JPET Fast Forward. Published on November 27, 2007 as DOI: 10.1124/jpet.107.128918

JPET# 128918

Celecoxib inhibits proliferation of retinal pigment epithelial and choroid-retinal endothelial cells by a Cox-2 independent mechanism

Aniruddha C Amrite and Uday B. Kompella

Department of Pharmaceutical Sciences (ACA* and UBK) Department of Ophthalmology and Visual Sciences (UBK), University of Nebraska Medical Center, Omaha, NE 68198-5840. *Current address: Quintiles Inc, Overland Park, KS 66211.

JPET Fast Forward. Published on November 21, 2007 as DOI: 10.1124/jpet.107.128918
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running Title: Cox-2 independent antiproliferative effects of celecoxib in the eye b) Corresponding author: Uday B. Kompella, PhD, 985840 Nebraska Medical Center, Omaha, NE 68198-5840, USA. Ph: (402) 559-2974; Fax: (402) 559-5368; E-mail: ukompell@unmc.edu

c) Number of text pages: 34

Number of tables: 4

Number of figures: 6

Number of references: 43

Number of words in the abstract: 247

Number of words in the introduction: 492

Number of words in the discussion: 1591

d) List of abbreviations

AIC: Akaike information criteria; ARMD: age related macular degeneration; VEGF: vascular endothelial growth factor; Cox: Cyclo-oxygenase; ATCC: American type culture collection; FACS: fluorescence-activated cell sorting; HUVEC: human umbilical vein endothelial cells

e) Recommended section: Chemotherapy, Antibiotics, and Gene Therapy

Abstract

Age related macular degeneration (ARMD) is a leading cause of blindness. The major reason for severe vision loss in ARMD is choroidal neovascularization due to an elevation in the expression of angiogenic factors such as vascular endothelial growth factor (VEGF). Drugs with anti-VEGF and anti-proliferative activities can be beneficial for the treatment of this disorder. We have previously demonstrated that celecoxib (a selective cyclooxygenase-2 (Cox-2) inhibitor) inhibits VEGF expression in retinal pigment epithelial cells. In this study, we investigated the anti-proliferative effects of celecoxib in retinal pigment epithelial (ARPE-19) and choroidal endothelial cells (RF/6A). The results indicate that celecoxib: 1) causes a dose dependent anti-proliferative effect in ARPE-19 and RF/6A cells (IC_{50}: 23 and 13 μM, respectively); 2) leads to a G2-M phase cell cycle arrest in these cell types 3) inhibits VEGF induced proliferation of RF/6A cells (IC_{50}: 20 μM); and 4) the concentrations of celecoxib required for anti-proliferative effects are lower than those required for the cytotoxicity. These effects of celecoxib are by mechanisms independent of its Cox-2 inhibitory activity as rofecoxib (another Cox-2 inhibitor) had no effects on the proliferation or cell cycle distribution of the two cell types, and flurbiprofen (an inhibitor of Cox-1 and Cox-2) had weak anti-proliferative effects on ARPE-19 cells with IC_{50} of 90 μM. In summary, celecoxib has potent anti-proliferative effects in RF/6A and ARPE-19 cells and thus can be a potential new treatment in proliferative disorders of the choroid-retina such as choroidal neovascularization in age related macular degeneration.

Introduction

Age related macular degeneration (ARMD) is a leading cause of blindness (Seddon and Chen, 2006). ARMD is broadly divided into two categories, wet and dry ARMD. Although about 80% of the cases with ARMD involve the dry form, most of the severe vision loss problems are associated with the wet form of ARMD (Ferris et al., 1984). The pathophysiology of the wet form of ARMD involves choroidal neovascularization and in some cases proliferation of the retinal pigment epithelium.

Current therapies for ARMD are limited in their efficacy, and lead to a modest improvement in the vision. There is therefore an ardent need to investigate new approaches for the treatment of ARMD, most importantly to prevent the vision loss due to neovascularization. Anti-proliferative drugs which act on specific phase of the cell cycle, and target the proliferating cells could be a beneficial approach for treatment of this advanced aggressive form of ARMD. Such drugs have been used in the treatment of neovascularization associated with malignant disorders. Potentially, a similar approach with or without currently existing treatment regimens might be useful in improving the therapy of choroidal neovascularization associated with ARMD.

Cyclo-oxygenase (Cox) is an important enzyme involved in the process of inflammation. This enzyme has two major isoforms, Cox-1 and Cox-2, which are believed to be the basal and the inducible isoforms respectively, and two lesser known variants termed Cox-3 and PCox-1a (Simmons et al., 2004). Cox inhibitors in general and selective Cox-2 inhibitors in particular have been investigated as potential anti-tumor drugs (Sarkar et al., 2007). Among the Cox-2 inhibitors, celecoxib has been most widely investigated as an anti-tumor agent. Indeed, celecoxib has been shown to have anti-

proliferative effects in several cancer cell types and also in human umbilical vein endothelial cells (HUVEC) cells (Lin et al., 2004; Niederberger et al., 2004). This anti-proliferative potential of celecoxib can be utilized in the treatment of ARMD, where there is proliferation of retinal endothelial cells and retinal pigment epithelial cells (Spaide, 2006).

We have previously demonstrated that celecoxib is capable of inhibiting VEGF expression in RPE cells (Amrite et al., 2006) and diabetic rat retinas (Ayalasomayajula and Kompella, 2003; Amrite et al., 2006). Further, we have shown that celecoxib inhibits diabetes induced elevation in retinal PGE_2 (Ayalasomayajula and Kompella, 2004). Others have demonstrated that celecoxib can prevent corneal neovascularization (Leahy et al., 2002). In the present study we investigated whether celecoxib has anti-proliferative effects on resting and vascular endothelial growth factor (VEGF) stimulated choroid endothelial cells and retinal pigment epithelial cells, the two major cell types involved in the progression of neovascular ARMD. We also investigated whether the anti-proliferative effects of celecoxib were due to inhibition of Cox-2 or due to a Cox-2 independent mechanism. We used ARPE-19 and RF/6A cell lines, which are models for retinal pigment epithelial and choroidal endothelial cells, respectively. For investigating the Cox-2 dependency we investigated the effects with another Cox-2 inhibitor rofecoxib, and a non-selective Cox inhibitor, flurbiprofen.

Methods

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl] benzenesulfonamide) and rofecoxib (4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one) were purchased from ChemPacific Corporation (Baltimore, MD). The anti-proliferative BrdU ELISA kit was purchased from EMD Biosciences Inc (La Jolla, CA). The culture medium DMEM/F12 and F12, trypsin-EDTA, penicillin-streptomycin, and fetal calf serum were purchased from Invitrogen (Carlsbad, CA). PGE_2 ELISA kit was purchased from Cayman Chemicals (Ann Arbor, MI). Recombinant human VEGF-165 was purchased from R& D systems (Minneapolis, MN). ARPE-19 (ATCC # CRL-2302: a spontaneously arising human retinal pigment epithelial cell line) and RF/6A (ATCC # CRL-1780: spontaneously transformed monkey choroid/retinal endothelium) cells were obtained from ATCC (Manassas, VA). All other chemicals including flurbiprofen (2-(3-fluoro-4-phenyl-phenyl)propanoic acid) were of analytical grade and obtained from Sigma (St. Louis, MO).

Cell culture: The ARPE-19 cells were grown in DMEM/F12 with 10% FCS and supplemented with L-glutamine and penicillin-streptomycin. The RF/6A cells were grown in Hams F12 with 10% FCS and supplemented with L-glutamine and penicillin-streptomycin. The medium was changed every other day. For ARPE-19 cells, cells with passage number between 21 and 30 were used for all the experiments. For RF/6A cells, cells of passage number between 40 and 60 were used for all the experiments.

