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Evaluation of the cytotoxicity of selected systemic and intravitreally dosed drugs in the cultures of human retinal pigment epithelial cell line and of pig primary retinal pigment epithelial cells

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Abstract

The cytotoxicity of the selected systemic and intravitreally dosed drugs tamoxifen, toremifene, chloroquine, 5-fluorouracil, gentamicin and ganciclovir was studied in retinal pigment epithelium (RPE) in vitro. The cytotoxicity was assayed in the human RPE cell line D407 and the pig RPE cell culture using the WST-1 test, which is an assay of cell proliferation and viability. The effects of experimental conditions on the WST-1 test (cell density, serum content in the culture medium, the exposure time) were evaluated. The EC_{50} values in tamoxifen-treated D407 cells ranged between 6.7 and 8.9 μmol/l, and in pig RPE cells between 10.1 and 12.2 μmol/l, depending on the cell density used. The corresponding values for toremifene were 7.4 to 11.1 μmol/l in D407 cells and 10.0 to 11.6 μmol/l in pig RPE cells. In chloroquine-treated cells, the EC_{50} values were 110.0 μmol/l for D407 cells and 58.4 μmol/l for pig RPE cells. Gentamicin and ganciclovir did not show any toxicity in micromolar concentrations. The exposure time was a significant factor, especially when the drug did not induce cell death, but was antiproliferative (5-fluorouracil). Serum protected the cells from the toxic effects of the drugs. Both cell cultures were most sensitive to tamoxifen and toremifene, and next to chloroquine. The drug toxicities obtained in the present study were quite similar in both cell tyupes; that is, the pig RPE cells and the human D 407 cell line, despite the differences in, for example, the growth rate and melanin contents of the cell types. Owing to the homeostatic functions important for the whole neuroretina, RPE is an interesting in vitro model for the evaluation of retinal toxicity, but, in addition to the WST-1 test, more specific tests and markers based on the homeostatic functions of the RPE are needed. © 2002 Published by Elsevier Science Ltd.

Keywords: Retinal pigment epithelial cultures; Drug effects; Cell proliferation

1. Introduction

The administration of systemic drugs and intravitreally dosed drugs can produce adverse side-effects on the retina. Tamoxifen and toremifene are anti-estrogen drugs used in the treatment of human breast cancer. Tamoxifen has been shown to cause changes in the neural retina and retinal pigment epithelium (RPE) (Pavlidis et al., 1992; Chern and Danis, 1993). Toremifene, which is structurally very close to tamoxifen, has shown no oculotoxic effects in vivo (Williams and Jeffrey, 1997). Chloroquine is an antirheumatic drug and also used in the treatment of malaria. It has been shown to accumulate in the retina and to degenerate photoreceptors after chronic exposure (Rosenthal et al., 1978). 5-Fluorouracil is used for inhibiting the growth of epiretinal membranes in the management of proliferative vitreoretinopathy. It has shown corneal and

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; RPE, retinal pigment epithelium.

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retinal toxicity depending on the dosage and the frequency of intra-ocular injection (Stern et al., 1983; Harstrick et al., 1998). Gentamicin is used in the treatment of bacterial endophthalmitis but may cause retinal disturbances that can limit the visual result (Olson et al., 1983). Ganciclovir is used in the treatment of cytomegalovirus retinitis in AIDS patients, but high-dose intravitreal ganciclovir may cause retinal toxicity (Saran and Maguire, 1994).

RPE is an important outer cell layer in the retina maintaining the retinal homeostatic functions (Mäenpää et al., 1997; Toimela et al., 1998; Mannerström et al., 2001). It is the outer blood–retinal barrier, and is specialised in different transport functions. It removes the outer parts of receptor cells with a continuous active phagocytosis process. Cultured RPE cells maintain the homeostatic functions, for example phagocytosis (Mannerström et al., 2001). The homeostatic functions of RPE are important for the whole neuroretina, and thus this cell type is a promising in vitro model for the evaluation of retinal toxicity.

In the present study, the toxicity of tamoxifen, toremifene, chloroquine, 5-fluorouracil, gentamicin and ganciclovir for RPE cells was studied in vitro. The toxicity of these drugs was compared in human RPE cell line D407 and pig RPE cell culture using the WST-1 test, which is an assay of cell proliferation and viability. The effects of cell density, serum content in the medium and incubation time on the cytotoxicity were also studied.

2. Materials and Methods

2.1. Materials

Tamoxifen, toremifene, chloroquine, 5-fluorouracil, gentamicin and ganciclovir were a gift from Orion Pharma (Turku, Finland). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and antibiotic–antimycotic solution (penicillin G sodium, streptomycin sulfate, amphotericin B) were purchased from Gibco (Paisley, UK). Culture flasks and 96-well plates were from Nunc (Roskilde, Denmark). The WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate} test was based on commercially available cell proliferation reagent WST-1 obtained from Boehringer (Mannheim, Germany).

