine concentrations. The lower sensitivity of the ATP test may be one reason here, since there was a marked difference between the EC_{50} values in the WST test and in the ATP measurement. The melanin pigment of the RPE is noteworthy when considering its protective role. The RPE was found to be a sensitive target for chloroquine (Bruinink et al. 1991) and chloroquine has a high affinity for melanin. However, conclusions regarding the drug binding to melanin have been inconsistent. It has been considered to be on one hand toxicity-increasing and on the other protective (Leblanc et al. 1998, Boulton 1998). Furthermore, the sparse pigmentation of the RPE cell line and the decreasing melanin content of pig RPE cells due to the several passages may generate confusion. In the Y79 cells, the decrease in cell viability was detected at concentrations several times higher than the 0.8-2.5 µM serum levels (Augustijns et al. 1992) (V). The EC_{50} for chloroquine has previously been found to be substantially lower in pig RPE cells (Mannerström et al. 2002) than in Y79 cells. This could be explained by the binding of chloroquine to melanin in pig RPE cells or by the more resistant character of the cell lines. In any case this would imply that the RPE is a target of the adverse effects of chloroquine. Considering the apparent low sensitivity of the retinoblastoma cells, the binding of chloroquine to the multidrug resistance protein (MRP) (Vezmar and Georges 1998) may also play a role.

5 Methodological considerations

5.1 Evaluation of drug effects in vitro

The advantage of an *in vitro* system is the possibility to control the chemical and physical environment of the cells studied. On one hand this improves the repeatability of tests and on the other enables controlled manipulations and exposures. Most often the aim is to provide circumstances for culturing which mimic as well as possible the *in vivo* environment of a specific cell type. Furthermore, in the case of unstable test compounds, these can be replaced frequently to stabilize exposure. However, *in vitro* assays also have essential limitations. To date, systemic distribution, binding and metabolism *in vivo* remain difficult to simulate (Freshney 2001). Moreover, the genetic instability of cultured cells, the lack of a tissue

microenvironment and the absence of barriers are constitute drawbacks of an *in vitro* system (Freshney 2001). The cultural microenvironment is more adequate in organotypic cultures, which reflect the *in vivo* situation better than cultured single cells (Hoff et al. 1999). Cell-specific evaluations are naturally compromised with these complex cultures. Co-cultures with few selected cell types and the usage of conditioned culture media are notable options between cultures of single cell type and organotypic cultures.

5.2 Estimation of cellular viability

There is considerable pressure to replace laboratory animals with cell and tissue cultures whenever possible when evaluating drug effects. A number of *in vitro* assays have been proposed as alternatives, but the choice of an applicable test for the correct prediction of toxicity is difficult. Assay batteries to measure several end points are under development (Sina et al. 1995, Bruner et al. 1997). The correlation of cytotoxicity end points with *in vivo* data is often unsatisfactory and more mechanism-based assays are hoped for (Sina et al. 1995). In the present study, the effects of drugs on the viability of Y79 cells were evaluated after 24-h drug exposures, which is a common time point for cytotoxicity tests (Cenni et al. 1999). The end points of cytotoxicity have been mitochondrial enzyme activity (Mosmann 1983) and the level of cellular ATP (Richter et al. 1996, Lu et al. 2000). The major advantages of cell viability measurements are good reproducibility, simple performance and easy handling of data. After 24-h exposures dose-dependent toxic effects were detected in the sub-confluent cultures, suggesting that the test is appropriate at least for rough toxicity screening. The disadvantage of cell viability assays is the incapability to distinguish between cytotoxicity and cytostasis and between metabolic inhibition and cell loss. For example, with tamoxifen both cytotoxic and cytostatic actions have been recognized (Etienne et al. 1989). Moreover, the type of cell death, apoptotic or necrotic, could only be guessed at. Mitochondrial damage and inhibition of ATP synthesis occur in both processes. In fact, these two forms of cell death are at present not classified as completely separate processes and many mechanisms are involved in the regulation of both (Doble 1999, Proskuryakov et al. 2003). Interestingly, however, the intracellular ATP content has been suggested to be a determinant of the mode of cell death (Richter et al. 1996, Eguchi et al. 1997, Nicotera et al. 1998). At low ATP levels cell death could thus be due to necrosis, whereas at higher ATP concentrations the mechanism could be mostly apoptotic. This inference is based on the findings that apoptotic signalling and apoptotic DNA fragmentation are ATP-dependent (Eguchi et al. 1997, Leist et al. 1997).

As seen with the glutamate transporter activity and expression, cell lines and cell cultures with a limited life span may produce divergent results. Risk is involved in relying on a cell line to represent the presumed phenotypes of normal cells. Parallel use of cell lines and primary cultures or subcultures of normal cells would increase the reliability of *in vitro* studies. However, in a rough cytotoxicity study, cell lines as a replacement for freshly established, time-consuming primary cultures can well be used in the general ranking of various compounds. It is of note, that some cell lines such as ARPE-19 retain many characteristics of normal RPE cells (Dunn et al. 1996, Dunn et al. 1998, Alizadeh et al. 2001, Fan et al. 2002).