Anti-proliferative effects of drugs on ARPE-19 and RF/6A cells: The anti-proliferative effects of the drugs in ARPE-19 and RF/6A cells were performed as described by Raghava and Kompella with some modifications (Raghava and Kompella, 2007). Briefly, for both cell types, 25,000 cells/well were plated in 96 well plates. One day post plating, the medium was removed and the cells were incubated with serum free medium with or without the drug (1 nM-100 µM). Along with the drug treatment, BrdU label was added to the culture medium. Following the treatments, the cells were processed for the anti-proliferative effects using BrdU ELISA with a kit supplied form the manufacturer and as per the manufacturer's instructions. In addition, the effects of VEGF-165 on proliferation of RF/6A and ARPE-19 cells were examined after incubating the cells for 24 hrs with 0.05-200 ng/ml of VEGF-165. The anti-proliferative effects of celecoxib in presence of VEGF-165 were examined by co-incubating the cells with VEGF-165 (50 ng/ml) and celecoxib (0-100 µM).

Cytotoxicity assessment using the MTT assay: For both cell types, about 10,000 cells per well were plated in 96-well plates and grown till confluency. Following confluency, the medium was removed and the cells were treated with drug solutions in serum free medium for 24 hrs. Following the treatments, the medium was removed and cells were incubated in serum free medium containing MTT for a period of 3 hrs. At the end of 3 hrs, the medium was removed and the formazan crystals formed were dissolved in DMSO and the absorbance was measured at 540 nm.

Pharmacodynamic modeling of toxicity and anti-proliferative effects: The data obtained from the cytotoxicity and anti-proliferative effects (% of control) was plotted against the drug concentrations. The data was fit to either the inhibitory or sigmoidal inhibitory models with or without the baseline effects. The model equations utilized are described below. The model selection was based on the precision of the parameter estimates (mean ± SEM), and the Akaike information criteria (AIC) which is a goodness of fit metric. When comparing two or more similar models, the lower the AIC value, the better the model fits the data. The IC_{50} values were obtained from the model fits. The curve fitting and parameter estimation was performed using WinNonlin 1.5 (Pharsight corporation, Mountain View, CA).

Inhibitory E_{max} model: $E=E_{max}*(1-(C/(C+IC_{50}))$ (1)

Inhibitory E_{max} model with a baseline effect: $E=E_{max}-(E_{max}-E_0)*(C/(C+IC_{50}))$ (2)

Sigmoidal inhibitory E_{max} model: $E=E_{max}*(1-(C^{\gamma}/(C^{\gamma}+IC_{50}{}^{\gamma}))$ (3)

Sigmoidal inhibitory E_{max} model with baseline effect: $E=E_{max}-(E_{max}-E_0)*(C^{\gamma}/(C^{\gamma}+EC_{50}{}^{\gamma}))$ (4)

Where E- pharmacodynamic response; E_{max}- maximum pharmacodynamic response; C- concentration; IC_{50}- concentration for 50% inhibitory effect; E_0- baseline pharmacodynamic response and γ- curve shape factor.

Cell cycle analysis: About 50,000 cells/well were plated in 6-well plates. The cells were allowed to attach and grow for 24 hrs in medium containing serum. The cells were then treated with the drugs in serum free medium (1 nM – 25 μM) for 24 hrs. Following the drug exposure, the medium was removed; the cells were trypsinized and pelleted with

centrifugation. The pellet was re-suspended in 1 ml of PBS and then 3 ml of ice-cold absolute ethanol was added drop-wise. The cells were incubated on ice for 1 hr and stored at -20 ^{o}C until analysis. The ethanolic cell suspension was centrifuged again, washed once with PBS and re-suspended in 1 ml of Telford's reagent overnight. The cell cycle phases were analyzed by FACSCalibur (BD biosciences, San Jose, CA).

PGE_2 secretion from ARPE-19 and RF/6A cells: The cells were grown in 48-well plates. After reaching confluency, the cells were incubated with the drugs (celecoxib, rofecoxib, and flurbiprofen) at varying concentrations (1 - 25 µM) for a period of 24 hrs in serum free medium. The LPS stimulated PGE_2 secretion was examined in ARPE-19 cells. The cells were treated for 24 hrs with 1-25µM of the drugs in serum free medium with LPS (1 µg/ml). At the end of the incubation period, the medium was removed and the PGE_2 secreted in the medium was analyzed using an ELISA kit.The PGE_2 secreted was normalized to the protein content of the cells measured using a Pierce BCA protein assay kit.

Statistical analysis: The statistical criteria used for the model selection have been described above. In order to compare cell cycle analysis and PGE_2 secretion between controls and drug treated cells a one-way ANOVA was used. Post hoc comparisons were made using the Tukey's post hoc analysis. The statistical comparisons were made using SPSS 11.0 (Chicago, IL). All results were considered statistically significant at a p value ≤ 0.05.

Results:

Pharmacodynamic model selection: The model selection criteria indicated that the sigmoidal E_{max} model is the best fit model for anti-proliferative effects as well as cytotoxicity. As an example, for the anti-proliferative effects of celecoxib in RF/6A cells, the final model selection diagnostic comparisons between the four models examined are given in Table 2. Based on the AIC criteria, the sigmoidal inhibitory E_{max} model without a baseline effect is the best model as far as statistical fits to the data are concerned. The IC_{50} values in this study were calculated using this model. The final parameter estimates for the model are shown in Table 2.

Celecoxib has anti-proliferative effects in RF/6A and ARPE-19 cells: The anti-proliferative effects of celecoxib on RF/6A and ARPE-19 cells are shown in Fig. 1. The data was fit to a sigmoidal inhibitory E_{max} pharmacodynamic model. The IC_{50} for the anti-proliferative effects of celecoxib in RF/6A and ARPE 19 cells are 12.17 ± 0.9 and 23.4 ± 0.8 µM, respectively. Neither rofecoxib (another selective Cox-2 inhibitor) (Fig. 2) nor flurbiprofen (a Cox-1 and Cox-2 inhibitor) (Fig. 3) had any anti-proliferative effects on ARPE-19 cells up to 100 µM. Flurbiprofen but not rofecoxib had some anti-proliferative effects on RF/6A cells with an IC_{50} close to 100 µM (Fig. 3C).

Celecoxib is less cytotoxic to non-proliferating cells: The MTT assay for cell survival is a measure of the cytotoxic effects of celecoxib in RF/6A and ARPE-19 cells (Figs. 1B and 1D). With a 24-hr exposure of celecoxib, no cytotoxicity was observed at concentrations up to about 20 µM. The IC_{50} values for the cytotoxic effect in RF/6A and

ARPE-19 cells are 30.2 ± 1.3 and 49.1 ± 2.6 µM, respectively. Rofecoxib and flurbiprofen did not have any cytotoxic effects in ARPE-19 or RF/6A cells up to 100 µM concentrations.

Celecoxib at higher concentrations causes a G2M phase cell cycle arrest: The cell cycle analysis after 24 hrs of exposure to celecoxib ranging in concentrations from 10 nM to 25 µM showed a dose dependent G2-M phase cell cycle arrest at doses >= 10 µM (Fig. 4). There was no significant difference in the cell cycle distribution at lower doses of celecoxib. Neither rofecoxib nor flurbiprofen had any effect on the cell cycle distribution of ARPE-19 cells for up to 25 µM concentration. A slight reduction in the S phase of RF/6A cells with a corresponding increase in the G0-G1 phase was observed with flurbiprofen at a concentration of 25 µM (Fig. 4F).

