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Title page

Induction of apoptosis in renal cell carcinoma by reactive oxygen species: involvement of ERK1/2, p38δ/γ, COX-2 downregulation and AIF translocation.

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Running title page

a) Apoptosis in renal cell carcinoma by reactive oxygen species.

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d) Abbreviations

RCC, renal cell carcinoma; HRE, human renal epithelial cells; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular-regulated kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH2-terminal kinase; NAC, N-acetyl L-cysteine; AIF, apoptosis inducing factor; ROS, reactive oxygen species; COX, cyclooxygenase; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; wt, wild type; DN, dominant negative; GC, guanylate cyclase.

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Abstract

Renal cell carcinoma (RCC) is the most common malignancy of the kidney. Unfortunately RCCs are highly refractory to conventional chemotherapy, radiation therapy and even immunotherapy. Thus, novel therapeutic targets need to be sought for the successful treatment of RCCs. We now report that 6-anilino-5, 8-quinolinequinone (LY83583) an inhibitor of cyclic guanosine 3´5´-monophosphate production induced growth arrest and apoptosis of the RCC cell line 786-0. It did not prove deleterious to normal renal epithelial cells, an important aspect of chemotherapy. To address the cellular mechanism(s) we employed both genetic and pharmacological approaches. LY83583 induced a time- and dose-dependent increase in RCC apoptosis through dephosphorylation of MEK1/2 and its downstream kinases ERK1 and -2. In addition we observed a decrease in Elk-1 phosphorylation and COX-2 downregulation. Surprisingly, we failed to observe an increase in either c-Jun NH2-terminal kinase or p38[alpha] and [beta] MAPK activation. Paradoxically, reintroduction of p38[delta] by stable transfection or overexpression of p38[gamma] dominant negative abrogated the apoptotic effect. Cell death was associated with a decrease and increase in Bcl-x_L and Bax expression, respectively, as well as cytochrome c release and AIF translocation. These events were associated with an increase in reactive oxygen species formation. The antioxidant, N-acetyl L-cysteine, however, opposed LY83583-mediated mitochondrial dysfunction, ERK1/2 inactivation as well as COX-2 downregulation, and apoptosis. In conclusion, our results suggest that LY83583 may represent a novel therapeutic agent for the treatment of RCC which remains highly refractory to antineoplastic agents. Our data provide a molecular basis for the anticancer activity of LY83583.

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Introduction

Renal cell carcinoma (RCC) is the sixth leading cause of cancer death with the worldwide incidence increasing at an annual rate of 2% (Boring et al., 1994). By the time it is diagnosed metastatic disease is present in approximately 30% of patients with an additional 30 to 40% developing metastases within a period of months or years following nephrectomy (Tourani et al., 2003). RCC has a poor prognosis owing to its late presentation and resistance to hormonal therapy (Motzer and Russo, 2000), chemotherapy (Amato, 2000) and radiotherapy (Bukowski, 1997). Currently, cytokine treatments of patients with RCC have produced insufficient response rates with complete remission being rare (Vuky and Motzer, 2000). Thus, novel therapeutic strategies need to be sought to improve the efficacy in treating RCC.

The naphthoquionolinedione compound LY83583 (6-anilino-5, 8-quinolinequinone) has previously been described as a guanylate cyclase (GC) inhibitor (Beasley et al., 1991). LY83583 competitively inhibits soluble GC, lowering the production of cyclic GMP in a wide range of tissues (Schmidt et al., 1985; O' Donnell and Owen, 1986) with the formation of reactive oxygen species (ROS) intermediates (Lee and Wurster, 1995). LY83583-induced ROS formation can be either in an enzymatic- or nonenzymatic-dependent fashion (Hasegawa et al., 2004). Increased GC activity with a corresponding increase in cGMP has previously been reported in renal tumors (Braughler et al., 1982). Furthermore, a role for this important second messenger has been implicated in cellular proliferation, differentiation and apoptosis (Chiche et al., 1998). This together with the fact that ROS formation may be pro- or anti-tumorigenic (Velarde et al., 2004; Gumpricht et al., 2005) prompted us to investigate a possible role for LY83583 as a therapeutic drug for RCC.

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The mitogen-activated protein kinase (MAPK) pathway is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation and apoptosis (Cobb, 1999). Members of the MAPK cascade include the extracellular signal related kinases (ERKs) of which there are now five family members, multiple c-Jun NH2-terminal kinase (JNK) and the p38 isoforms (Cobb, 1999). In general the ERK pathway is associated with cellular proliferation, differentiation and survival while the JNK and p38 pathways are generally, but not exclusively, associated with inflammation, apoptosis, and cellular differentiation. We have focused on the MAPK cascade in our investigation because of the reported constitutive activation of the ERKs in human malignancies including RCC (Oka et al., 1995; Barry et al., 2001) with this factor being attributed to the almost complete failure of systemic drug therapy regimes for RCC (Motzer et al., 1996). In addition numerous reports have linked alterations in MAPKs to oxidative stress (Dong et al., 2004; Kamata et al., 2005). Thus, we have looked at the possibility that LY83583 may play a role in MAPK signaling in RCC, transcription factor activation, as well as downstream gene regulation.

Using complementary genetic and pharmacological approaches, we have now explored the possible therapeutic potential of LY83583 for the treatment of RCC. We have focused our attention on elucidating the signaling pathways involved in LY83583-induced RCC apoptosis. Inhibition of RCC cell growth was found to be ERK1/2- and MEK1/2-dependent but did not involve JNK activation. In contrast, however, while p38α and -β do not appear to be involved, disparate roles for p38γ and -δ are implicated. In addition, the dephosphorylation of ERK1/2 correlated with Elk-1 dephosphorylation and COX-2 downregulation. Furthermore these events are associated with changes in the expression of the Bcl-2 family members, Bcl-x_L and Bax.

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Together these important findings suggest that LY83583 warrants attention in RCC treatment as well as perhaps other malignancies as a novel therapeutic approach.

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Materials and Methods

Reagents: EDTA, EGTA, leupeptin, aprotinin, sodium orthovanadate, sodium flouride, RPMI, fetal calf serum (FCS), antibiotics, anti-FLAG^R M2 mAb, 1', 2', 3'-dihydrorhodamine, HA 14-1 and monobromobimane were purchased from Sigma (Dublin, Ireland). 2', 7'-dichlorofluorescein diacetate was purchased from Molecular Probes (Eugene, OR). LY83583 (6-anilino-5, 8-quinolinequinone), 8-bromo-cGMP, zaprinast, Rp-8-cGMPs, zVAD-fmk, Ac-DEVD-CHO and the Bax channel blocker were purchased from Calbiochem (Nottingham, United Kingdom). Cytochrome-c mAb was purchased from Pharmingen (San Diego, CA). Phosphorus p42/p44 MAPK, ERK5, MKK3/MKK6, Akt (Ser 473), Akt (Thr 308), MEK1/2, JNK, p38 MAPK, Elk-1 antibody kits as well as Bcl-x_L, Bcl-2, BAD and Bax were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibody p53 (Pab 1801), and mAb AIF (E-1) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse mAb p38α and -δ were purchased from BD Transduction Laboratories (Oxford, UK), rabbit polyclonal p38γ antibody from Upstate (Lake Placid, NY) and mouse mAb p38β antibody from Zymed Laboratories Inc. (San Francisco, CA). COX-1 and –2 mAb were purchased from Cayman Chemicals (Ann Arbor, MI). si*GENOME*™ *SMART*pool® AIF siRNA and si*Control*™ RISC-Free siRNA were purchased from Dharmacon RNA Technologies (Lafayette, CO).

Cell culture - The RCC cell line 786-0 purchased from the ATCC (Rockville, MD) was cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cells were washed twice with RPMI 1640, and 3×10^4 cells/well were plated into 6-well tissue culture plates. After 24 hr cells were cultured in medium supplemented with antibiotics and 0.5% FCS for all experiments performed unless otherwise stated. Normal human renal epithelial cells (HRE) purchased from Cambrex BioScience

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(Wokingham, Berkshire) were grown according to the manufacturer's instructions. Briefly, cells were grown in renal epithelial cell growth medium (BulletKit^{R}) which contained renal epithelial cell basal medium (REBM^{TM}) and a range of supplements. Cells were plated in 6-well tissue culture plates as described above.