2.2. Cell cultures

The pig RPE cell culture was established as previously described by Mäenpää et al. (1997). Pig eyes were obtained from a local slaughterhouse. During transport they were kept in ice-cold saline containing 1% antibiotic–antimycotic solution, and used within 3–4 h after death. The eyes were opened aseptically by a circumferential incision just behind the ora serrata, and then the vitreous body and neuroretina were discarded from the eyecups. The RPE cells were isolated by incubating the eyecups filled with 0.25% trypsin buffer for 2 h. The cells were collected and plated at densities of $4–6\times10^4$/cm^2 in 75 cm^2 culture flasks. 20–30 pig eyes were used to start one RPE culture. The cells were cultured in DMEM and supplied with 20% FCS and 1% antibiotic–antimycotic solution in an incubator containing a humidified athmosphere with 5% CO_2 at 37 °C. Half of the medium was replaced after 24 h and then twice a week with DMEM containing 10% FCS. The cells reached confluency after 1 week and were subcultured. After two passages, the cells were plated into 96-well plates either 5×10^4 cells/cm^2 or 1.3×10^5 cells/cm^2 in DMEM supplied with 10% FCS and 1% antibiotic–antimycotic solution. The cells were grown for 24 h before drug exposure. The human RPE cell line D407 was a generous gift from Dr. Hunt (University of South Carolina, USA). The D407 cells were plated into 96-well plates at the density of 1×10^4, 3.3×10^4 or 5×10^4 cells/cm^2 in DMEM, supplied with 3% FCS and 1% antibiotic–antimycotic solution. The cells were grown for 24 h before drug exposure.

2.3. Drug exposure

Before the experiment, the culture medium of the 96-well plates was changed into DMEM containing either 0, 3 or 10% FCS, and preincubated for 20 min. The cells were exposed to different concentrations of tamoxifen (1–25 μM), toremifene (1–25 μM), chloroquine (1–1000 μM), gentamicin (0–90 mM), 5-fluorouracil (0–5000 μM) and ganciclovir (0–40 mM) in a total volume of 100 μl. Chloroquine and ganciclovir were dissolved in water, 5-fluorouracil and gentamicin were dissolved in cell culture medium, and tamoxifene and toremifene were dissolved in water containing 10% DMSO. The final DMSO concentration in the wells was 0.5%. The controls were incubated with equal volumes of drug solvents to avoid changes that could be due to solvent. The cells were incubated with the drugs for 24, 48, 72 and 96 h, depending on the drug.

2.4. Cell viability and proliferation

After the drug treatment, 10 μl of WST-1 reagent was added to each well. The plates were agitated for 1 min, and the cells were incubated with the WST-1 reagent for 1 h in 5% CO_2 at 37 °C. Following incubation, the absorbance at 450 nm was determined using a microplate reader (Victor 1420 Multilabel Counter, Wallac, Finland). Cell viability was expressed as a percentage of absorbance obtained in the treated wells relative to that in the untreated control wells.

2.5. Statistics

In this study design, each test generally consisted of three experiments performed in separate 96-well plates with six parallel concentrations on each plate. The whole of the data from the three experiments was pooled together. The mean±S.E.M. at each concentration level was calculated from 18 observations. Statistical differences between treated cells and controls were evaluated using one-way ANOVA with Dunnett's post test (GraphPad Prism). Sigmoidal dose–response curves were drawn when possible. The dose–response curves were obtained by fitting the data by a non-linear regression analysis to the four-parameter sigmoidal function, and the EC_{50} values were calculated (Sigmaplot).

3. Results

The discontinuous pig RPE cells proliferated slower than the human D407 cell line. In order to achieve about 90% confluency, generally the cell density was 5×10^4 cells/cm^2 for D407 cells and 1.3×10^5 cells/cm^2 for pig RPE cells. In trials where the effect of cell density on the cytotoxicity of the compound (tamoxifen and toremifene) was tested, several cell densities were applied.

3.1. Tamoxifen and toremifene

Cell density affected the cytotoxic effect of drugs. In D407 cells the EC_{50} for tamoxifen was 6.7 μmol/l when 3.3×10^4 cells/cm^2 were used, but 8.9 μmol/l when the cell density was 5×10^4 cells/cm^2 (Fig. 1a). The corresponding values for toremifene were 7.4 μmol/l and 11.1 μmol/l (Fig. 2a). In pig RPE cells, the EC_{50} for tamoxifen was 10.1 μmol/l when 5×10^4 cells/cm^2 were used, and 12.2 μmol/l when the cell amount was 1.3×10^5 cells/well (Fig. 1b). The corresponding values for toremifene were 10.0 μmol/l and 11.6 μmol/l, respectively (Fig. 2b). Serum prevented the cytotoxic effects of the drugs. In D407 cells, when 3% FCS was present, only the highest tamoxifen and toremifene concentrations (25 μM) led to considerable cell death. In pig RPE cells, no cytotoxicity was detected in the whole concentration range (1–20 μM), when 10% FCS was present.

3.2. Chloroquine

Pig RPE cells (Fig. 3b) were more sensitive to chloroquine than D407 cells (Fig. 3a). In both cell cultures the protective effect of serum against chloroquine toxicity was minor. In D407 cells (3.3×10^4 cells/cm^2) the EC_{50} values were 110 μmol/l in serum-free conditions and 122.7 μmol/l in the presence of 3% FCS. The EC_{50} values for pig RPE cells (1.3×10^5 cells/cm^2) were 58.4 μmol/l in serum-free conditions and 60.9 μmol/l in the presence of 10% serum.