Celecoxib inhibits VEGF stimulated proliferation of RF/6A cells: We examined the proliferation of RF/6A cells that were supplemented with recombinant human VEGF-165 at concentrations ranging from (0.05-200 ng/ml). VEGF-165 induced proliferation of RF/6A cells at concentrations > 10 ng/ml (Fig. 5A). When the cells were incubated with VEGF-165 (50 ng/ml) in the presence of varying concentrations of celecoxib, no proliferation of RF/6A cells was seen. The IC_{50} for the anti-proliferative effects of celecoxib in the presence of VEGF was 20.4 ± 1.1 µM (Fig. 5). There was no statistically significant difference in the IC_{50} for anti-proliferative effects of celecoxib in RF/6A cells in the presence or absence of VEGF-165. ARPE-19 cells did not respond to the

stimulation by VEGF. There was no cell proliferation up to a VEGF-165 concentration of 200 ng/ml.

Celecoxib, rofecoxib and flurbiprofen inhibit PGE_2 secretion from ARPE-19 and RF/6A cells: The PGE_2 secretion in ARPE-19 cells and RF/6A cells was 1688 ± 866 and 715 ± 519 pg/mg tissues, respectively. Celecoxib, rofecoxib as well as flurbiprofen significantly inhibited the PGE_2 secretion from RF/6A and ARPE-19 cells, at concentrations 10 µM and above. Lipopolysaccharide (LPS; 1 µg/ml) stimulated the PGE_2 secretion from ARPE-19 cells by 2.5 folds and the LPS stimulated PGE_2 secretion was significantly inhibited by all three drugs at the concentrations tested (Table 4).

Discussion

In the present study we demonstrate for the first time that celecoxib, a clinically used Cox-2 inhibitor, has anti-proliferative effects on choroid-retinal endothelial cells and retinal pigment epithelial cells. We also demonstrate that the anti-proliferative effects of celecoxib on these cell types are independent of its Cox-2 inhibitory action. In addition, celecoxib can inhibit VEGF-165 induced proliferation of choroid-retina endothelial cells. Celecoxib also causes a G2-M phase cell cycle arrest in the endothelial and RPE cells with greater anti-proliferative and cell cycle arrest potency in endothelial cells as compared to the RPE cells.

VEGF is thought to be a primary growth factor responsible for the proliferation of endothelial cells and neo-angiogenesis in several pathologies including cancer, diabetic retinopathy and ARMD (Jonas and Neumaier, 2007). VEGF is known to be a survival factor for endothelial cells both in vitro as well as in vivo (Gerber et al., 1998a; Gerber et al., 1998b; Benjamin et al., 1999). VEGF, through VEGF receptor 2 signaling, can induce proliferation and migration of endothelial cells. VEGF also prevents the induction of apoptosis in endothelial cells and this is mediated through the PI3/Akt pathway (Gerber et al., 1998b) .

We have previously demonstrated that celecoxib inhibits VEGF secretion from RPE cells, and also inhibits diabetes induced VEGF secretion from the retina (Amrite et al., 2006). In the present study we found that celecoxib has an anti-proliferative effect in the choroid-retinal endothelial cells with an IC_{50} of ~13 µM. This value is in agreement with the IC_{50} value of 10.6 ± 6.3 µM observed in HUVEC cells, another type of endothelial cells (Niederberger et al., 2004). Thus, celecoxib can have a dual beneficial

effect in ARMD by inhibiting VEGF secretion from retinal pigment epithelial cells and preventing proliferation of endothelial cells. Our results indicate that celecoxib can be an effective anti-proliferative agent for these cell types even in the presence of external VEGF-165 (50 ng/ml), probably due to inhibition of some process downstream of VEGF receptors.

It is interesting to note that statistically significant inhibition in VEGF secretion from ARPE-19 cells is obtained with celecoxib concentrations in the nanomolar range (10 nM or higher) (Amrite et al., 2006). The concentration required for statistically significant anti-proliferative effects of celecoxib on ARPE-19 cells is much higher (~5 µM). Also, the slope for VEGF inhibition is much shallower than the slope for the anti-proliferative effects. Thus, two different mechanisms could be involved in the anti-VEGF and anti-proliferative effects of celecoxib on the ARPE-19 cells.

The possible mechanisms for the anti-proliferative effect could be inhibition of Akt signaling in these cells by celecoxib. Celecoxib is shown to inhibit Akt signaling in several cell types including endothelial cells (Hsu et al., 2000; Lai et al., 2003; Kulp et al., 2004; Wu et al., 2004; Zhang et al., 2004), (Basu et al., 2005; Barnes et al., 2007), (Nam et al., 2004), (Arico et al., 2002) (Lin et al., 2004; Yang et al., 2004). In addition to inhibiting Akt signaling, celecoxib can induce apoptosis in the cells through interaction with extracellular signal-regulated kinase 2 (ERK2) and endoplasmic reticulum calcium ATPase signaling pathways (Hsu et al., 2000; Johnson et al., 2001).

The retinal pigment epithelial proliferation is involved in late age related macular degeneration as well as in proliferative retinopathy (Spaide, 2006). Celecoxib inhibited proliferation of RPE cells in our in vitro studies. For both the cell types, the anti-

proliferative IC_{50} values are less than the IC_{50} for cytotoxicity. Thus, celecoxib is more toxic to proliferating cell types than cells which are non-dividing. However, it is important to note that the cytotoxic and anti-proliferative potencies of celecoxib on these cell types differ only by 2-folds. Thus, with drug level fluctuations associated with an immediate release dosage form (Amrite et al., 2007), it is possible that there could be significant toxicity to normal cells and this might be a limitation in developing celecoxib as an antiproliferative therapeutic agent for CNV. In addition, the mechanisms involved in both the anti-proliferative and the cytotoxic effects might be similar.

We also found that rofecoxib, another Cox-2 selective inhibitor with a Cox-2 selectivity greater than that of celecoxib, and Cox-2 inhibition potency similar to that of celecoxib has no anti-proliferative effects on the choroid-retinal endothelial or the ARPE-19 cells up to a concentration of 100 μM, which is more than 4-7 folds higher concentration than the anti-proliferative IC_{50} concentration of celecoxib in these cell lines. It has been previously demonstrated in HUVEC cells that celecoxib is much more potent than rofecoxib in its anti-proliferative effects (Lin et al., 2004; Niederberger et al., 2004). Also we demonstrate here that flurbiprofen, a non-selective Cox inhibitor with Cox-2 inhibitory potency similar to the selective Cox-2 inhibitors rofecoxib and celecoxib, did not have a significant anti-proliferative effect in the choroidal endothelial cells. Further, in vivo studies indicate that celecoxib inhibits corneal neovascularization (Leahy et al., 2002) but flurbiprofen is unable to do so (Riazi-Esfahani et al., 2006). These observations suggest that not all Cox-2 inhibitors can have anti-angiogenic potential.

While the Cox-2 IC_{50} for celecoxib is in the range of 40-70 nM (Smith et al., 1998), the observed IC_{50} for the anti-proliferative effects is much higher (~10-25 µM) for the two cell types. At these concentrations, there is a significant inhibition of Cox-2 by the drugs tested, as evidenced by the PGE_2 secretion from the two cell types with or without the treatment with the Cox inhibitors. These huge differences in IC_{50} values also point to a Cox-2 independent mechanism for the anti-proliferative effects of celecoxib. With all the three drugs tested, the decrease in PGE_2 secretion occurs at concentrations much higher than the reported Cox-1 and Cox-2 IC_{50} values of the drugs. However, the IC_{50} values reported in the literature were for the purified enzyme and not in a cell culture system (Barnett et al., 1994; Smith et al., 1998; Prasit et al., 1999). The unbound concentrations within the cell could be much lower than the concentrations in the culture medium, and hence, this discrepancy in IC_{50} values can arise. In addition, the two cell types examined, ARPE-19 and RF/6A are pigmented and contain melanin. We have demonstrated that there is significant binding of celecoxib to melanin (Cheruvu et al., 2007). This binding can further reduce the available unbound celecoxib. The effect of celecoxib is not likely due to physicochemical property differences between the three drugs. All three drugs are similar in their water solubility and lipophilicity (as shown in Table 1). Hence, the observed differences are not likely due to differential drug levels achieved by the three drugs within the cells. Also, it is important to note that all the three drugs reduced PGE_2 secretion from RF/6A and ARPE-19 cells, indicating that at the concentrations tested, the drugs were able to inhibit the Cox enzymes (Table 3).