Proliferation-cytotoxicity assay - Cells were plated at a density of 2×10^4 cells/well into a 6-well tissue culture plate. Twenty four hours later cells were treated with various concentrations of LY83583, including 0.03, 0.1, 0.3, and 1 μM and after 24, 48, 72 and 96 hr, the cell viability was assessed by trypan blue (0.4% w/v) exclusion assay. *MTT assay* - to determine cell viability MTT (5 μg/ml) was added to each well of a six well plate and the reduction of MTT was assayed to calculate viable cell numbers as previously described (Barry et al., 2001).

Immunoblot Analysis - 786-0 cells were cultured in RPMI supplemented with antibiotics and 0.5% FCS. Cells were lysed in ice-cold lysis buffer as previously described (Barry et al., 2001). Supernatants were used for immunoblotting with specific antibodies for the phosphorylated or total p42/p44 MAPK, MEK1/2, ERK/5, JNK1/2, p38, MKK3/MKK6 or Elk-1 using the experimental conditions described by the manufacturer (New England Biolabs, Beverly, MA). Similarly, membranes were incubated with antibodies for p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Bcl-2, Bcl-x_L, BAD, and Bax (New England Biolabs, Beverly, MA), cytochrome c (Pharmingen, San Diego, CA), caspase–3 (BD Transduction Laboratories, Oxford, UK), or COX-1 and –2 (Cayman Chemicals, Ann Arbor, MI). Chemiluminescent detection was performed using the SuperSignal immunodetection system (Pierce Chemical, Dublin, Ireland) to reveal positive bands that were visualized after exposure to Hyperfilm ECL (Amersham Biosciences, Bucks, UK).

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Stable transfection of cells - The generation of stable 786-0 cell lines expressing FLAG-tagged wild type (wt) and dominant negative (DN) forms of human p38α, -β, -δ, and -γ have been previously described (Pramanik et al., 2003). These plasmids were a kind gift from Professor J. Han (Scripps Research Institute, La Jolla, CA). The constitutively active MEK1 EE plasmid was a kind gift from Professor M.G. Kazanietz (University of Pennsylvania, Philadelphia). Briefly, electroporations were performed using a rectangle pulse EPI 2500 electroporator (Fischer, Heidelberg, Germany). A 100 µl aliquot of 786-0 cells (2×10^6) were mixed with 10 µg DNA in a 0.2 mm gap cuvette (8 x 8 ms (milliseconds), 330 V, 8 x 1s time interval). Following 15-min recovery period at room temperature (RT), cells were diluted twenty fold in culture medium and incubated at 37°C, and 5% CO_2. Using this protocol, we routinely observed less than 10% of dead cells after electroporation. Following 48-72 hr after the addition of DNA, cells were washed twice in phosphate-buffered saline, and split 1:3 into 35-mm diameter dishes. The transfected cells were selected in growth medium containing 600 µg/ml Geneticin (G418; Life Technologies Inc., Grand Island, NY). After 4-8 weeks, individual cell colonies were transferred for clone expansion and maintained in culture medium supplemented with 600 µg/ml G418. Protein expression levels were determined by Western blot analysis of G418-selected cell foci using anti-FLAG^R M2 mAb (Sigma, Dublin, Ireland). All blots were visualized with the procedure outlined in the enhanced chemiluminescence kit (Amersham Biosciences, Bucks, UK).

Measurement of reactive oxygen species - Formation of ROS was detected using the redox sensitive dye 1', 2', 3'-dihydrorhodamine (DHR) as previously described (Halliwell and Whiteman, 2004). This dye is lipophilic and readily diffuses across cell membranes where it is oxidized to the green fluorescent rhodamine 123 (Halliwell and Whiteman, 2004). Briefly, 786-0 cells (3×10^6 cells/ml) in 6-well plates were pre-incubated in the dark with DHR (30µM) for 30

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min at 37ºC and subsequently treated with LY83583 (1 μM). In the absence of drug, vehicle (0.1% Me_2SO) was utilized. Fluorescence was measured using the TECAN GENios microplate reader at λ 485nm and λ 535nm excitation and emission respectively.

Mitochondrial and cytosolic extraction - Treated and untreated 786-0 cells (6×10^7) were harvested by centrifugation at 850 × g for 2 min at RT. Mitochondrial and cytosolic extraction was carried out using the Mitochondrial Isolation Kit (Pierce Chemical, Dublin, Ireland) according to manufacturer's instructions.

Immunofluorescence - AIF detection was carried out by immunofluorescence. Briefly, nontransfected 786-0 cells or 786-0 cells transfected with empty vector (pcDNA3), p38δ wt, p38δ DN, p38γ wt or p38γ DN (3×10^4) were grown on coverslips and were or were not treated with LY83583 (1 μM) for 96 hr in the presence or absence of N-acetyl L-cysteine (NAC), Z-VAD-fmk or calpeptin. Cells were then washed with PBS, fixed with 1.5% paraformaldehyde for 30 min at RT, and permeabilized with 2ml of 0.2% Triton X-100. Non-specific binding was blocked using 10 mg/ml BSA in PBS for 15 min followed by incubation with anti-AIF (E-1) mAb (1:100) diluted in blocking buffer for 1 h at 37^0C. Following addition of secondary anti-mouse FITC conjugated IgG (1:100), slides were treated with anti-Fade (DAKO) mounting media and cells were visualized under a fluorescence microscope.

Flow Cytometry - Apoptotic cells were identified by double supravital staining with recombinant FITC-conjugated annexin V and propidium iodide (PI) as previously described (Bowden et al., 2002). The annexin V-FITC apoptosis detection kit (BD Pharmingen™) was used to visualize DNA according to the manufacturer's instructions. Briefly, following LY83583 (1 μM), or U0126 (20 μM) treatment floating and attached cells were pooled and washed twice with cold

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PBS. HRE and 786-0 cells (1 X 10^5) were incubated with 5 µl of Annexin V-FITC and 5 µl of PI in the dark for 45 mins at RT. Cells were analyzed on a Becton Dickinson FACScalibur. The fraction of cells in each quadrant was calculated using Cell Quest (Becton Dickinson) program.

Terminal Deoxynucleotide Transferase-Mediated dUTP Nick End Labeling (TUNEL) - TUNEL of fragmented DNA was performed according to the manufacture's instructions (Roche Molecular Biochemicals).

AIF siRNA - si*GENOME*™ *SMART*pool® AIF siRNA and si*Control*™ RISC-Free siRNA were purchased from Dharmacon, Inc. (Lafayette, CO). In brief, 786-0 (3×10^6) cells were seeded in six well plates in RPMI containing 10% FCS and without antibiotics. Transfections using Dharma*Fect*™ 4 reagent and *SMART*pool® AIF siRNA (100 nM) or si*Control*™ RISC-Free siRNA (100 nM) were performed 24 hr later as per the manufacturer's instructions. The optimal time for protein/RNA depletion after transfection was determined. 786-0 cells were treated 48hr after transfection with LY83583 (1µM) for 48 hr.

Statistical Analysis

Results are expressed as mean ± S.E. Statistical comparisons were made by using analysis of variance with subsequent application of Student's *t* test, as appropriate.

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Results

LY83583 induces a time and dose-dependent cell growth inhibition in 786-0 but not HRE cells. To determine whether LY83583 inhibits 786-0 RCC proliferation, cells were treated with or without LY83583 (0.03, 0.1, 0.3 and 1 μM) for 0, 24, 48, 72, and 96 hr. Control cells were treated with 0.1% Me_2SO alone. 786-0 cells demonstrated a time- and dose-dependent decrease in cell number (Fig. 1*A*). Similar results were also obtained when two other RCC cell lines, ACHN and Caki-1, were treated with LY83583 (data not shown). Normal human renal epithelial cells (HRE) were also treated with LY83583 and counted. These cells, however, proved refractory to LY83583 treatment even at 96 hr (Fig. 1*B*).