3.3. 5-Fluorouracil

5-Fluorouracil showed a quite similar but minor effect on D407 cells (5×10^4 cells/cm^2) (Fig. 4a) and pig RPE cells (1.3×10^5 cells/cm^2) (Fig. 4b). Concentrations of up to 1 mM of 5-fluorouracil decreased the cell viability only about 50% after 24 h of incubation. The responses of cells to 5-fluorouracil were similar in serum-free and in serum-containing (3%) culture medium in both cell cultures. As longer incubation times (48, 72 and 96 h)

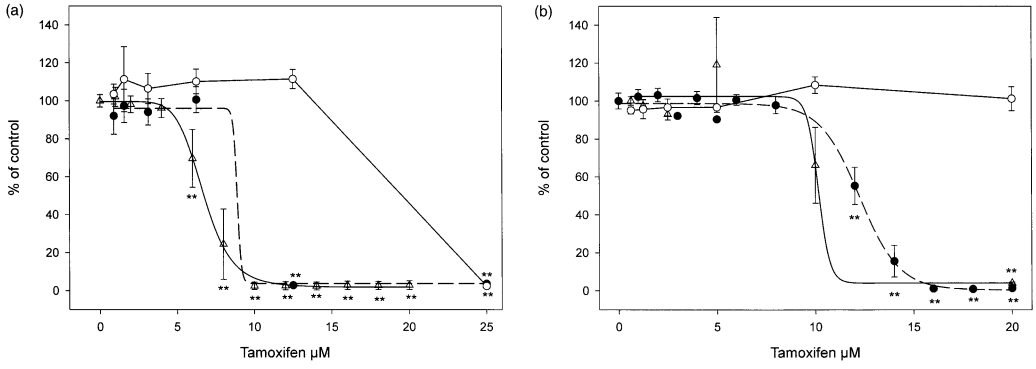

Fig. 1. The viability of human D407 cells and pig RPE cells assayed with the WST-1 test after exposure to various concentrations of tamoxifen for 24 h. (a) Human D407 cell culture: 3.3×10^4 cells/cm^2 without serum in the medium, EC_{50} 6.7 μmol/l (Δ), 5×10^4 cells/cm^2 without serum in the medium, EC_{50} 8.9 μmol/l (●, dashed line), 5×10^4 cells/cm^2 in the presence of 3% serum (○). (b) Pig RPE cell culture: 5×10^4 cells/cm^2 without serum in the medium, EC_{50} 10.1 μmol/l (Δ), 1.3×10^5 cells/cm^2 without serum in the medium, EC_{50} 12.2 μmol/l (●, dashed line), 5×10^4 cells/cm^2 in the presence of 10% serum (○). In all figures each point represents the mean±S.E.M. of three experiments with six samples in each concentration. The significant reductions in viability as compared to control are indicated as $^*P<0.05$ and $^{**}P<0.01$.

and smaller cell amounts (1×10^4 cells/cm^2) were used, there was an initial decrease in cell viability, and a plateau thereafter (Fig. 4c). The viability decreased along with increasing exposure time: after 48 h of exposure with 5-fluorouracil concentrations up to 5 mM, the D407 cell viability was approximately 60%. After 96 h of exposure the amount of viable cells was below 20%, starting from 40 μM 5-fluorouracil concentration.

3.4. Gentamicin

Gentamicin did not contribute to the cell toxicity in micromolar concentrations in either D407 cells (5×10^4 cells/cm^2) (Fig. 5a) or pig RPE cells (5×10^4 cells/cm^2) (Fig. 5b). In D407 cells some reduction in cell viability was seen, starting from 10 mM concentration. Pig RPE cells were less sensitive; any reduction in cell viability was detected at first with 40 mM gentamicin. Serum (3% for D407 cells and 10% for pig RPE cells) seemed to have a slightly protective effect on both cell cultures.

3.5. Ganciclovir

Ganciclovir did not show toxicity in the micromolar concentration range. Pig RPE cells (1.3×10^5 cells/cm^2) (Fig. 6b) seemed to be slightly more sensitive to ganciclovir than D407 cells (5×10^4 cells/cm^2) (Fig. 6a). There was an initial increase in WST-1 in D407 cells, and a reduction started in the 25 mM concentration of ganciclovir when serum was present, and in the 40 mM concentration in the absence of serum. The viability of pig RPE cells showed a slight dose–dependent decrease in the absence of serum. No effect of ganciclovir on pig RPE cell viability was seen in the presence of serum.