We observed that celecoxib causes a G2-M phase cell cycle arrest in the retinal endothelial cells as well as the retinal pigment epithelial cells. There is also an S phase

cell cycle arrest at the highest concentrations tested (25 µM). The effect is observed at celecoxib concentrations greater than 10 µM. On the other hand, rofecoxib and flurbiprofen have no such effect on the cell cycle. The potency of the cell cycle arrest with celecoxib is greater in RF/6A cells as compared to the ARPE-19 cells. Celecoxib has been shown to selectively inhibit the growth of malignant cells by causing a G2-M phase cell cycle arrest (Dvory-Sobol et al., 2006).

The in-vitro anti-proliferative IC_{50} for celecoxib on these cell types is in the range of 10s of µM. The therapeutically achievable C_{max} levels lie in the 3-8 µM range in the plasma (Davies et al., 2000). The tissue levels could be slightly higher. In this study there was an acute exposure of celecoxib to the cells for 24 hrs. A more sustained exposure over a longer period of time might have anti-proliferative effects at lower concentrations of celecoxib. This is supported by the fact that celecoxib exerts anti-angiogenic and anti-proliferative effects in vivo in cancers in several animal models as well as some human studies (Basu et al., 2005; Soo et al., 2006). Also, celecoxib has been demonstrated to be an effective chemotherapeutic for colon polyps and is in clinical trials as an adjunct therapy for several cancers (Arber et al., 2006; Bertagnolli et al., 2006; Gadgeel et al., 2007; Heath et al., 2007). Development of more permeable analogs of celecoxib with similar anti-proliferative effects could be another strategy to achieve the therapeutically effective levels in the body.

Although this study illustrates some beneficial effects of celecoxib in proliferative retinal disorders, some limitations of this study have to be taken into consideration. The study investigated the effects of the drug on individual cell types without the consideration of the extracellular matrix and the tissue milieu. This can have significant

effects on cell signaling and cell survival, hence the results observed in vitro cannot be directly extrapolated in vivo. It is likely that mechanisms other than those proposed are involved in the anti-proliferative effects. Toxic effects need to be given special attention as the anti-proliferative and cytotoxic potencies of celecoxib on these cell types were close.

Thus, in summary, celecoxib can have anti-proliferative effects in ARPE-19 as well as RF/6A cells and can cause cell cycle arrest in the G2-M phase in these cells. This, together with its anti-inflammatory and anti-VEGF effects provides a strong rationale for potential use of celecoxib by itself or as an adjunct to currently existing therapies in the proliferative disorders of the eye.

Acknowledgements

The authors like to thank the UNMC flow cytometry core facility for assistance with the cell cycle studies.

References

Amrite AC, Ayalasomayajula SP, Cheruvu NP and Kompella UB (2006) Single periocular injection of celecoxib-PLGA microparticles inhibits diabetes-induced elevations in retinal PGE2, VEGF, and vascular leakage. *Invest Ophthalmol Vis Sci* **47**:1149-1160.

Amrite AC, Edelhauser HF and Kompella UB (2007) Modeling of corneal and retinal pharmacokinetics following periocular administration. *Invest Ophthalmol Vis Sci*:In Press.

Arber N, Eagle CJ, Spicak J, Racz I, Dite P, Hajer J, Zavoral M, Lechuga MJ, Gerletti P, Tang J, Rosenstein RB, Macdonald K, Bhadra P, Fowler R, Wittes J, Zauber AG, Solomon SD and Levin B (2006) Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med* **355**:885-895.

Arico S, Pattingre S, Bauvy C, Gane P, Barbat A, Codogno P and Ogier-Denis E (2002) Celecoxib induces apoptosis by inhibiting 3-phosphoinositide-dependent protein kinase-1 activity in the human colon cancer HT-29 cell line. *J Biol Chem* **277**:27613-27621.

Ayalasomayajula SP and Kompella UB (2003) Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model. *Eur J Pharmacol* **458**:283-289.

Ayalasomayajula SP and Kompella UB (2004) Retinal delivery of celecoxib is several-fold higher following subconjunctival administration compared to systemic administration. *Pharm Res* **21**:1797-1804.

Barnes NL, Warnberg F, Farnie G, White D, Jiang W, Anderson E and Bundred NJ (2007) Cyclooxygenase-2 inhibition: effects on tumour growth, cell cycling and lymphangiogenesis in a xenograft model of breast cancer. *Br J Cancer* **96**:575-582.

Barnett J, Chow J, Ives D, Chiou M, Mackenzie R, Osen E, Nguyen B, Tsing S, Bach C, Freire J and et al. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim Biophys Acta* **1209**:130-139.

Basu GD, Pathangey LB, Tinder TL, Gendler SJ and Mukherjee P (2005) Mechanisms underlying the growth inhibitory effects of the cyclo-oxygenase-2 inhibitor celecoxib in human breast cancer cells. *Breast Cancer Res* **7**:R422-435.

Benjamin LE, Golijanin D, Itin A, Pode D and Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* **103**:159-165.

Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB and Hawk ET (2006) Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* **355**:873-884.

Cheruvu NP, Amrite AC and Kompella UB (2007) Effect of eye pigmentation on trans-scleral drug delivery. *Invest Ophthalmol Vis Sci*. In Press.

Davies NM, McLachlan AJ, Day RO and Williams KM (2000) Clinical pharmacokinetics and pharmacodynamics of celecoxib: a selective cyclo-oxygenase-2 inhibitor. *Clin Pharmacokinet* **38**:225-242.

Desai KGH, Kulkarni AR and Aminbhavi TM (2003) Solubility of Rofecoxib in the Presence of Methanol, Ethanol, and Sodium Lauryl Sulfate at (298.15, 303.15, and 308.15) K. *J Chem Eng Data* **48**:942-945.

Dvory-Sobol H, Cohen-Noyman E, Kazanov D, Figer A, Birkenfeld S, Madar-Shapiro L, Benamouzig R and Arber N (2006) Celecoxib leads to G2/M arrest by induction of p21 and down-regulation of cyclin B1 expression in a p53-independent manner. *Eur J Cancer* **42**:422-426.

Ferris FL, 3rd, Fine SL and Hyman L (1984) Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* **102**:1640-1642.

Gadgeel SM, Ruckdeschel JC, Heath EI, Heilbrun LK, Venkatramanamoorthy R and Wozniak A (2007) Phase II study of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), and celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, in patients with platinum refractory non-small cell lung cancer (NSCLC). *J Thorac Oncol* **2**:299-305.

Gerber HP, Dixit V and Ferrara N (1998a) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* **273**:13313-13316.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V and Ferrara N (1998b) Vascular endothelial growth factor regulates endothelial cell

survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* **273**:30336-30343.

Heath EI, Canto MI, Piantadosi S, Montgomery E, Weinstein WM, Herman JG, Dannenberg AJ, Yang VW, Shar AO, Hawk E and Forastiere AA (2007) Secondary chemoprevention of Barrett's esophagus with celecoxib: results of a randomized trial. *J Natl Cancer Inst* **99**:545-557.

Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM and Chen CS (2000) The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* **275**:11397-11403.

Johnson AJ, song X, Hsu A and Chen C (2001) Apoptosis signaling pathways mediated by cyclooxygenase-2 inhibitors in prostate cancer cells. *Adv Enzyme Regul* **41**:221-235.

Jonas JB and Neumaier M (2007) Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Exudative Age-Related Macular Degeneration and Diffuse Diabetic Macular Edema. *Ophthalmic Res* **39**:139-142.

Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH, Fowble JW, Ward PJ and Chen CS (2004) 3-phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. *Cancer Res* **64**:1444-1451.

Lai GH, Zhang Z and Sirica AE (2003) Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth in vitro through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol Cancer Ther* **2**:265-271.

Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT and Masferrer JL (2002) Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* **62**:625-631.

Li P and Zhao L (2003) Solubilization of flurbiprofen in pH-surfactant solutions. *J Pharm Sci* **92**:951-956.

Lin HP, Kulp SK, Tseng PH, Yang YT, Yang CC, Chen CS and Chen CS (2004) Growth inhibitory effects of celecoxib in human umbilical vein endothelial cells are mediated through G1 arrest via multiple signaling mechanisms. *Mol Cancer Ther* **3**:1671-1680.

Nam DH, Park K, Park C, Im YH, Kim MH, Lee S, Hong SC, Shin HJ, Kim JH, Eoh W and McDonnell TJ (2004) Intracranial inhibition of glioma cell growth by cyclooxygenase-2 inhibitor celecoxib. *Oncol Rep* **11**:263-268.

Niederberger E, Manderscheid C, Grosch S, Schmidt H, Ehnert C and Geisslinger G (2004) Effects of the selective COX-2 inhibitors celecoxib and rofecoxib on human vascular cells. *Biochem Pharmacol* **68**:341-350.

Prasit P, Wang Z, Brideau C, Chan CC, Charleson S, Cromlish W, Ethier D, Evans JF, Ford-Hutchinson AW, Gauthier JY, Gordon R, Guay J, Gresser

M, Kargman S, Kennedy B, Leblanc Y, Leger S, Mancini J, O'Neill GP, Ouellet M, Percival MD, Perrier H, Riendeau D, Rodger I, Zamboni R and et al. (1999) The discovery of rofecoxib, [MK 966, Vioxx, 4-(4'-methylsulfonylphenyl)-3-phenyl-2(5H)-furanone], an orally active cyclooxygenase-2-inhibitor. *Bioorg Med Chem Lett* **9**:1773-1778.

Raghava S and Kompella UB (2007) AQ4, an antitumor anthracenedione, inhibits endothelial cell proliferation and vascular endothelial growth factor secretion: Implications for the therapy of ocular neovascular disorders. *Eur J Pharmacol*.

Riazi-Esfahani M, Peyman GA, Aydin E, Kazi AA, Kivilcim M and Sanders DR (2006) Prevention of corneal neovascularization: evaluation of various commercially available compounds in an experimental rat model. *Cornea* **25**:801-805.

Sarkar FH, Adsule S, Li Y and Padhye S (2007) Back to the Future: COX-2 Inhibitors for Chemoprevention and Cancer Therapy. *Mini Rev Med Chem* **7**:599-608.

Seddon JM and Chen CA (2006) Epidemiology of age related macular degeneration, in *Retina* (Ryan SJ ed) pp 1017-1028, Elsevier-Mosby, Philadelphia, PA.

Seedher N and Bhatia S (2003) Solubility enhancement of Cox-2 inhibitors using various solvent systems. *AAPS PharmSciTech* **4**:E33.

Simmons DL, Botting RM and Hla T (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* **56**:387-437.

Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K and Isakson PC (1998) Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc Natl Acad Sci U S A* **95**:13313-13318.

Soo RA, Wu J, Aggarwal A, Tao Q, Hsieh W, Putti T, Tan KB, Low JS, Lai YF, Mow B, Hsu S, Loh KS, Tan L, Tan P and Goh BC (2006) Celecoxib reduces microvessel density in patients treated with nasopharyngeal carcinoma and induces changes in gene expression. *Ann Oncol* **17**:1625-1630.

Spaide R (2006) Etiology of late age related macular disease, in *Age related macular degenration* (Alfaro VD, Liggett PE, Mieler WF, Quiroz-Mercado H, Jager R and Tano Y eds) pp 23-39, Lippincot Williams & Wilkins Philadelphia, PA.

Wu T, Leng J, Han C and Demetris AJ (2004) The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. *Mol Cancer Ther* **3**:299-307.

Yang HM, Kim HS, Park KW, You HJ, Jeon SI, Youn SW, Kim SH, Oh BH, Lee MM, Park YB and Walsh K (2004) Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. *Circulation* **110**:301-308.

Zhang Z, Lai GH and Sirica AE (2004) Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology* **39**:1028-1037.

Footnotes

This work was supported by NIH grants R24 EY017045, R01 DK064172, R03 EY013842, and R21 EY017360.

Uday B. Kompella, PhD, 985840 Nebraska Medical Center, Omaha, NE 68198-5840, USA. Ph: (402) 559-2974; Fax: (402) 559-5368; E-mail: ukompell@unmc.edu

Legends for Figures

Fig 1: Effects of celecoxib on cell proliferation and cytotoxicity in ARPE-19 and RF/6A cells. A) Cell proliferation by BrdU assay in ARPE-19 cells; B) Cytotoxicity assessment by MTT assay in ARPE-19 cells; C) Cell proliferation by BrdU assay in RF/6A cells; D) Cytotoxicity assessment by MTT assay in RF/6A cells. Data are presented as mean ± s.d for n=6. The IC_{50} values are calculated using the inhibitory sigmoid E_{max} model. The solid line is the fit using the model.

Fig 2: Effects of rofecoxib on cell proliferation and cytotoxicity in ARPE-19 and RF/6A cells. A) Cell proliferation by BrdU assay in ARPE-19 cells; B) Cytotoxicity assessment by MTT assay in ARPE-19 cells; C) Cell proliferation by BrdU assay in RF/6A cells; D) Cytotoxicity assessment by MTT assay in RF/6A cells. Data are presented as mean ± s.d for n=6. The IC_{50} values are calculated using the inhibitory sigmoid E_{max} model. The solid line is the fit using the model.

Fig 3: Effects of flurbiprofen on cell proliferation and cytotoxicity in ARPE-19 and RF/6A cells. A) Cell proliferation by BrdU assay in ARPE-19 cells; B) Cytotoxicity assessment by MTT assay in ARPE-19 cells; C) Cell proliferation by BrdU assay in RF/6A cells; D) Cytotoxicity assessment by MTT assay in RF/6A cells. Data are presented as mean ± s.d for n=6. The IC_{50} values are calculated using the inhibitory sigmoid E_{max} model. The solid line is the fit using the model.

Fig 4: Effect of drugs on cell cycle of ARPE-19 and RF/6A cells. A) Effect of celecoxib on cell cycle of ARPE-19 cells; B) Effect of celecoxib on cell cycle of RF/6A cells. C) Effect of rofecoxib on cell cycle of ARPE-19 cells; D) Effect of rofecoxib on cell cycle of RF/6A cells; E) Effect of flurbiprofen on cell cycle of ARPE-19 cells; F) Effect of flurbiprofen on cell cycle of RF/6A cells. Data are expressed as mean ± s.d for n=3.

Fig 5: VEGF-165 induces proliferation in B)RF/6A but not A) ARPE-19 cells at concentrations greater than 10 ng/ml. Data are expressed as mean ± s.d. for n=6-8. Celecoxib inhibits proliferation of C) ARPE-19 and D) RF/6A cells in presence of VEGF-165 (50 ng/ml) stimulus.

Fig 6: Proposed mechanism for the use of celecoxib in choroidal neovascularization and RPE proliferation. (-) Indicates inhibition by celecoxib.