LY83583 induces a time-dependent increase in 786-0 cell apoptosis but not HRE cells. A previous report identified cellular senescence of human fibroblasts upon LY83583 treatment (Lodykin et al., 2002). Using β-galactosidase staining we failed to observe 786-0 cellular senescence in the presence of this drug (Lodykin et al., 2002 data not shown). We did, however, observe a time-dependent increase in 786-0 cell apoptosis (Fig. 2). Fluorescein-conjugated annexin V (FL1-H) and PI (FL2-H) staining (detected by flow cytometry) were used as criteria for distinguishing cycling, early apoptotic, and late apoptotic cells. Quadrants I, II, III, and IV in Fig. 2*A* and *C* represent different cell groups: area I represents unlabelled viable cells ($Ann\text{-}V^-/PI^-$), area II contains early apoptotic cells ($Ann\text{-}V^+/PI^-$), area III indicates late apoptotic cells ($Ann\text{-}V^+/PI^+$) and area IV represents cells destroyed during experimental workup as previously described (Bowden et al., 2002). Control (0.1% Me_2SO) treated 786-0 cells demonstrate 87.2%, 8.2%, 3.6% and 1.0% for areas I, II, III, and IV respectively. However, following treatment with LY83583 (1 μM) there is a dramatic time-dependent increase in the number of both early and late apoptotic cells (Fig. 2*A*). Similar apoptotic results were obtained

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with ACHN and Caki-1 upon treatment with LY83583 (data not shown). TUNEL analysis was also performed to confirm the effects of LY83583 on 786-0 cell death. Consistent with the flow cytometry data control (0.1% Me_2SO) treated 786-0 cells do not demonstrate any positive TUNEL staining (Fig. 2*B*). Cells treated with LY83583 (1 μM), however, demonstrate a time-dependent increase in TUNEL staining (Fig. 2*B*). Changes in cell morphology such as cell cytoplasm destruction and chromatin condensation are evident upon LY83583 treatment (Fig. 2*B*). At 96 hr the cell cytoplasm has been destroyed (Fig. 2*B*). In contrast, however, HRE cells do not demonstrate an increase in apoptosis by either flow cytometry (Fig. 2*C*) or TUNEL staining (Fig. 2*D*) upon addition of LY83583 (1 μM).

LY83583-induced 786-0 apoptosis is ROS-dependent but guanylate cyclase-independent. Previous reports have demonstrated that LY83583 competitively inhibits GC lowering intracellular levels of cGMP (Schmidt et al., 1985; O'Donnell and Owen, 1986) with the formation of ROS intermediates (Lee and Wurster, 1995). Therefore, we decided to focus our next set of experiments on investigating the mechanism(s) of action of LY83583-induced 786-0 apoptosis. Again, using flow cytometric analysis of annexin V/PI staining as described above 786-0 cells were or were not treated with LY83583 (1 μM) in the presence or absence of two different cGMP-dependent protein kinase activators 8-bromo-cGMP (8-Br-cGMP) and 8-(4-chlorophenylthio)cGMP (8-pCPT-cGMP). Both were added 1 hr prior to treatment with LY83583. In addition a previous report (O' Donnell and Owen, 1986) outlined that 8-Br-cGMP was insufficient to block the actions of LY83583 unless a phosphodiesterase inhibitor was also added. Thus, in addition to treating cells with 8-Br-cGMP we also added a specific phosphodiesterase inhibitor zaprinast. As observed above (Fig. 2*A*) LY83583 induced a time-dependent decrease in cell viability (Table 1*A*). We did not see a change in cell viability when

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cells were pretreated with 8-Br-cGMP and zaprinast (1 mM, and 20 μM respectively for 24-96 hr) prior to the addition of LY83583 (Table 1*B*). Similarly, 8-pCPT-cGMP (250 μM, 24-96 hr) failed to reverse the apoptotic response (data not shown). To further strengthen the absence of a role for GC in the observed apoptotic response, cells were also treated with a potent and selective sGC inhibitor – 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Again, this specific inhibitor failed to alter the observed apoptotic response (Table 1*C*). Thus, the apoptotic effect of LY83583 on RCC is not due to the inhibition of GC. Thus, we investigated whether ROS scavengers could reverse the apoptotic effect observed in RCC. Cells were treated with NAC (10 mM) and LY83583 (1 μM). The decrease in cell viability was reversed at all time points (Table 1*D*). Similarly, treatment of 786-0 cells with four other different antioxidants- vitamins C (200 μM), and E (50 μM), L-NAME (1 mM), and melatonin (150 μM) attenuated LY83583 (1 μM)-mediated lethality (Table 1*E-H*).

LY83583 stimulates a dramatic increase in ROS production in 786-0 cells. Following the inhibitory effects of a range of antioxidants on LY83583-induced apoptosis as described above we next turned our attention to ROS formation, employing the fluorescent dye 1', 2', 3'-dihydrorhodamine (DHR). DHR is a probe widely used to detect several reactive species (Halliwell and Whiteman, 2004). It is oxidized to rhodamine 123 which is highly lipophilic and positively charged, and accumulates in mitochondria, held there by the membrane potential (Halliwell and Whiteman, 2004). We observed a time-dependent increase in ROS formation following LY83583 (1 μM) addition (Fig. 3). A significant increase in ROS formation was observed as early as 1 min following addition of LY83583 (1 μM) to 786-0 cells. This is in agreement with a pervious report where LY83583-induced ROS formation in PC12 cells was observed by electron paramagnetic resonance analysis following a 2 min incubation period

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(Hasegawa et al., 2004). A similar increase in ROS production was detected using a second fluorescent probe 2´, 7´-dichlorofluorescein diacetate (data not shown). There was also a time-dependent increase in ROS formation upon treatment of ACHN and Caki-1 with LY83583 (1 μM) (data not shown).

Effect of LY83583 and ROS formation on MAPK expression. Members of the MAPK cascade have been implicated in both pro- and anti-apoptotic effects (Cobb, 1999). In addition oxidative stress is known to alter MAPK cascades (Dong et al., 2004; Kamata et al., 2005). Thus, we investigated the effect(s) of LY83583 on the different MAPKs, using the phosphorylation of ERK1/2, ERK5, MEK1/2, JNK, p38, and MKK3/6 as an index of kinase activation (Barry et al., 2001). Constitutive activation of ERKs in human malignancies has been previously documented both by ourselves and others (Oka et al., 1995; Barry et al., 2001). We now report that 786-0 cells also demonstrate constitutive ERK1/2 activation (Fig. 4*A*). However, upon addition of LY83583 we observed dephosphorylation of ERK1/2 as early as 5 mins with complete loss of phosphorylation at 30 mins (Fig. 4*A*). Total MAPK levels were not changed, as judged with a MAPK antibody that recognized both the phosphorylated and unphosphorylated forms of the enzyme (Barry et al., 2001). We also observed ERK1/2 inactivation when ACHN and Caki-1 were treated with LY83583 (1 μM) (data not shown). Since MEK is the upstream activator of ERKs (Oka et al., 1995), we addressed the role of MEK1/2. When 786-0 cells were treated with LY83583 (1 μM) we observed a decrease in MEK1/2 phosphorylation prior to ERK1/2 dephosphorylation (Fig. 4*A*). Another member of the mammalian MAPK pathway, ERK5 (also known as BMK1) is known to be activated by oxidant stress as well as having a role in many physiological processes including carcinogenesis (Wang and Tournier, 2006). Unlike ERK1/2 we did not observe constitutive ERK5 phosphorylation in 786-0 cells (Fig. 4*A*). Furthermore we

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did not observe an increase in ERK5 phosphorylation when 786-0 cells were treated with LY83583 (1 μM) (Fig. 4*A*). Addition of sorbitol (20 μM) was used as a positive control to ensure phospho-ERK5 antibody functionality. The central role of the PI3K-Akt pathway in renal cancer cell survival prompted us to investigate whether LY83583-induced apoptosis involves any changes in this signaling pathway. Treatment of 786-0 cells with LY83583 (1 μM) leads to a rapid dephosphorylation of Akt as revealed by Western blotting using a phospho-specific anti-Akt antibody (phospho-Thr308) (Fig. 4*A*). We did not observe Akt phosphorylation on Ser473 in 786-0 cells (data not shown). To address the question of whether other members of the MAPK cascade could also be involved in LY83583-induced 786-0 apoptosis we examined the phosphorylation (activation) of both JNK and p38 MAPK. Unlike ERK1/2 786-0 cells do not express constitutive JNK and p38 MAPK activation (Fig 4*A*). Upon treatment with LY83583 (1 μM) we surprisingly failed to see an increase in either JNK or p38 MAPK phosphorylation (Fig. 4*A*), two putative MAPKs previously linked to apoptosis (Cobb, 1999). Cells were treated with anisomycin (1 μg/ml), a JNK activator, and sorbitol (20 μM), a p38 MAPK activator, as positive controls to ensure antibody functionality (Fig 4*A*). Furthermore using antibodies against MKK3 and MKK6 we investigated whether the pan-p38 pathway MAPKKs are activated by LY83583. We did not observe an increase in MKK3 or MKK6 phosphorylation upon treatment with LY83583 (1 μM) for all time points studied (Fig. 4*A*). Sorbitol (20 μM) treated cells were again used to ensure antibody functionality.