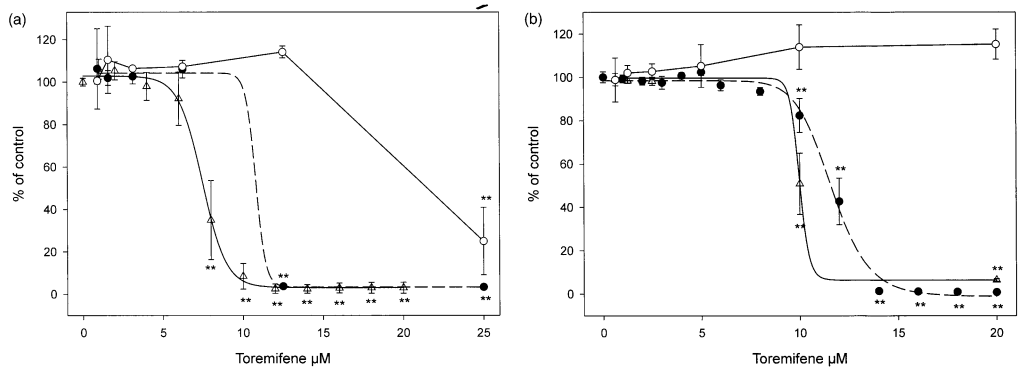

Fig. 2. The viability of human D407 cells (a) and pig RPE cells (b) assayed with the WST-1 test after exposure to various concentrations of toremifene for 24 h. (a) Human D407 cell culture: 3.3×10^4 cells/cm^2 without serum in the medium, EC_{50} 7.4 μmol/l (Δ), 5×10^4 cells/cm^2 without serum in the medium, EC_{50} 11.1 μmol/l (●, dashed line), 5×10^4 cells/cm^2 in the presence of 3% serum (○). (b) Pig RPE cell culture: 5×10^4 cells/cm^2 without serum in the medium, EC_{50} 10.0 μmol/l (Δ), 1.3×10^5 cells/cm^2 without serum in the medium, EC_{50} 11.6 μmol/l (●, dashed line), 5×10^4 cells/cm^2 in the presence of 10% serum (○).

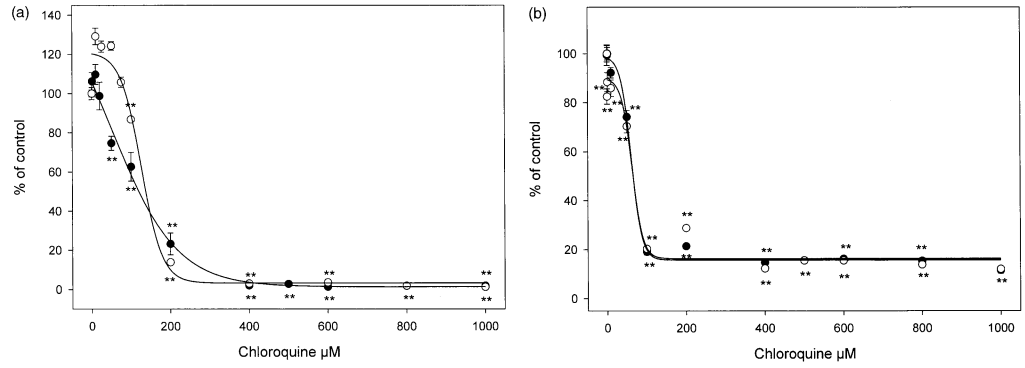

Fig. 3. The viability of human D407 cells (a) and pig RPE cells (b) assayed with the WST-1 test after exposure to various concentrations of chloroquine for 24 h. (a) Human D407 cell culture: 3.3×10^4 cells/cm^2 without serum in the medium, EC_{50} 110.0 μmol/l (●) and 3.3×10^4 cells/cm^2 in the presence of 3% serum, EC_{50} 122.7 μmol/l (○). (b) Pig RPE cell culture: 1.3×10^5 cells/cm^2 without serum in the medium, EC_{50} 58.4 μmol/l (●), and 1.3×10^5 cells/cm^2 in the presence of 10% serum, EC_{50} 60.9 μmol/l (○).