JPET Fast Forward. Published on November 21, 2007 as DOI: 10.1124/jpet.107.128918
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: properties of drugs used in the study

Drug	Log D (pH 7) (Sci Finder)	Solubility (25 ^{0}C) (µg/ml)	Cox-1 IC_{50} (µM)	Cox-2 IC_{50} (µM)
Celecoxib	4.21	7 (Seedher and Bhatia, 2003)	15 (Smith et al., 1998)	0.04 (Smith et al., 1998)
Rofecoxib	1.34	8.19 ± 0.03 (Desai et al., 2003)	>15 (Prasit et al., 1999)	0.02 (Prasit et al., 1999)
Flurbiprofen	4.12	9.9 (Li and Zhao, 2003)	0.04 (Barnett et al., 1994)	0.51 (Barnett et al., 1994)

Table 2: Pharmacodynamic model selection for anti-proliferative effects of celecoxib on RF/6A cells.

Model	Parameter Estimate (Mean ± SEM)				Correlation Coefficient	AIC[a]
	E_{max}	IC_{50}	γ	E_0		
1) Inhibitory E_{max}	100.5 ± 3.3	8.1 ± 1	NA	NA	0.93	764.7
2) Inhibitory E_{max} with baseline effect	100.2 ± 3	10.7 ± 2.2	NA	$8x10^{-5}$ ± 4.12	0.94	752
3) Sigmoidal inhibitory E_{max}	94 ± 2.6	12.7 ± 0.9	2.1 ± 0.3	NA	0.95	741.2
4) Sigmoidal inhibitory E_{max} with baseline effect	93.9 ± 2.6	12.9 ± 1.1	2.2 ± 0.4	$9x10^{-6}$ ± 4.7	0.95	743.0

([a]AIC: Akaike Information Criteria; statistical goodness of fit criteria used to select among models for a particular dataset. The lower the AIC value, better is the statistical fit to the data)

JPET Fast Forward. Published on November 21, 2007 as DOI: 10.1124/jpet.107.128918
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3: Celecoxib, rofecoxib, and flurbiprofen inhibit PGE_2 secretion from ARPE-19 and RF/6A cells at concentrations used in the study

Treatment	PGE_2 secretion (pg/mg protein)	
	ARPE-19	RF/6A
Controls	1688 ± 866	715 ± 519
Celecoxib 1 µM	1540 ± 712	272 ± 545*
Celecoxib 10 µM	413 ± 827*†	ND†
Celecoxib 25 µM	ND†	ND†
Rofecoxib 1 µM	982 ± 1330	332 ± 664*
Rofecoxib 10 µM	2125 ± 227	160 ± 321†*
Rofecoxib 25 µM	ND†	ND†
Flurbiprofen 1 µM	1513 ± 1164	245 ± 490†*
Flurbiprofen 10 µM	1065 ± 1965*	ND†
Flurbiprofen 25 µM	508 ± 1017†*	ND†

The data are presented as mean ± s.d. for n = 4. ND: not detected. Levels below the limit of detection of the assay (36 pg/ml) were considered as zero.*Detected in only one out of the 4 samples. †Statistically significant difference with respect to untreated control cells ($P \leq 0.05$).

JPET Fast Forward. Published on November 21, 2007 as DOI: 10.1124/jpet.107.128918
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4: Celecoxib, rofecoxib, and flurbiprofen inhibit lipopolysaccharide (LPS; 1 µg/ml) stimulated PGE_2 secretion from ARPE-19 cells at concentrations used in the study

Treatment	LPS stimulated PGE_2 secretion in ARPE-19 cells (% untreated control)
LPS	244 ± 56*
LPS + Celecoxib 1 µM	95 ± 32‡
LPS + Celecoxib 10 µM	53 ± 23*‡
LPS + Celecoxib 25 µM	ND*‡
LPS + Rofecoxib 1 µM	85 ± 22‡
LPS + Rofecoxib 10 µM	47 ± 11*‡
LPS + Rofecoxib 25 µM	ND*‡
LPS + Flurbiprofen 1 µM	73 ± 18‡
LPS + Flurbiprofen 10 µM	48 ± 7*‡
LPS + Flurbiprofen 25 µM	ND*‡

The data are presented as mean ± s.d. for n = 4-6. ND: not detected. * Significantly different compared to untreated controls. ‡Significantly different compared to LPS treatment