To further evaluate the effect of LY83583 on 786-0-induced apoptosis we investigated its effect on both the transcription factor Elk-1 and its downstream target COX-2. Previous reports have documented that COX-2 is overexpressed in RCC and plays a role in the tumorigenesis of these cells (Chen et al., 2004). Thus, COX-2 may be a therapeutic target for the treatment of

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RCC. We observed that 786-0 cells also express COX-2 as well as constitutive Elk-1 phosphorylation (Fig. 4*A*). Upon treatment of 786-0 cells with LY83583 (1 μM) we observed a decrease in Elk-1 phosphorylation as early as 30 minutes consistent with the inactivation of upstream ERK1/2. Not surprisingly we also observed a decrease in COX-2 expression following Elk-1 dephosphorylation (Fig. 4*A*). No changes in COX-1 expression were observed upon addition of LY83583 (1 μM) (Fig. 4*A*).

Since we observed that ROS scavengers could null the LY83583-induced apoptotic effect we investigated whether they could also have an effect on the MAPK signaling cascade. 786-0 cells were treated with NAC (10 mM) alone or NAC (10 mM) and LY83583 (1 μM). NAC (10 mM) alone does not quench ERK1/2 phosphorylation as has been observed in some tumor cell lines. It did, however, prevent the dephosphorylation of ERK1/2, MEK1/2, and the transcription factor Elk-1 as well as the downregulation of COX-2 expression following LY83583 (1 μM) treatment at all time points studied i.e. 5, 15 and 30 minutes and 2-96 hr (only the 96 hr time point is shown) (Fig. 4*B*).

p38δ and γDN rescued LY83583-induced 786-0 cell death is ROS-dependent. The involvement of p38 MAPK in apoptosis is well documented (Cobb, 1999). In addition previous studies have shown that ROS formation can activate p38 MAPK depending on the cell type (Alder et al., 1999). Surprisingly, we did not observe an increase in p38 MAPK phosphorylation (activation) in LY83583-induced 786-0 apoptosis (Fig. 4*A*). The p38 MAPK antibodies (New England Biolabs) used in Fig. 4*A*, however, only identify the phosphorylation of p38α and β and lack specificity in terms of identifying the different p38 isoforms. Therefore, to investigate the role(s) of p38 MAPK in more detail we employed the use of antibodies specific for each of the

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p38 isoforms (Yang et al., 1999; Ho et al., 2004; Kuma et al., 2004). We observed that the RCC cell line 786-0 expresses the p38 isoforms- α, -β, and -γ, but not δ (Fig. 5*A*). In contrast normal human renal epithelial (HRE) cells express all four isoforms (Fig. 5*A*). This begs the question of whether or not the loss of p38δ may play a role in the tumorigenicity of RCC. To address this issue we developed 786-0 stable cell lines expressing each of the p38 MAPK isoforms and their dominant negative (DN) mutants, confirmed by Western blot analysis of the FLAG-tagged plasmids (Fig. 5*B*). All p38 MAPK plasmids have been previously described in detail (Pramanik et al., 2003). pcDNA3 (empty vector) was used as a control. Not surprisingly since we failed to see p38α/β MAPK phosphorylation above (Fig. 4*A*), 786-0 cells stably transfected with p38α, p38β, or their DN mutants displayed a similar reduction in cell numbers and apoptosis when compared to nontransfected cells in the presence of LY83583 (1 μM) (Fig. 5*C* and *D*). Surprisingly, however, we observed that reintroduction of p38δ could abrogate the apoptotic effect of LY83583 (Fig. 5*C* and *D*). As expected cells expressing the p38δ DN isoform behaved as non-transfected cells in the presence of LY83583 (as 786-0 cells do not express p38δ) (Fig. 5*C* and *D*). In addition we observed that overexpression of the p38γ DN mutant also rescued the cells from LY83583-induced apoptosis (Fig. 5*C* and *D*). Cells overexpressing the p38γ isoform displayed a similar reduction in cell numbers and apoptosis compared to nontransfected cells in the presence of LY83583 (Fig. 5*C* and *D*).

To further investigate the possible mechanism(s) involved in the role of p38δ and p38γ DN in reversing LY83583-induced apoptosis we examined whether there could possibly be cross talk between the different MAPK pathways. We examined whether the mutant cells could alter the constitutive ERK1/2 activation observed in RCC. While RCC cells overexpressing p38δ and

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p38γ DN did not display an altered pattern of ERK1/2 phosphorylation we did observe that treatment of these cells with LY83583 (1 μM) did not result in ERK1/2 dephosphorylation (inactivation) (Fig. 5*E*). This is in contrast to p38δ DN or p38γ wt (used as controls in this experiment) which display complete loss of ERK1/2 dephosphorylation following LY83583 (1 μM) treatment (Fig. 5*E*). Since we did not observe MKK3 or MKK6 phsophorylation when nontransfected 786-0 cells (Fig. 4A) or transfected 786-0δ or 786-0γDN were treated with LY83583 (1 μM) (data not shown) then it is probable that p38δ and -γ are altering ERK1/2 function through mass action effects. In summary, we can conclude that p38δ and -γ have opposing roles in LY83583-induced 786-0 apoptosis. While p38γ appears to confer sensitivity of 786-0 cells to LY83583-induced apoptosis, p38δ confers resistance.

U0126 mimics the effect of LY83583. To correlate the above data with the mechanisms of RCC-induced apoptosis we investigated whether MEK1/2 inactivation can bring about the observed effects of LY83583-induced apoptosis. Using a specific and potent MEK1/2 inhibitor U0126 we observed a time-dependent decrease in 786-0 cell viability (Fig. 6). This is in agreement with a previous report using colorectal cancer cell lines with constitutively active MEK/MAPK where cell death by U0126 was only found in p53 wild-type cells as is the case with 786-0 cells (Wang *et al.*, 2004). We also examined the effect of U0126 on cells overexpressing p38δ and p38γDN. As we observed for LY83583 we again observed a reduction in U0126-induced loss of cell viability upon treatment with U0126 albeit not completely. This is obvious at 72hr and 96hr (Fig. 6). In contrast to inactivating MEK (using U0126 and observing a loss in cell viability), stable 786-0 cells expressing active MEK (kindly donated by Prof. M.G. Kazanietz, University of Pennsylvania, Philadelphia) mimicked the effect of p38δ and p38γ DN.

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No loss in cell viability was observed when 786-0 MEK cells were treated with LY83583 (1 μM) (Fig. 6).

Effect of LY83583 on apoptotic-related gene expression is ROS-dependent. In view of the role of the expression and subcellular localization of Bcl-2 family proteins in apoptosis regulation, expression of these proteins was investigated in LY83583 treated 786-0 cells. No major changes in expression was observed for Bcl-2, or BAD. We did, however, observe a decrease in the expression of the anti-apoptotic member Bcl-x_L and an increase in the expression of the pro-apoptotic member Bax following treatment with LY83583 (1 μM) albeit at the later time points of 48, 72 and 96 hr (Fig. 7*A*). These changes in expression were abrogated in the presence of NAC (10 mM) (Fig. 7*B*). To investigate whether the observed effect of LY83538 on the downregulation of Bcl-x_L expression is a secondary effect of caspase activation cells were treated with the pan-caspase inhibitor z-VAD-fmk (50 μM). Since caspase inhibitors are unstable the drug was re-supplemented every 24hr. We did not observe a change in Bcl-x_L downregulation or in Bax upregulation in the presence of the caspase inhibitor (Fig. 7*B*). We also analyzed p53 wild type expression but did not observe a change in its expression following LY83583 treatment (Fig. 7*A*). In agreement with a previous report we did observe $p21^{waf1/cip1}$ upregulation when 786-0 cells were treated with this drug, albeit in a p53-independent manner (data not shown) (Lodykin et al., 2002). To examine further the role(s) of Bcl-2 family members and Bax in the apoptotic response we used two novel pharmacological inhibitors, HA 14-1 (An et al., 2004) and a Bax channel blocker (Bombrun et al., 2003) respectively. HA14-1 (ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxo-ethyl)-4H-chromene-3-carboxylate) is a synthetic cell permeable molecule which specifically competes with Bak BH3 domain-derived peptide in binding Bcl-2 family members (An et al., 2004). The Bax channel blocker (3,6-

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dibromocarbazole piperazine derivatives of 2-propanol) inhibits Bax channel–forming activity (Bombrun et al., 2003). Thus, if members of the anti-apoptotic Bcl-2 family are involved in the apoptotic response then addition of an inhibitor should further increase the apoptosis induced by LY83583. Conversely, if the pro-apoptotic Bax is involved then the use of a Bax channel blocker should decrease the observed apoptosis. We used both inhibitors at concentrations which alone do not induce apoptosis as previously reported (Bombrun et al., 2003). We observed a time-dependent increase and decrease respectively in the presence of HA-1 and the Bax channel blocker (Table 2*B* and *C*). We did not observe, however, complete suppression of apoptosis in the presence of the latter drug (Table 2*C*). This is not surprising since Bax can bind to pre-exisiting mitochondrial membrane pores (i.e. the permeability transition pore (PTP)) while the inhibitor is operational during Bax pore formation).