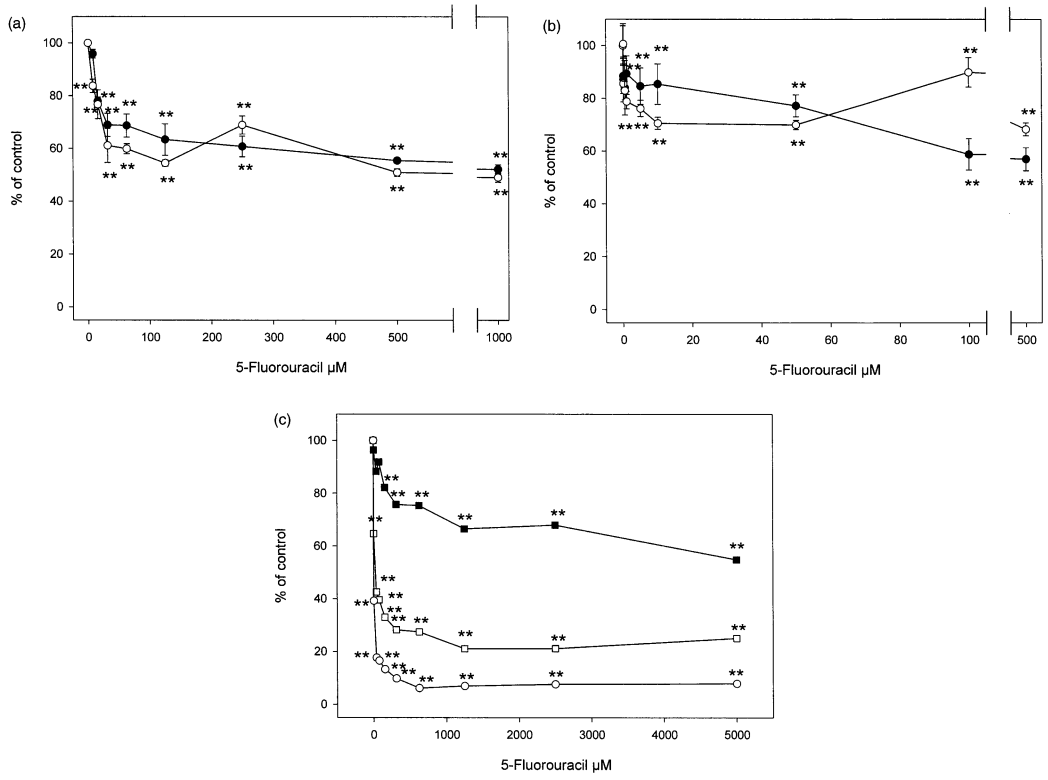

Fig. 4. The viability of human D407 cells (a,c) and pig RPE cells (b) assayed with the WST-1 test after exposure to various concentrations of 5-fluorouracil for 24 h. (a) Human D407 cell culture: 5×10^4 cells/cm^2 without serum in the medium (●), and 5×10^4 cells/cm^2 in the presence of 3% serum (○). (b) Pig RPE cell culture: 1.3×10^5 cells/cm^2 without serum in the medium (●), and 1.3×10^5 cells/cm^2 in the presence of 3% serum (○). (c) Human D407 cell culture: 1×10^4 cells/cm^2 in the presence of 3% serum, exposure time 48 h (■), 1×10^4 cells/cm^2 in the presence of 3% serum, exposure time 72 h (□), and 1×10^4 cells/cm^2 in the presence of 3% serum, exposure time 96 h (○).

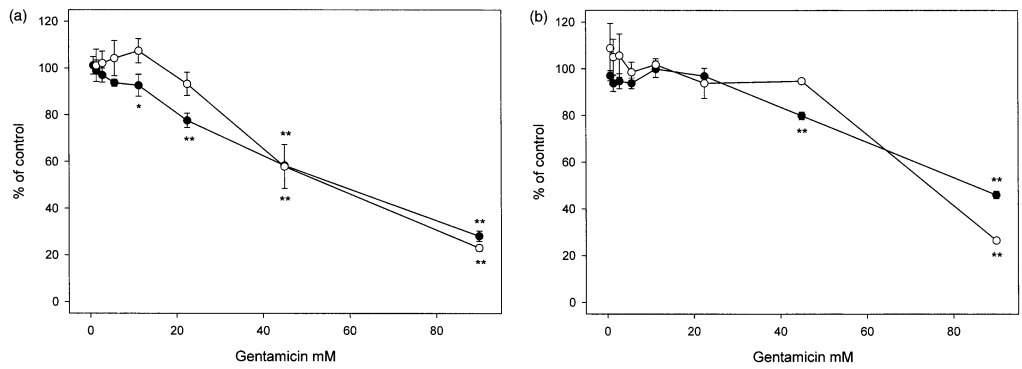

Fig. 5. The viability of human D407 cells (a) and pig RPE cells (b) assayed with the WST-1 test after exposure to various concentrations of gentamicin for 24 h. (a) D407 cell culture: 5×10^4 cells/cm^2 without serum in the medium (●) and 5×10^4 cells/cm^2 in the presence of 3 serum (○). (b) Pig RPE cell culture: 5×10^4 cells/cm^2 without serum in the medium (●), and 5×10^4 cells/cm^2 in the presence of 10% serum (○).

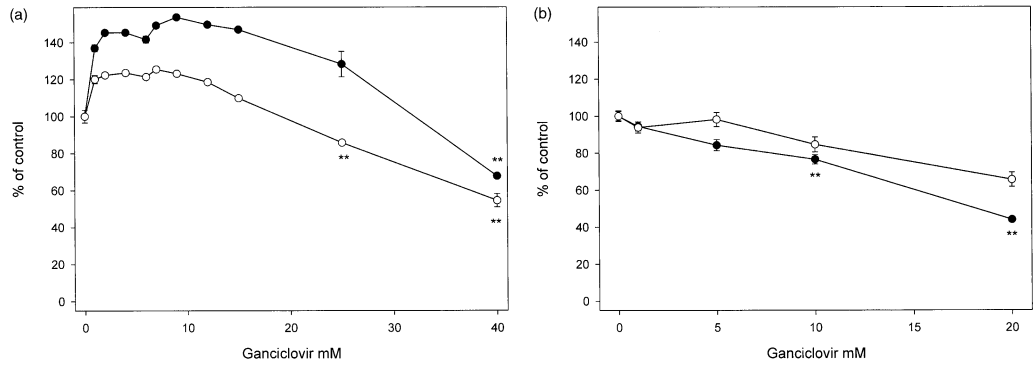

Fig. 6. The viability of human D407 cells (a) and pig RPE cells (b) assayed with the WST-1 test after exposure to various concentrations of ganciclovir for 24 h. (a) Human D407 cell culture: 5×10^4 cells/cm^2 without serum in the medium (●) and 5×10^4 cells/cm^2 in the presence of 3% serum (○). (b) Pig RPE cell culture: 1.3×10^5 cells/cm^2 without serum in the medium (●), and 1.3×10^5 cells/cm^2 in the presence of 3% serum (○).