Figure 1

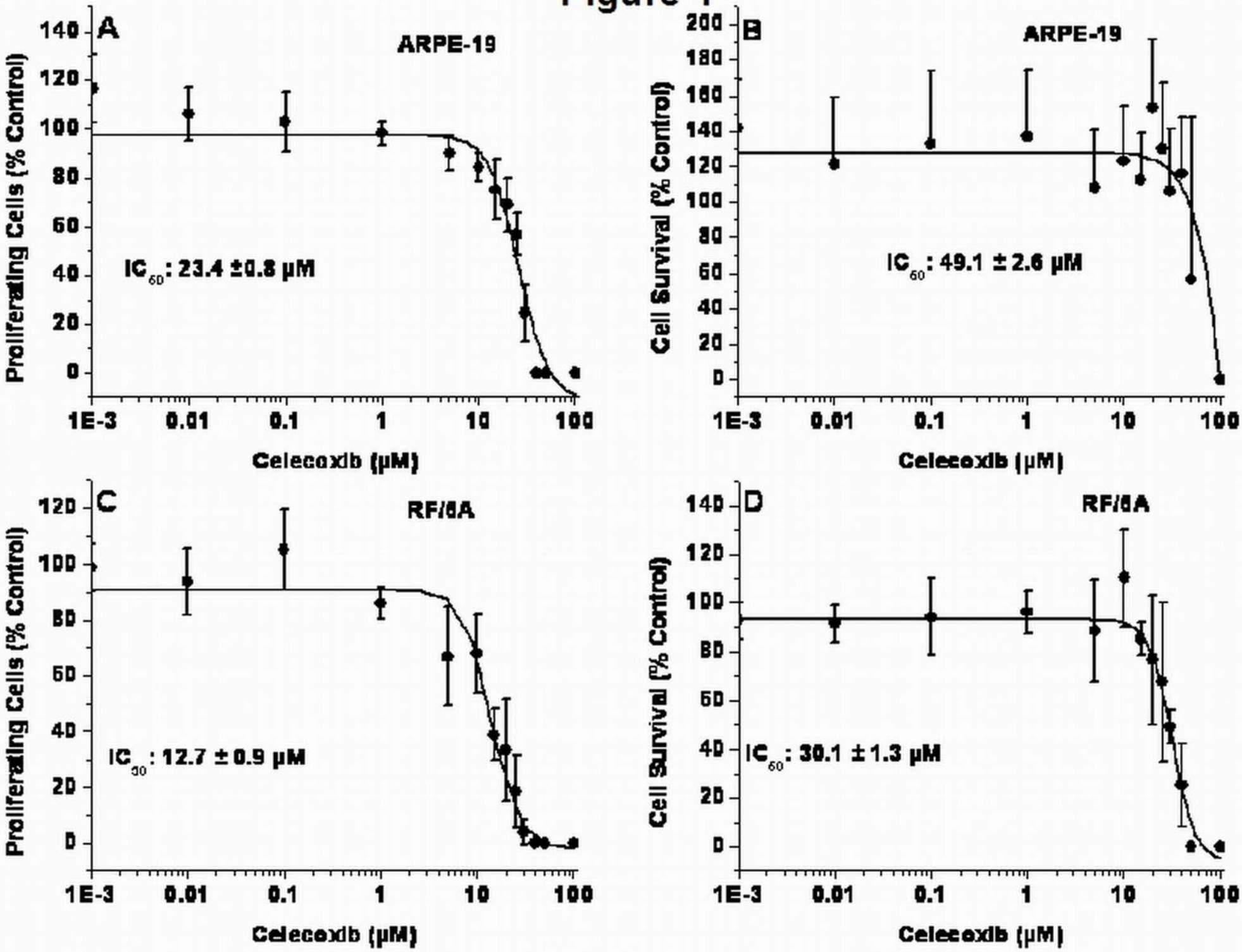


Figure 2

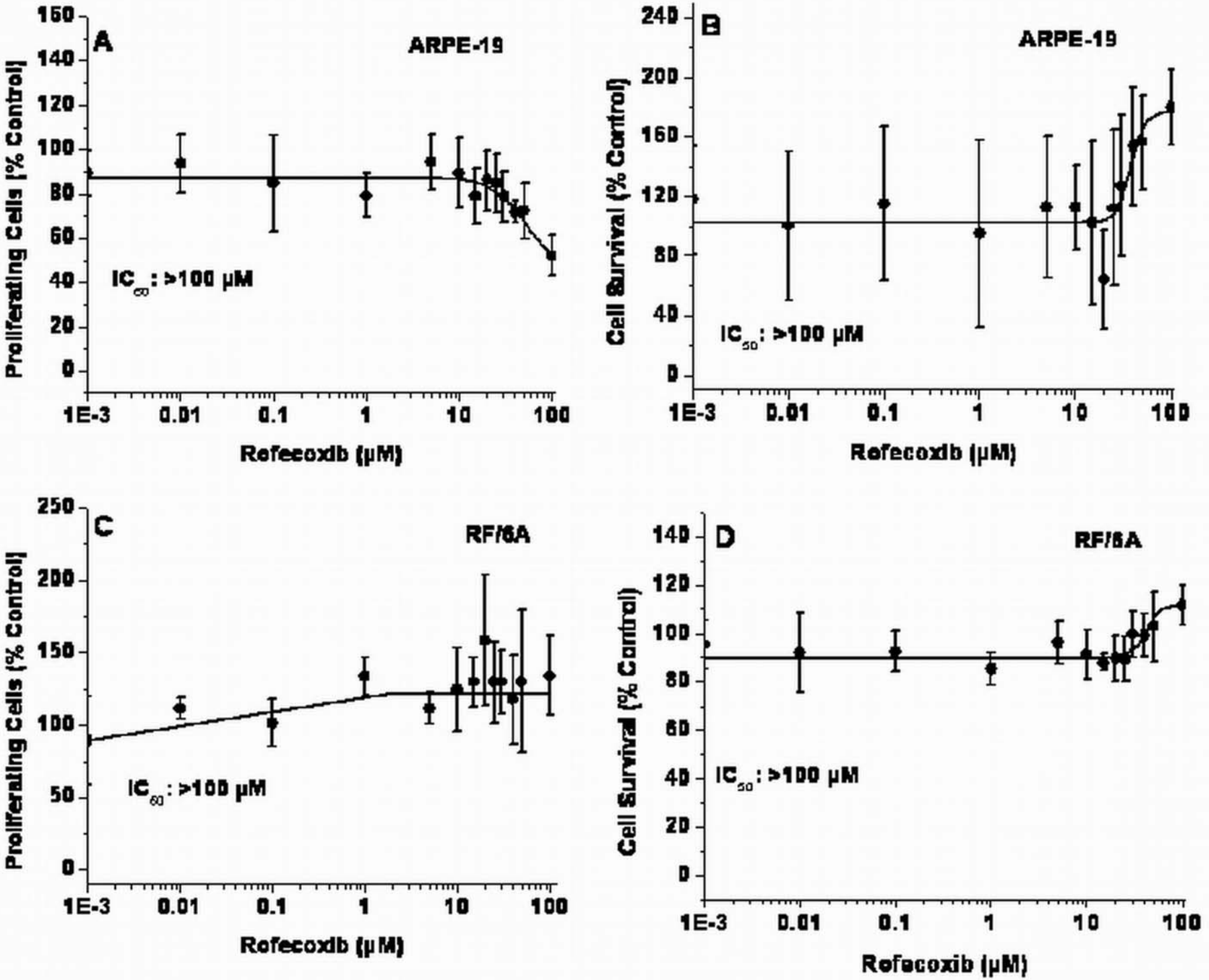
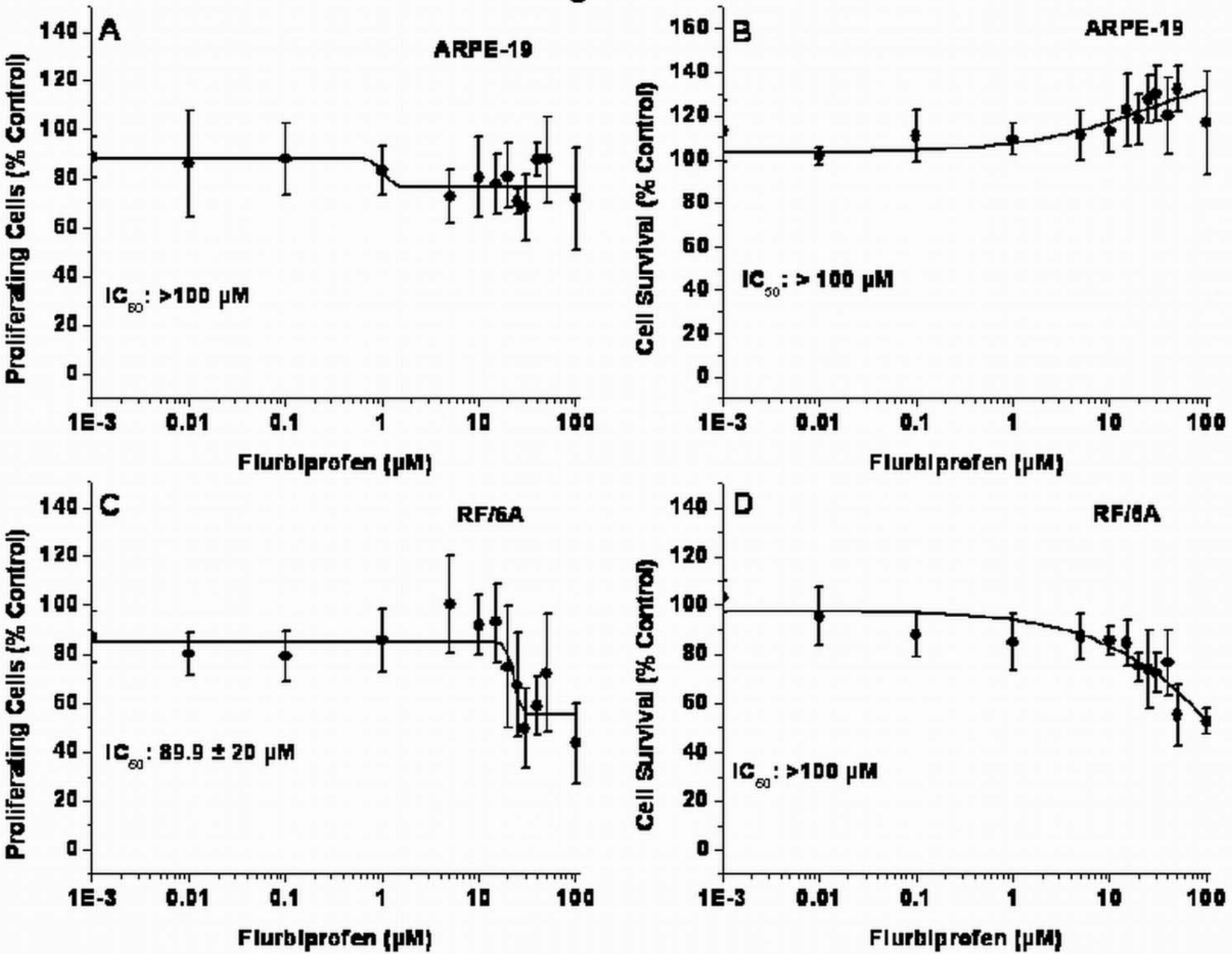


Figure 3



JPET Fast Forward. Published on November 21, 2007 as DOI: 10.1124/jpet.107.128918
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4