LY83583 induced cytochrome c release, caspase-3 activation and AIF translocation in a ROS-dependent manner. Mitochondria play a crucial role in apoptosis by releasing apoptogenic factors. The pro-apoptotic family member Bax plays a role in the release of such factors as cytochrome c, apoptosis inducing factor (AIF), and Smac/DIABLO. Since we observed an increase in Bax expression we examined whether these apoptotic associated factors could also play a role in LY83583-induced RCC apoptosis. Upon subcellular fractionation we observed a release into the cytosol of cytochrome c following treatment with LY83583 (1 μM) (Fig. 8*A*). This translocation was evident at 24 and continued to 96 hr. Only results for 96 hr are shown (Fig. 8*A*).This effect was blocked in the presence of NAC (10 mM) (Fig. 8*A*). We consistently did not observe Smac/DIABLO in the 786-0 cell line (data not shown). This is agreement with a recent report demonstrating downregulation of Smac/DIABLO in RCC (Mizutani et al., 2005). Cytochrome c released into the cytosol forms part of the apoptosome

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which leads ultimately to the activation of downstream procaspases such as procaspase-3. We observed processing of procaspase 3 by Western blotting with the formation of the p20 form of the large subunit following addition of LY83583 (Fig. 8*B*). This was ROS dependent since addition of NAC (10 mM) prevented the cleavage of this caspase (Fig. 8*B*). To investigate the role of caspase-3 in 786-0-induced apoptosis cells were treated with the pan-caspase inhibitor z-VAD-fmk (50 μM) and the specific caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) (50 μM). We observed that both inhibitors were able to prevent processing of procaspase-3 (data not shown) but surprisingly failed to abrogate LY83583-induced apoptotic cell death (Fig. 8*C*). Both caspase inhibitors have been shown to prevent 786-0-induced apoptosis (G.C.O'Sullivan and O.P.Barry manuscript in preparation and data not shown). We decided not to look further at other caspases since the pan-caspase inhibitor was without effect (Fig. 8*C*). Thus, LY83583-induced 786-0 apoptotic cell death appears to occur in a caspase-independent fashion. Cellular apoptosis, however, can proceed without the involvement of caspases. LY83583-induced cell death may progress by an alternative route originating from mitochondrial permeability transition (MPT) mediated by oxidative changes in critical redox-sensing mitochondrial membrane proteins. To investigate this alternative pathway we used monobromobimane (MBB), a protein that prevents changes in mitochondrial membrane proteins that are required to initiate MPT. We observed, however, that MBB (1 μM) did not alter the apoptotic response of 786-0 cells in the presence of LY83583 (Fig. 8*C*). Similar results were observed when MBB was also used at 10 μM (data not shown). Another factor that is reported to be released during the apoptotic response is AIF. AIF normally resides in the mitochondrial intermembrane space whereby it translocates to the nucleus during apoptosis. The release of AIF from the mitochondria can be mediated by a caspase-dependent or independent pathway. Using

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immunofluorescence we observed that treatment of 786-0 cells with LY83583 (1 μM) causes translocation of AIF from the cytoplasm to the nucleus in a caspase-independent manner (Fig. 8*D*). Recently AIF translocation was also reported to be calpain-dependent. However, upon treatment of our cells with calpeptin (20 μM) we still observed AIF in the nucleus (Fig. 8*D*). In addition we found that calpeptin administration to 786-0 cells prior to the addition of LY83583 failed to reverse the apoptotic effect (Fig. 8*C*). We did, however, observe that treatment of 786-0 cells with NAC (10 mM) prevented both the apoptotic effect as well as the translocation of AIF to the nucleus (Fig. 8*C* and *D*). A role for p38 MAPK in AIF translocation has recently been reported (Yu et al., 2004). Thus, we sought to investigate whether similar control exists in 786-0 cells treated with LY83583 (1 μM). This was indeed the case with 786-0 cells transfected with p38δ and p38γ DN opposing AIF translocation to the nucleus while 786-0 p38δ DN and p38γ wt cells behaved as non-transfected cells with a corresponding migration of AIF to the nucleus following LY83583 treatment (Fig. 8*D*). Finally, to further strengthen a role for AIF in LY83583-induced RCC apoptosis we used an siRNA approach. AIF levels were reduced by ~ 90% (by densitometric analysis) in 786-0 cells upon transfection with *SMART*pool® AIF siRNA for 48hr (Fig. 8*E*). si*Control*™ RISC-Free siRNA used as a control did not alter the levels of AIF protein expression in 786-0 cells. AIF siRNA transfected and non-transfected 786-0 cells were then treated with LY83583 (1 μM) for 48 hr (levels of AIF were still suppressed at this time point i.e. 96hr following the start of transfection). Using flow cytometric analysis we observed an increase in cell viability in AIF depleted cells after LY83583 (1 μM) addition albeit not to the level of untreated cells (Fig. 8*F*). Nontransfected 786-0 cells displayed a 53.2 ± 4.3% loss in cell viability after treatment with LY83583 (1 μM) for 48 hr while *SMART*pool® AIF siRNA transfected cells displayed a loss of 24.2 ± 1.9% (Fig. 8*F*).

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Discussion

The lack of effective therapy for the treatment of RCC has recently led to the investigation and development of various immunological treatment strategies (Vuky and Motzer, 2000). Despite this, however, overall response rates of 6 to 20% remain inadequate (Vuky and Motzer, 2000). These poor clinical response rates together with the toxicity associated with such treatment has meant that identification of other less toxic biological molecules is imperative. Unfortunately few antineoplastic compounds have been identified in a recent specific search for agents active in this disease (Mertins et al., 2001). In the present study, however, we have identified an anti-tumorigenic agent, a naphthoquinolinedione compound, LY83583, for RCC, a compound which proved refractory to normal human renal epithelial cells. Despite some observations (Lee and Wurster, 1995; Lodygin et al., 2002) work pertaining to this drug as a useful therapeutic agent is lacking. We now report on the molecular mechanisms that underlie the sensitivity of renal tumor cells to this possible future therapeutic agent.

Proliferation and programmed cell death are key features of oncogenesis and tumor progression. Despite the fact that ROS can act as mediators of proliferation or act as key players in apoptosis, cell cycle arrest and cellular senescence (Velarde et al., 2004; Gumpricht et al., 2005), a defined mechanistic model of how ROS contribute to the cascade of apoptotic events is still lacking. In this report we document the mechanisms involved in LY83583-induced 786-0 apoptosis through ROS formation. Both DCFH and DHR have been used as indicators of respiratory burst in different cell types (Halliwell and Whiteman, 2004). However, their fluorescence is an assay of generalized oxidative stress rather than of any particular reactive species. We observed an increase in the oxidation of both DHR and DCFH in a time-dependent fashion when 786-0 cells were treated with LY83583 (Fig. 3 and data not shown). As outlined in

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a recent review by Halliwell and Whiteman (2004) this reflects an increase in the reactive species $OH^{\bullet}$, $NO_2^{\bullet}$, and $ONOO^-$ rather than H_2O_2 or $O2^-$.

In contrast to previous studies whereby the elimination of ROS by chemical or enzymic antioxidants decreases the tumorigenicity of various types of tumor cells, we now demonstrate that excessive production of ROS in RCC can have an anti-tumorigenic effect. Depending on the cell type, different MAPK family members, such as ERK1/2, p38 MAPK, and JNK have been shown to possess ROS-sensitive kinase activity (Dong et al., 2004; Kamata et al., 2005). In addition constitutive activation of the Ras/ERK/MAPK pathway has been implicated in the progression of and often the de-differentiated phenotype of RCC (Oka et al., 1995) which contributes to the refractory nature of these tumors to conventional treatment strategies (Motzer et al., 1996). We have now shown that members of the MAPK cascade act as targets for ROS-induced apoptosis in RCC. Early inactivation of MEK1/2 and ERK1/2 was observed on treatment of 786-0 cells with LY83583 with a corresponding inactivation of the downstream transcription factor Elk-1 as well as downregulation of COX-2. In addition we observed a role for the serine threonine kinases Akt and Pak1 (which was found to be constitutively activated in these cells, data not shown) in mediating LY83583-induced apoptosis. In the presence of ROS scavengers, however, we failed to observe apoptosis as well as kinase inactivation.