4. Discussion

In the present study, the effects of tamoxifen, toremifene, chloroquine, 5-fluorouracil, gentamicin and ganciclovir were studied in pig RPE cell culture and human D407 cell line using WST-1 test. In the WST-1 assay, the number of viable cells is proportional to the absorbance. The various dehydrogenase enzymes in mitochondria cleave the tetrazolium salt producing formazan that is detected at the wavelength of 450 nm. Unlike the MTT test, which is widely used to measure cell viability and proliferation, the WST-1 test yields water-soluble cleavage products and thus does not require any additional solubilisation steps before photometric measurements. Ideally, the cell number should be high enough to enable cell-to-cell interactions in the culture. Moreover, the cells should be in an exponential growth phase, but the confluency should not be exceeded. In such a case, WST-1 test can be used to evaluate both the toxic and the antiproliferative effects of compounds on the cells. To achieve the optimal growth phase (90% confluency) for RPE cell cultures with different proliferation rates, different pig RPE and D407 cell densities were used at plating.

Tamoxifen and toremifene are well-known antiproliferative anticancer drugs. There is also evidence of the acute toxicity of tamoxifen. For example, in breast cancer cells tamoxifen increases DNA fragmentation (apoptosis) (Perry et al., 1995; El Etreby et al., 1998). The present in vitro data show that RPE cells were most sensitive to tamoxifen and toremifene. Unlike in tamoxifen treatment, no oculotoxic side effects associated with toremifene treatment have been reported. However, our data suggest quite similar toxicity of these drugs in RPE cells in vitro. When the toxicity of these drugs was tested on human hepatoblastoma cell line HepG2, it was found that the HepG2 cells were far more sensitive to tamoxifen (EC_{50} 10 μmol/l) than to toremifene (EC_{50} 29 μmol/l) (Mäntylä, unpublished data). This is supported by Williams et al. (1998), who have shown that tamoxifen is genotoxic and produces tumours in rat liver, while toremifene is not genotoxic. Therapeutic doses of tamoxifen and toremifene cause serum concentrations of 3.0–3.5 μM for tamoxifen (Stuart et al., 1992) and 2 μM for toremifene (Anttila et al., 1990). As the tissue levels of tamoxifen can be 10–60 times higher than their serum levels (Lien et al., 1991), the concentrations used in the present study (0–25 μM) are within a relevant range.

Chloroquine was less toxic to the RPE cells than were tamoxifen and toremifene. However, a clear reduction of cell viability in both RPE cultures after 24 h of incubation with chloroquine was detected. Chloroquine accumulates within the lysosomes of RPE cells. This leads to inhibition of lysosomal enzymes (Toimela et al., 1998), and possibly to cell injury. Therapeutic doses of chloroquine can cause serum levels of 0.8–2.5 μM (Augustijns et al., 1992). Chloroquine has a high affinity to melanin, which can result in high chloroquine concentrations in RPE (Tsuchiya et al., 1987).

In 5-fluorouracil-treated cells the differences in viability between controls and treated cells became more clear along increasing incubation time, but the dose–response curves did not shift towards a more toxic direction. Therefore we assume that the effect of 5-fluorouracil on the RPE cell cultures was more likely antiproliferative than acutely toxic. This is in accordance with several previous in vivo results, where no toxicity of 5-fluorouracil in the retina was detected (Barrada et al., 1984; Vernot et al., 1985; Joondeph et al., 1988). Instead, the antiproliferative effect of 5-fluorouracil has been reported in RPE cells (Stern et al.,

1983). We used 5-fluorouracil concentrations of up to 650 μg/ml. The maximal daily 5-fluorouracil dosage clinically used is 1000 mg.

In the present study, gentamicin and ganciclovir did not show any toxicity in RPE cells in micromolar concentrations. The highest concentrations used (up to 37 mg/ml for gentamicin and 11 mg/ml for ganciclovir) to find any effects on cells were clearly above the upper limit (100 μg/ml) recommended for the toxicity testing of drugs in vitro. As the optimal plasma concentration applied clinically is 25 μg/ml for gentamicin, and 50 μg/ml for ganciclovir, the concentrations used in our study cover the therapeutic range. There are controversial reports concerning the eye toxicity of gentamicin and ganciclovir. It has been shown that gentamicin causes retinal toxicity, especially in the photoreceptor outer segment/retinal pigment epithelium complex (D'Amico et al., 1985; Talamo et al., 1985). We did not find any relevant toxicity of gentamicin in RPE cultures, which is supported by the results of Brown et al. (1990) and Hines et al. (1993), who have shown that despite crucial damage on retina, the retinal pigment epithelium stays intact after gentamicin treatment in vivo. It has been suggested that the retinal damage associated with ganciclovir treatment is a secondary effect related to the frequent administration procedure rather than due to the toxicity of the substance itself. High doses of intravitreal ganciclovir has been shown to cause retinal damage and visual loss (Saran and Maguire, 1994), but when injected in liposomal encapsules or applied with a scleral plug, no retinal toxicity was observed (Díaz-Llopis et al., 1992; Hashizoe et al., 1994).