5.3 Establishment of retinal co-culture

In addition to *in vitro* studies with single cell types, more complex culture systems such as co-cultures and organotypic cultures can also be applied (Stacey and Viviani 2001, Tähti et al. 2003). Co-culture with separate chambers makes it possible to investigate a single cell type and, on the other hand, cellular interactions via paracrine secretion through the semipermeable filter. There are several filters of this type commercially available. One should consider for example pore size, cell attachment on the filter and microscopic examination properties. It was possible here to investigate the ARPE-19 cell layer microscopically with the polytetrafluoroethylene filter, but the disadvantage was the collagen coating needed for the attachment of ARPE-19 cells. The coating and pore size might again affect drug penetration through the filter, a conception supported by the results with tamoxifen and the cell-free filter (V).

During the past few years, co-cultures have been introduced for different purposes, one of the most advanced being the blood-brain barrier model (Cestelli et al. 2001, Gaillard et al. 2001, Tähti et al. 2003). RPE cells have a major role as a model of the blood-retinal barrier. Apparently it would be possible to develop further the present retinal co-culture for studies on the barrier properties. This would require, first of all, evaluation of barrier integrity by means of leakage of a fluorescent tracer or by measuring transepithelial resistance (Dunn et al. 1996, Gaillard et al. 2001, Tähti et al. 2003). The RPE cell type used should be carefully chosen, since ARPE-19 cells have not been reported to have such favorable and fast-developing barrier properties (Dunn et al. 1996). Unfortunately, primary cells are also reported to lose some of their differentiated features rapidly upon sub-culturing (Grisanti and Guidry 1995, Stacey and Viviani 2001). It is in fact, difficult to attain consensus, since the use of primary cultures is likewise far from effortless. An additional methodological discrepancy arises from the long-lasting tight junction formation (weeks) and from the possible senescence of the RPE after prolonged periods *in vitro*. Furthermore, the lack of nutrients may cause bias in the toxicity estimation of investigated compounds if the cell density is high and the culture media is not changed frequently enough.

SUMMARY AND CONCLUSIONS

(1) The glutamate transporter in RPE cells exhibits features of the high-affinity glutamate transporter in the CNS. This conclusion is based on functional and expression studies on the glutamate transporter in the RPE. Glutamate uptake proved to be a saturable, sodium-dependent process and β-HA and aspartate were also seen to be substrates of the glutamate transporter. Pig RPE cells and both RPE cell lines, D407 and ARPE-19, expressed the neuronal glutamate transporter EAAC1. Glutamate transporter EAAT4 was detected only in the cell lines, pointing to strong expression of EAAT4 in rapidly proliferating cells. In addition, the human RPE cell line D407 exhibited higher uptake capacity compared to pig RPE cells. The results suggest that efficient glutamate transport in RPE cells may have a vital role in regulating retinal glutamate homeostasis, especially if the function of Müller cells in the retina is compromised. Furthermore, the strong expression and activity of the glutamate transporter in RPE cell lines may reflect a role in cell proliferation and migration.

(2) In spite of the robust, cell-line-restricted expression of glutamate transporter EAAT4, manipulations of cell line proliferation did not directly alter the expression levels. Tamoxifen, retinoic acid and insulin were not capable of changing the total levels of EAAT4 in the ARPE-19 cells. This unaltered expression implies the involvement of mechanisms independent of protein synthesis in the regulation of EAAT4.

(3) The antiestrogens tamoxifen and toremifene competitively inhibited glutamate uptake in pig RPE cells and in the human RPE cell line D407. The inhibitory effect was already seen below cytotoxic drug concentrations and was more pronounced in the cell line. Both tamoxifen and toremifene seemed to have the same inhibitory potential in glutamate uptake. Both also reduced the viability of retinoblastoma Y79 cells, but tamoxifen was evidently slightly more toxic to retinoblastoma cells than toremifene. The effect on glutamate uptake and cell viability was seen at concentrations presumably attainable in tissues during normal drug administration. The results suggest that impairment of glutamate uptake may be linked to the tamoxifen-induced retinal defects.

Chloroquine did not affect the glutamate uptake capacity of RPE cells. Furthermore, reduction in the viability of retinoblastoma cells was seen at relatively high drug concentrations. In the retinal co-culture, modification of the detrimental effect of chloroquine was discernible, suggesting a protective role of the RPE. However, the exact reason for this reduction remains open.

(4) A co-culture was constructed with an ARPE-19 cell line growing on a semipermeable filter and Y79 cells growing in the lower chamber. Penetration of the drug through the filter, attachment of cells to the filter, and sufficient amounts of nutrients are pivotal factors when developing a viable co-culture. If it is sought to ascertain the formation of a dense, barrier-like RPE layer on the filter, simple microscopic investigations would not be sufficient.

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