The roles played by the p38 subfamily of MAPKs in apoptosis are more complex than previously thought with distinct members appearing to have different roles. While a role for p38α and -β was not apparent in our studies, we did observe, however, that p38δ and -γ appear to have disparate roles in LY83583-induced apoptosis. Cells expressing the former isoform or a dominant negative mutant of the latter isoform conferred resistance to LY83583 with a

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concomitant decrease in AIF translocation. This resistance, however, was not related to their activation and may be due to their mass action effects on altering ERK1/2 function. It is not clear how p38δ and -γ possess different functions in the apoptosis observed in our system. One obvious possibility is that the two isoforms have differing downstream substrates. Further studies are presently underway to address this issue in an attempt to delineate their role(s) in LY83583-induced apoptosis.

There are at present incongruent results with regard to the relevance of the Bcl-2 gene family, apoptotic induction, and cellular proliferation in the development and progression of RCC (Kallio et al., 2004). In general both Bcl-2 and Bcl-x_L have been shown to antgonise ROS production in apoptosis and to protect cells from exogenous oxidant-induced apoptosis. In agreement with published data for RCC we have observed high levels of expression of anti-apoptotic Bcl-2 as well as pro-apoptotic Bax in 786-0 (and ACHN and Caki-1 cells, data not shown). Despite this, however, Bax expression was further increased upon addition of LY83583 with a corresponding decrease in Bcl-x_L expression. We observed a change in Bax and Bcl-x_L expression at 48, 72 and 96 hr (Fig. 7*A*) yet apoptosis occurred at 24 hr (Fig. 2*A* and *B*). Their role in the apoptotic response at the earlier time of 24 hr may not be evident by Western blotting as early small changes in their expression may not be discernable against high basal background expression. The additional experiments, however, using the Bax channel blocker as well as the Bcl-2 inhibitor (HA14-1) (Table 2) confirms the role of both Bax and Bcl-x_L at 24 to 96 hr in LY83583-induced RCC apoptosis.

One of the most puzzling paradigms related to cell death at present is the release of AIF. In agreement with recent reports involving p38 MAPK and ERK in AIF translocation (Yu et al., 2004) we have demonstrated that ERK1/2 inactivation as well as the specific isoforms p38δ and

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-γ are involved in AIF translocation. A review of the literature has demonstrated conflicting reports regarding the role of caspases in its release. Both caspase-dependent and -independent effects have been ascribed to the release of AIF. To make the process somewhat more complicated a role for calpain I mediated Bid cleavage has been associated with AIF release. In our studies, however, we have found that neither caspases nor calpain I appear to affect AIF translocation to the nucleus during apoptosis. This does not, however, preclude the involvement of other endogenous proteases present in the mitochondrial intermembrane space which may be required for proteolysis of AIF prior to its translocation. We are presently investigating this intriguing mechanism.

RCC is one of the most difficult malignancies to treat due mainly to the fact that it is reported to express, except for adrenocortical cancer, the highest levels of P-glycoprotein among tumors (Goldstein et al., 1989). We now report on a therapeutic drug which does not appear to be influenced by the high levels of this protein (O.P. Barry, unpublished observation). In addition the lack of cytotoxicity of LY83583 in normal human renal epithelial cells as outlined in this study is an obvious advantage. One explanation may be the lack of constitutive ERK1/2 activation in HRE cells (O.P. Barry, unpublished observation) which appears to play a central role in mediating the apoptotic process of LY83583 in RCC. In addition the presence of p38δ which we found to confer resistance to LY83583-induced apoptosis in 786-0 cells may be another factor contributing to the resistance of HRE cells to this drug. Present studies are now focusing on the effect of this novel compound on RCC growth *in vivo* using animal models. It is our hope that this body of work may pave the way to the future usage of this compound as a therapeutic agent in a tumor which presently has few effective therapies.

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Footnotes

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Figure Legends

Figure 1. LY83583 induces a time- and dose-dependent inhibition of RCC 786-0 cell proliferation but not normal human renal epithelial cells (HRE). 786-0 (*A*) and HRE (*B*) cells (2 x 10^4) were treated with LY83583 (0.03, 0.1, 0.3, and 1 μM) for 24-96 hr and counted. When cells were untreated vehicle alone (0.1% Me_2SO) was added. The results shown are mean ± S.E. of four independent experiments.

Figure 2. LY83538 induces a time-dependent increase in apoptosis in 786-0 but not HRE cells. Following treatment of 786-0 (*A* and *B*) and HRE (*C* and *D*) cells with LY83538 (1 μM, 24-96 hr) apoptotic and non-apoptotic cells were detected either by flow cytometry following staining with FITC-conjugated Annexin V and PI (*A* and *C*) or by TUNEL analysis (*B* and *D*) as described in *Materials and Methods.* 10, 000 cells were counted by FACScalibur analysis to determine the distribution of cells (*A* and *C*). Quadrants I, II, III and IV represent different cell groups; I represents unlabelled viable cells (Ann-V^-/PI^-), II represents early apoptotic cells (Ann-V^+/PI^-), III represents late apoptotic cells (Ann-V^+/PI^+) and IV represents cells destroyed during experimental workup (*A* and *C*). The figures are representative of five independent experiments.

Figure 3. Exposure of 786-0 cells to LY83583 leads to a dramatic increase in ROS production. 786-0 cells were preincubated with 1′, 2′, 3′, DHR (30 μM) for 30 mins and, thereafter, stimulated with LY83583 (1μM). Results are expressed as % increase in relative fluorescence units compared to control (0.1% Me_2SO) treated 786-0 cells. Significant (***: $p<0.001$, *:$p<0.05$) changes from control (0.1% Me_2SO) treated 786-0 cells. Data represent the mean ± S.E. of five independent experiments.

Figure 4. Effect of LY83583 on 786-0 MAPK, Elk-1 and COX-2 expression is ROS-dependent. 786-0 cells were treated with and without LY83583 (1 μM) for the indicated times (*A*). Aliquots of 786-0 cells containing 30 μg of protein lysate were loaded on a 10% SDS-PAGE gel and analyzed by immunoblot using total and phospho-anti-ERK1/2 (*A* and *B*) and -5 (*A*), anti-MEK1/2 (*A* and *B*), anti-Akt (*A*), anti-p38α/β (*A*), anti-JNK1/2 (*A*), anti-MKK3/MKK6 and anti-Elk-1 (*A* and *B*) antibodies. Treatment of cells with anisomycin (1 μg/ml) was included as a positive control for phosphorylated JNK1/2, or sorbitol (20 μM) for phosphorylated-p38α/β MAPK, MKK3/MKK6 and ERK5 (*A*). 40 μg of protein lysate was used for analysis of COX-2 and 20 μg for COX-1, and 7% and 3% was used, respectively, for the separating and stacking gels (*A* and *B*). Cells were or were not treated with N-acetyl L-cysteine (NAC) (10 mM) and LY83583 (1 μM) for 96 hr (*B*). All membranes were probed with β-actin as a loading control. The figures are representative of four independent experiments.

Figure 5. p38δ and p38γDN rescue LY83583-mediated 786-0 apoptosis in a ROS-dependent manner. Western blot analysis of p38 MAPK isoform expression in HRE and 786-0 cells (*A*). 786-0 cells were or were not (control C) stably transfected with pcDNA3 (empty vector), p38

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MAPK wt isoforms (α, -β, -γ and -δ) and p38 MAPK dominant negative (DN) isoforms. Sixty μg of protein was probed with anti-FLAG^{R} M2 mAb which recognizes the FLAG tag on the pcDNA3 empty vector and p38 constructs (*B*). Transfected and non-transfected cells were or were not treated with LY83583 (1 μM, 96 hr) (*C* and *D*). Cells were counted (*C*) and viable cells were detected by flow cytometry following staining with FITC-conjugated Annexin V and PI (*D*) as described in *Materials and Methods.* Significant (***: $p<0.001$) changes from non-transfected 786-0 cells treated with LY83583 (1 μM) (*C* and *D*). Western blot analysis of ERK1/2 and phospho-ERK1/2 in p38δ, δDN, γ and γDN transfected and nontransfected cells in the presence or absence of LY83583 (1 μM, 96 hr) (*E*). Membranes were probed with β-actin as a loading control (*B* and *E*). The figures are representative of four independent experiments.