In general, pig RPE and D407 cells showed quite consistent responses to the drugs studied. However, some differences were detected: pig RPE cells were somewhat more sensitive to chloroquine and ganciclovir, and less sensitive to gentamicin. The different sensitivities of D407 and pig RPE cells to chloroquine can be due at least partly to the differences in the melanin contents in the cell cultures studied. D407 cells lack melanin almost completely, but in the pig RPE cells it is abundant. Chloroquine has a high affinity to melanin (Tsuchiya et al., 1987; Leblanc et al., 1998; Eves et al., 1999), which may increase the intracellular chloroquine concentration and the toxic effects of chloroquine. This affinity has not been reported, for example, for tamoxifen (Eves et al., 1999). There are also controversial findings of the effect of melanin on the toxic effects of drugs. It has been shown that ocular pigmentation protects retina from gentamicin toxicity (Zemel et al., 1995), which would explain the greater sensitivity of D407 cells to gentamicin compared to pig RPE cells. The significance of the cell density was mainly tested in tamoxifen- and toremifene-treated cells, which showed that the toxicity of the drug was indirectly proportional to the amount of cells used. However, the magnitude of EC_{50} values did not alter, ranging between 6.7 and 12.2 μmol/l for tamoxifen, and 7.4 and 11.6 μmol/l for toremifene. The exposure time also had some significance on the toxicity as discovered in 5-fluorouracil-treated cells. Serum is known to interfere with toxicity assays, since it may bind compounds and therefore reduce the concentrations of free compounds in the culture medium. In the present study, the effect of serum was clearly protective in tamoxifen- and toremifene-treated cell cultures, possibly due to the estrogens in serum, which may compete with these anti-estrogens. Serum was slightly protective in chloroquine and gentamicin-treated cell cultures. In ganciclovir-treated pig RPE cell culture, serum was slightly protective, but in the D407 cell culture, the opposite situation was found. In 5-fluorouracil-treated cells no clear effect of serum was detected. It is known that the binding of aminoglycosides to serum proteins is minor. The binding of drugs to serum differs due to their different physicochemical properties, which in turn dictates the effect of serum in toxicity assays.

In conclusion, tamoxifen and toremifene were most toxic to both of the RPE cell cultures, followed by chloroquine. 5-Fluorouracil did not cause cell death, but it was antiproliferative. Gentamicin and ganciclovir did not show any toxicity in micromolar concentrations. Our findings on the selected drugs support the adverse drug reactions obtained in connection with the clinical use of these compounds. When evaluating the retinal toxicity of chemicals in more detail, however, the homeostatic regulatory functions of RPE should be studied in addition to the simple cytotoxicity measurements. Also other retinal cell cultures, and perfusion cultures of the whole retina should be considered.

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References

Anttila, M., Valavaara, R., Kivinen, S., Mäenpää, J., 1990. Pharmacokinetics of toremifene. Journal of Steroid Biochemistry 36, 249–252.

Augustijns, P., Geusens, P., Verbeke, N., 1992. Chloroquine levels in blood during chronic treatment of patients with rheumatoid arthritis. European Journal of Clinical Pharmacology 42, 429–433.

Barrada, A., Peyman, G.A., Case, J., Fishman, G., Thomas, A., Fiscella, R., 1984. Evaluation of intravitreal 5-fluorouracil, vincristine, VP 16, doxorubicin, and thiotepa in primate eyes. Ophthalmic Surgery 15, 767–769.

Brown, G.C., Eagle, R.C., Shakin, E.P., Gruber, M., Arbizio, V.V., 1990. Retinal toxicity of intravitreal gentamicin. Archives of Ophthalmology 108, 1740–1744.

Chern, S., Danis, R.P., 1993. Retinopathy associated with low-dose tamoxifen. American Journal of Ophthalmology 116, 372–373.

D'Amico, D.J., Caspers-Velu, L., Libert, J., Shanks, E., Schrooyen, M., Hänninen, L.A., Kenyon, K.R., 1985. Comparative toxicity of intravitreal aminoglycoside antibiotics. American Journal of Ophthalmology 100, 264–275.

Díaz-Llopis, M., Martos, M.J., Espana, E., Cervera, M., Vila, A.O., Navea, A., Molina, F.J., Romero, F.J., 1992. Liposomally-entrapped ganciclovir for the treatment of cytomegalovirus retinitis in AIDS patients (Eperimental toxicity and pharmacokinetics, and clinical trial). Documenta Ophthalmologica 82, 297–305.

El Etreby, Liang Y, Wrenn R. W. and Schoenlein P. V. 1998. Additive effect of mifepristone and tamoxifen on apoptotic pathways in MCF-7 human breast cancer cells. Breast Cancer Research and Treatment, *51*, 149–168.

Eves, P., Smith-Thomas, L., Hedley, S., Wagner, M., Balafa, C., Mac Neil, S., 1999. A comparative study of the effect of pigment on drug toxicity in human choroidal melanocytes and retinal pigment epithelial cells. Pigment Cell Research 12, 22–35.