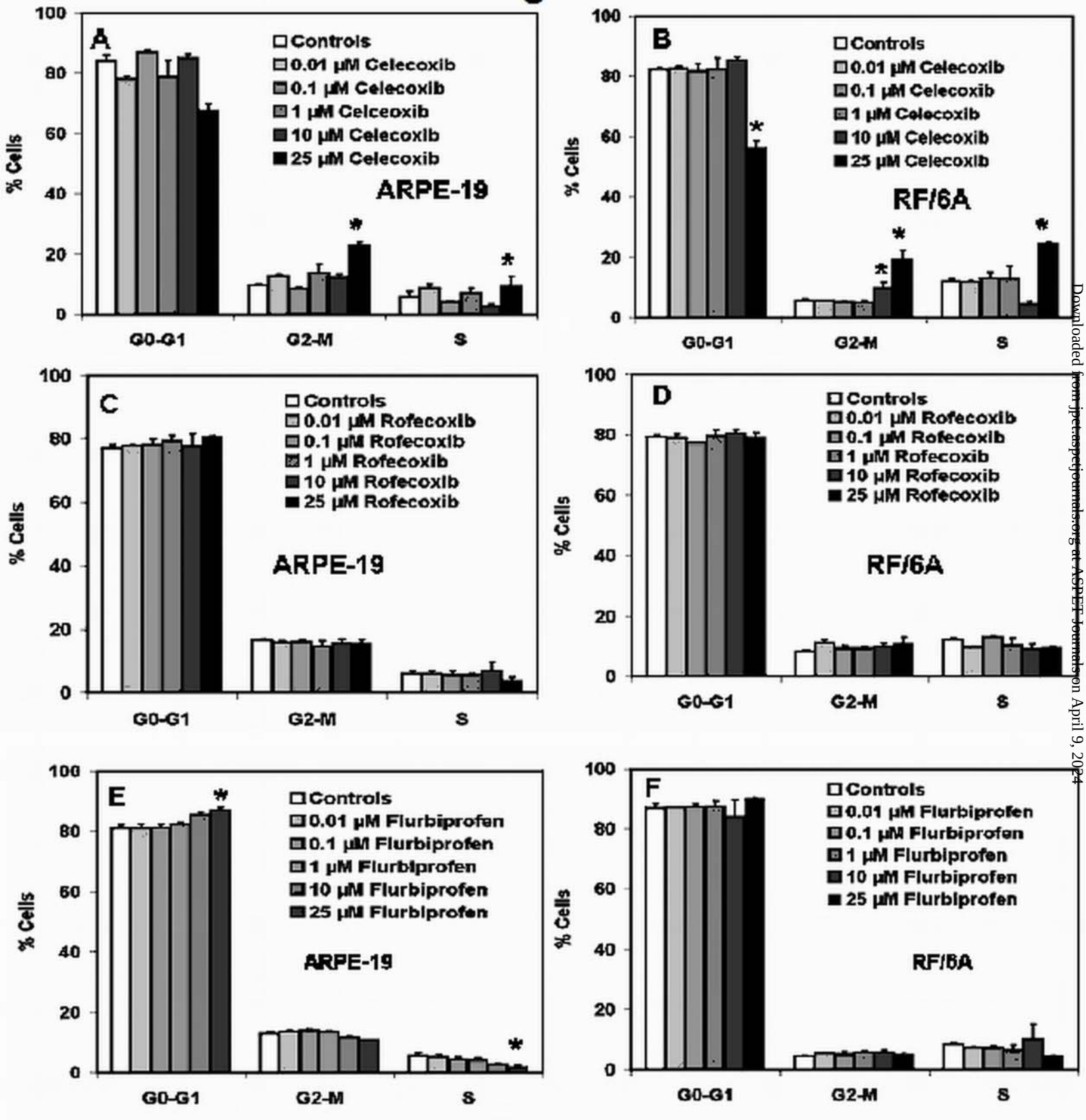


Figure 5

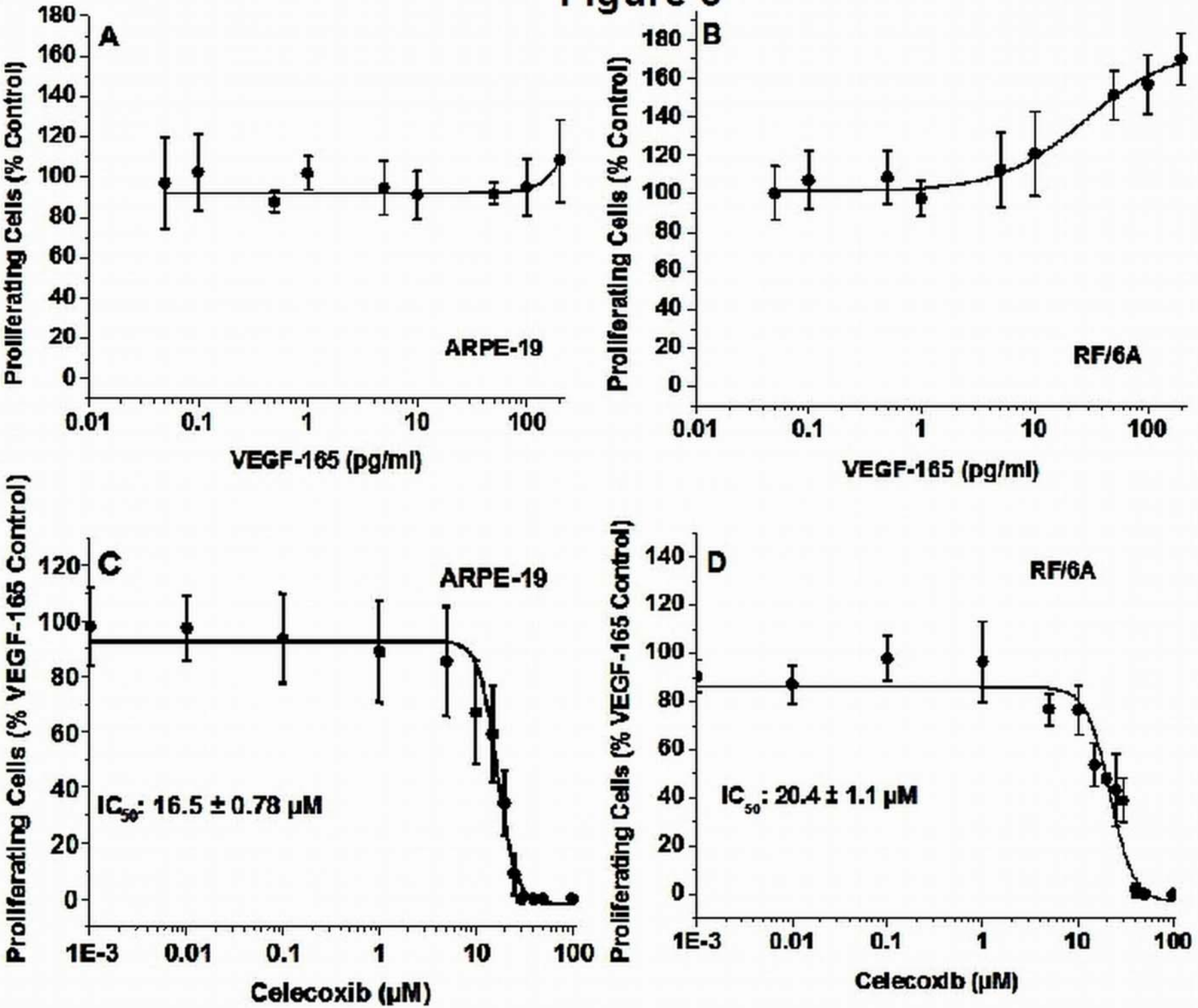


Figure 6