Figure 6. The MEK inhibitor U0126 mimics the effect of LY83583 while active MEK opposes its effect. 786-0, 786-0δ and 786-0γDN cells were treated with and without U0126 (20 μM) for 24-96 hr. 786-0 MEK cells were treated with and without LY83583 (1 μM) for 24-96 hr. Cell viability was detected by flow cytometry following staining with FITC-conjugated Annexin V and PI as described in *Materials and Methods.* Significant (***: $p<0.001$, **:$p<0.01$) changes from non-transfected 786-0 cells treated with U0126 (20 μM). Data represent the mean ± S.E. of three independent experiments.

Figure 7. Effect of LY83583 on 786-0 apoptosis-related gene expression is ROS-dependent. 786-0 cells were treated with and without LY83583 (1 μM) for the indicated time points. Equal amounts of cellular protein (30 μg) were fractionated on 15% SDS-PAGE gels and immunobloted with Bcl-2, Bcl-x_L, BAD, Bax, and p53 monoclonal antibodies (*A*). Cells were or were not treated with N-acetyl L-cysteine (NAC) (10 mM), z-VAD-fmk (50 μM) and LY83583 (1 μM) (*B*). Membranes were probed with β-actin as a loading control. The figures are representative of four independent experiments.

Figure 8. LY83583-induced cytochrome c release, caspase-3 activation and AIF translocation is ROS-dependent. Cytochrome c was measured in the mitochondrial (M) and cytosolic (C) fractions (*A*) and proteolytic processing of procaspase-3 was evident (*B*) when 786-0 cells were treated with LY83583 (1 μM) for 96 hr. Forty μg of protein was fractionated by 10% and 15% SDS-PAGE gels respectively for cytochrome c and caspase-3. Cells were treated with N-acetyl L-cysteine (NAC) (10 mM) and LY83583 (1 μM) for 96 hr (*A-D*). The specific caspase-3 inhibitor, DEVD-CHO (50 μM), the pan-caspase inhibitor, z-VAD-fmk (50 μM), an inhibitor of MPT formation, monobromobimane (MBB) (1 μM) or the calpain I inhibitor, calpeptin (20 μM), were added as indicated 1hr prior to the addition of LY83583 (1 μM) (*C* and *D*). Immunostaining for AIF in transfected or non-transfected 786-0 cells the presence or absence of LY83583 (1 μM) (*D*). Western blot analysis showing depletion of AIF following transfection of 786-0 cells with AIF siRNA for 48hr (*E*). siRNA AIF transfected and non-transfected 786-0 cells were treated with LY83583 (1 μM) for 48hr. Flow cytometric analysis was performed following staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot

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plot. Percentage of control (0.1% Me_2SO treated) 786-0 cells are represented as viable cells (quadrant I) (*F*). Significant (*: $p<0.05$) changes from non-transfected 786-0 cells treated with LY83583 (1 μM) (*F*).The figures are representative of five independent experiments.

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TABLE 1

Time-dependent decrease in 786-0 cell viability following LY83583 treatment is ROS-dependent but guanylate cyclase independent. Flow cytometric analysis was performed following staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot plot. Percentage of control (0.1% Me_2SO treated) 786-0 cells are represented as viable cells (quadrant I). Cells were treated with LY83583 (1 μM) (*A*) or pretreated with 8-Br-cGMP (1 mM) and zaprinast (20 μM) (*B*), ODQ (20 μM) (*C*), N-acetyl L-cysteine (10 mM) (*D*), vitamin C (200 μM) (*E*), vitamin E (50 μM) (*F*), L-NAME (1 mM) (*G*), or melatonin (150 μM) (*H*) for 1 hr followed by treatment with LY83583 (1 μM) for 24-96 hr. Data represent the mean ± S.E. of five independent experiments.

	Cell viability (%)							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
0hr	90 ± 5	95 ± 5	91 ± 9	87 ± 5	98 ± 6	89 ± 5	87 ± 5	97 ± 4
24hr	65 ± 6	61 ± 7	69 ± 4	81 ± 4	94 ± 5	93 ± 4	81 ± 4	91 ± 6
48hr	45 ± 10	38 ± 10	40 ± 5	80 ± 13	91 ± 10	95 ± 3	88 ± 7	90 ± 3
72hr	16 ± 6	8 ± 8	10 ± 6	89 ± 8	89 ± 5	91 ± 8	95 ± 8	94 ± 6
96hr	10 ± 10	3 ± 5	4 ± 5	83 ± 7	90 ± 3	93 ± 9	93 ± 3	93 ± 7

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TABLE 2

LY83583-induced 786-0 apoptosis is Bcl-2- and Bax-dependent. Flow cytometric analysis was performed following staining with FITC-conjugated Annexin V and PI and gated onto a fluorescent dot plot. Percentage of control (0.1% Me_2SO treated) 786-0 cells are represented as viable cells (quadrant I). Cells were treated with LY83583 (1 μM) alone (*A*) or pretreated with the Bcl-2 inhibitor HA 14-1 (1 μM) (*B*) or a Bax channel blocker (5 μM) (*C*) for 1 hr followed by treatment with LY83583 (1 μM) for 24-96 hr. Data represent the mean ± S.E. of three independent experiments.

	Cell viability (%)		
	A	**B**	**C**
0hr	85 ± 7	93 ± 6	94 ± 9
24hr	66 ± 4	41 ± 9	89 ± 10
48hr	48 ± 11	28 ± 13	84 ± 5
72hr	15 ± 10	6 ± 8	64 ± 10
96hr	11 ± 10	3 ± 5	50 ± 7

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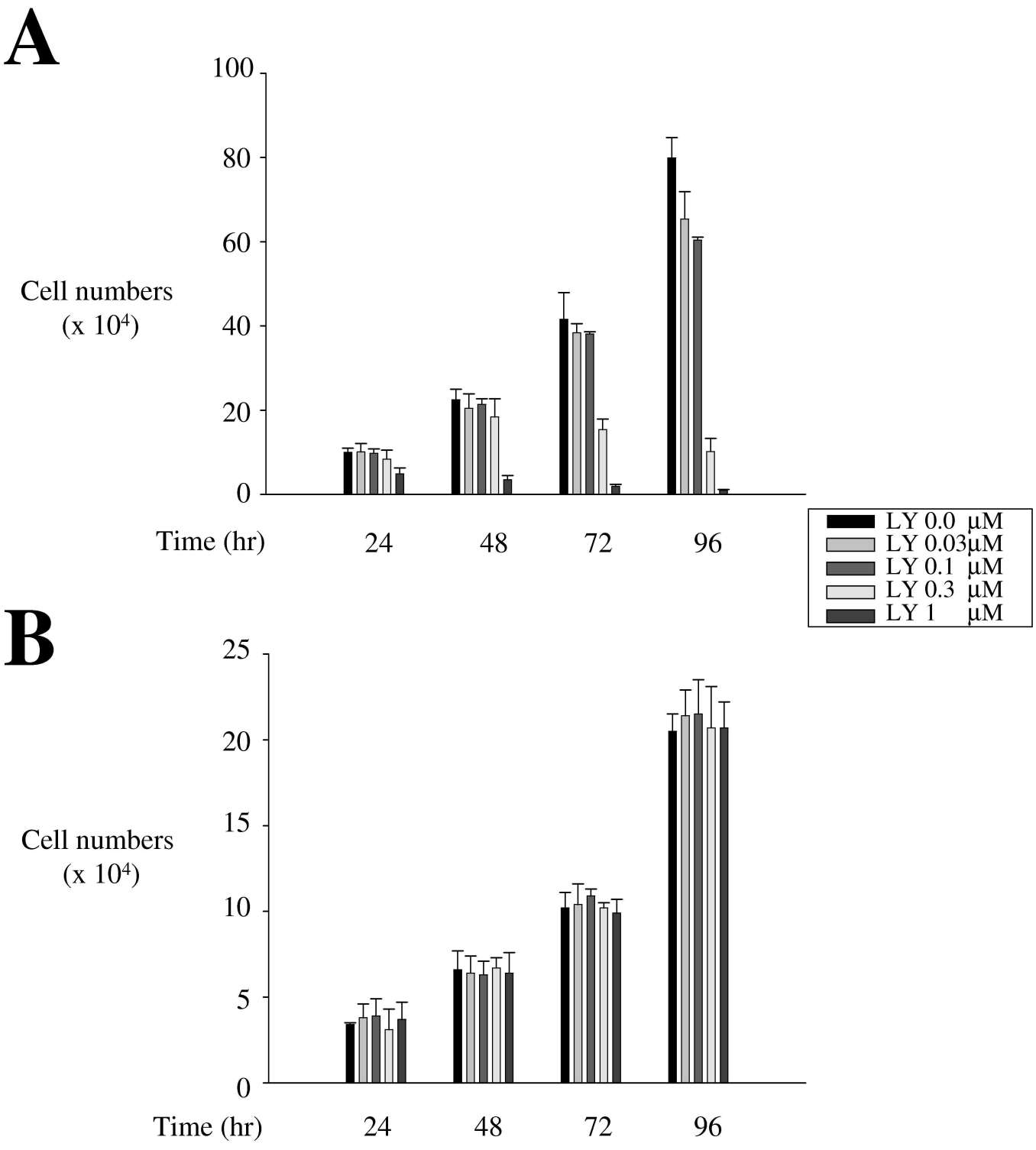