Harstrick, A., Gonzales, A., Schleucher, N., Vanhoefer, U., Lu, K., Formento, J.L., Milano, G., Wilke, H., Seeber, S., Rustum, Y., 1998. Comparison between short or long exposure to 5-fluorouracil in human gastric and colon cancer cell lines: biochemical mechanism of resistance. Anti-Cancer Drugs 9, 625–634.

Hashizoe, M., Ogura, Y., Kimura, H., Moritera, T., Honda, Y., Kyo, M., Hyon, S.H., Ikada, Y., 1994. Scleral plug of biodegradable polymers for controlled drug release in the vitreous. Archives of Ophthalmology 112, 1380–1384.

Hines, J., Vinores, S.A., Campochiaro, P.A., 1993. Evolution of morphologic changes after intravitreous injection of gentamicin. Current Eye Research 12, 521–529.

Joondeph, B.C., Peyman, G.A., Khoobehi, B., Yue, B.Y., 1988. Liposome-encapsulated 5-fluorouracil in the treatment of proliferative vitreoretinopathy. Ophthalmic Surgery 19, 252–256.

Leblanc, B., Jezequel, S., Davies, T., Hanton, G., Taradach, C., 1998. Binding of drugs to eye melanin is not predictive of ocular toxicity. Regulatory Toxicology and Pharmacology 28, 124–132.

Lien, E.A., Solheim, E., Ueland, P.M., 1991. Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. Cancer Research 51, 4837–4844.

Mannerström, M., Mäenpää, H., Toimela, T., Salminen, L., Tähti, H., 2001. The phagocytosis of rod outer segments is inhibited by selected drugs in retinal pigment epithelial cell cultures. Pharmacology and Toxicology 88, 27–33.

Mäenpää, H., Toimela, T., Saransaari, P., Salminen, L., Tähti, H., 1997. Mechanism of tamoxifens retinal toxicity studied in pig pigment epithelial cell cultures. ATLA 25, 297–302.

Olson, J.C., Flynn, H.W., Forster, R.K., Culbertson, W.W., 1983. Results in the treatment of postoperative endophthalmitis. Ophthalmology 90, 692–699.

Pavlidis, N.A., Petris, C., Briassoulis, E., Klouvas, G., Psilas, C., Rempapis, J., Petroutsos, G., 1992. Clear evidence that long-term, low-dose tamoxifen treatment can induce ocular toxicity. A prospective study of 63 patients. Cancer 69, 2961–2964.

Perry, R.R., Kang, Y., Greaves, B.R., 1995. Relationship between tamoxifen-induced transforming growth factor beta 1 expression, cytostasis and apoptosis in human breast cancer cells. British Journal of Cancer 72, 1441–1446.

Rosenthal, A.R., Kolb, H., Bergsma, D., Huxsoll, D., Hopkins, J.L., 1978. Chloroquine retinopathy in the rhesus monkey. Investigative Ophthalmology and Visual Science 17, 1158–1175.

Saran, B.R., Maguire, A.M., 1994. Retinal toxicity of high dose intravitreal ganciclovir. Retina 14, 248–252.

Stern, W.H., Guerin, C.J., Erickson, P.A., Lewis, G.P., Anderson, D.H., Fisher, S.K., 1983. Ocular toxicity of fluorouracil after vitrectomy. American Journal of Ophthalmology 96, 43–51.

Stuart, N.S., Philip, P., Harris, A.L., Tonkin, K., Houlbrook, S., Kirk, J., Lien, E.A., Carmichael, J., 1992. High-dose tamoxifen as an enhancer of etoposide cytotoxicity. Clinical effects and in vitro assessment in p-glycoprotein expressing cell lines. British Journal of Cancer 66, 833–839.

Talamo, J.H., D'Amico, D.J., Hänninen, L.A., Kenyon, K.R., Shanks, E.T., 1985. The influence of aphakia and vitrectomy on experimental retinal toxicity of aminoglycoside antibiotics. American Journal of Ophthalmology 100, 840–847.

Toimela, T., Salminen, L., Tähti, H., 1998. Effects of tamoxifen, toremifene and chloroquine on the lysosomal enzymes in cultured retinal pigment epithelial cells. Pharmacology and Toxicology 83, 246–251.

Tsuchiya, M., Hayasaka, S., Mizuno, K., 1987. Affinity of ocular acid-insoluble melanin for drugs in vitro. Investigative Ophthalmology and Visual Science 28, 822–825.

Vernot, J., Peyman, G.A., Gailitis, R., Fiscella, R., Woodhouse, M., Weinberg, D., 1985. Effects of selected repeated intravitreal chemotherapeutic agents. International Ophthalmology 8, 193–198.

Williams, G.M., Jeffrey, A.M., 1997. Safety assessment of tamoxifen and toremifene. Oncology 11 (5 Suppl. 4), 41–47.

Williams, G.M., Ross, P.M., Jeffrey, A.M., Karlsson, S., 1998. Genotoxicity studies with the antiestrogen toremifene. Drug and Chemical Toxicology 21, 449–476.

Zemel, E., Loewenstein, A., Lei, B., Lazar, M., Perlman, I., 1995. Ocular pigmentation protects the rabbit retina from gentamicin-induced toxicity. Investigative Ophthalmology and Visual Science 36, 1875–1884.