Figure 1

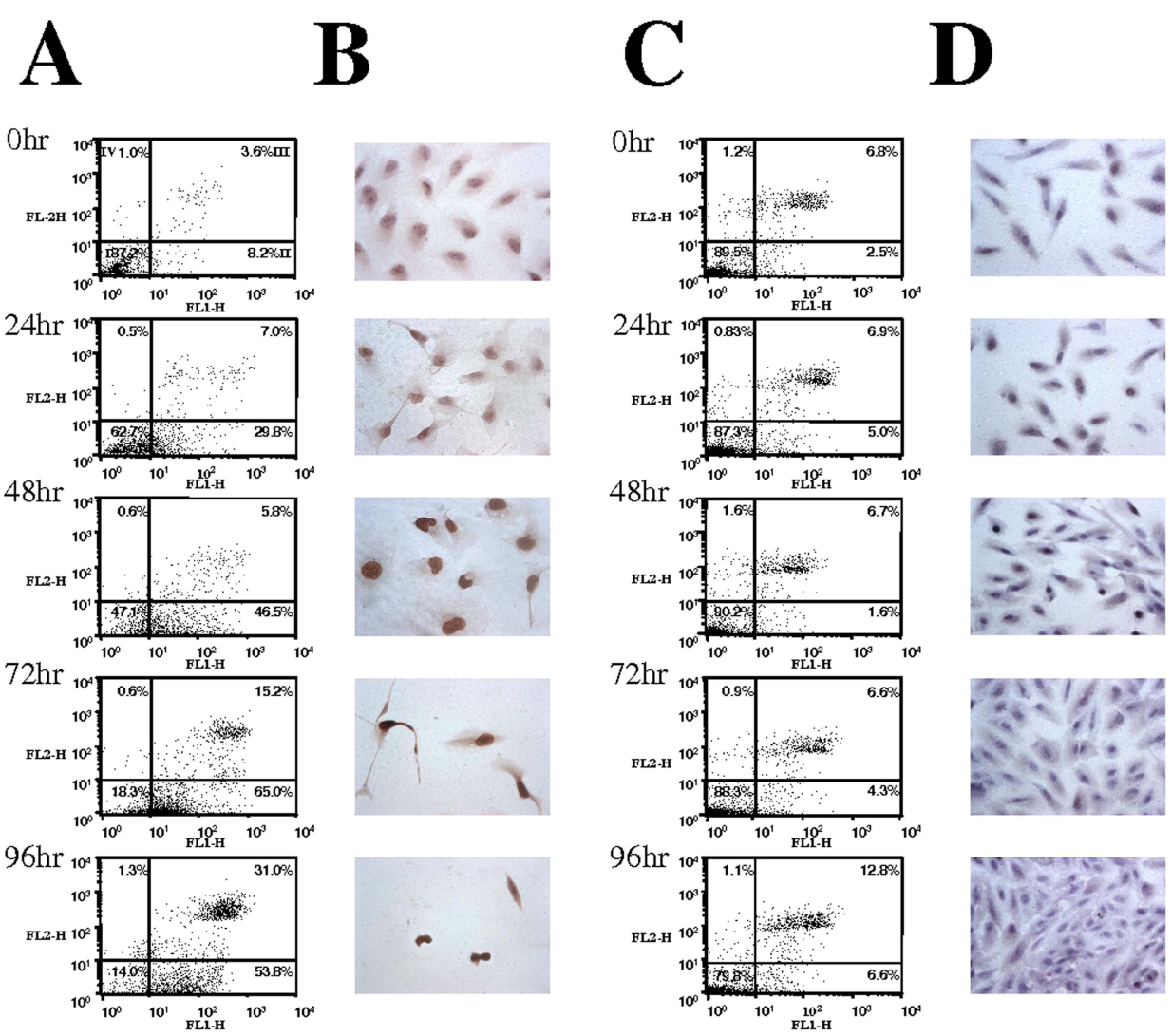
A
B
C
D
0hr
24hr
48hr
72hr
96hr
FL-2H
FL2-H
FL1-H
IV 1.0%
3.6%III
187.2%
8.2%II
0.5%
7.0%
62.7%
29.8%
0.6%
5.8%
47.1%
46.5%
0.6%
15.2%
18.3%
65.0%
1.3%
31.0%
14.0%
53.8%
1.2%
6.8%
89.5%
2.5%
0.83%
6.9%
87.3%
5.0%
1.6%
6.7%
90.2%
1.6%
0.9%
6.6%
88.3%
4.3%
1.1%
12.8%
79.8%
6.6%

Figure 2

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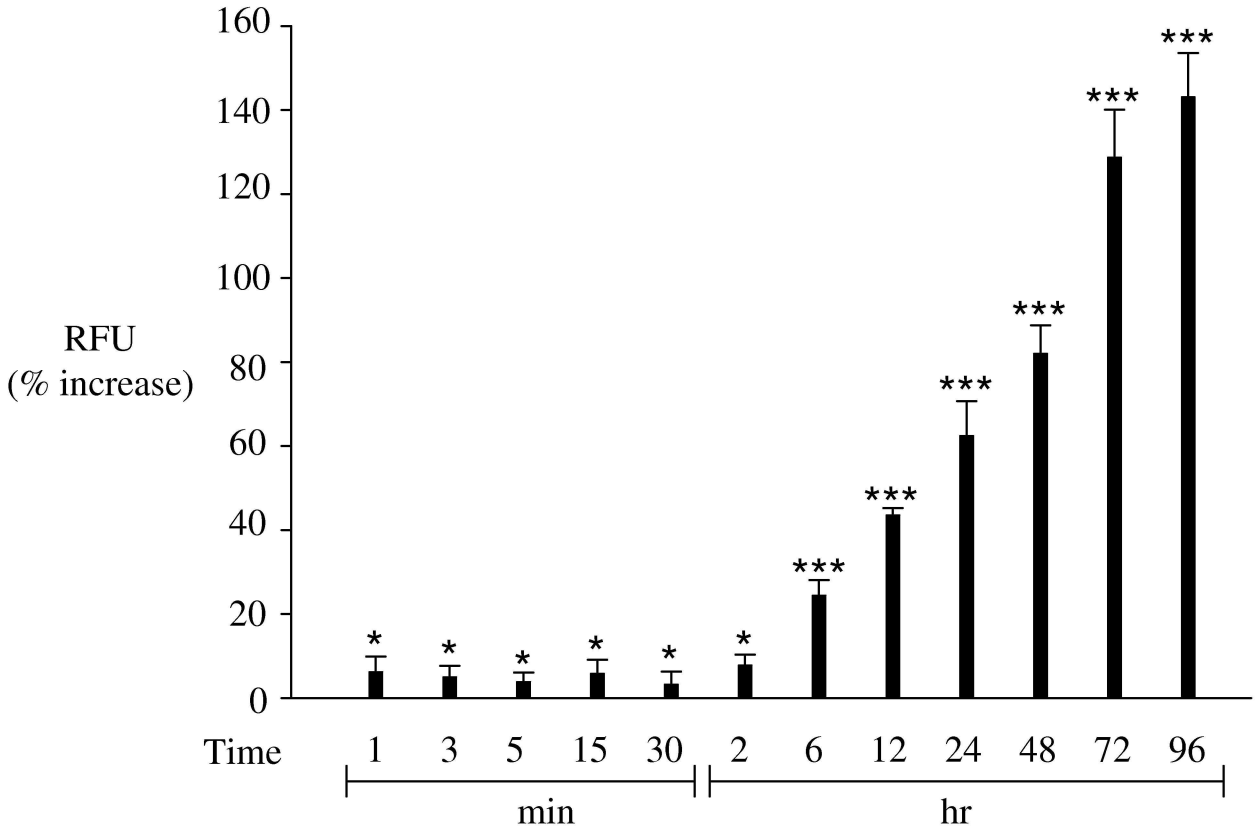

Figure 3

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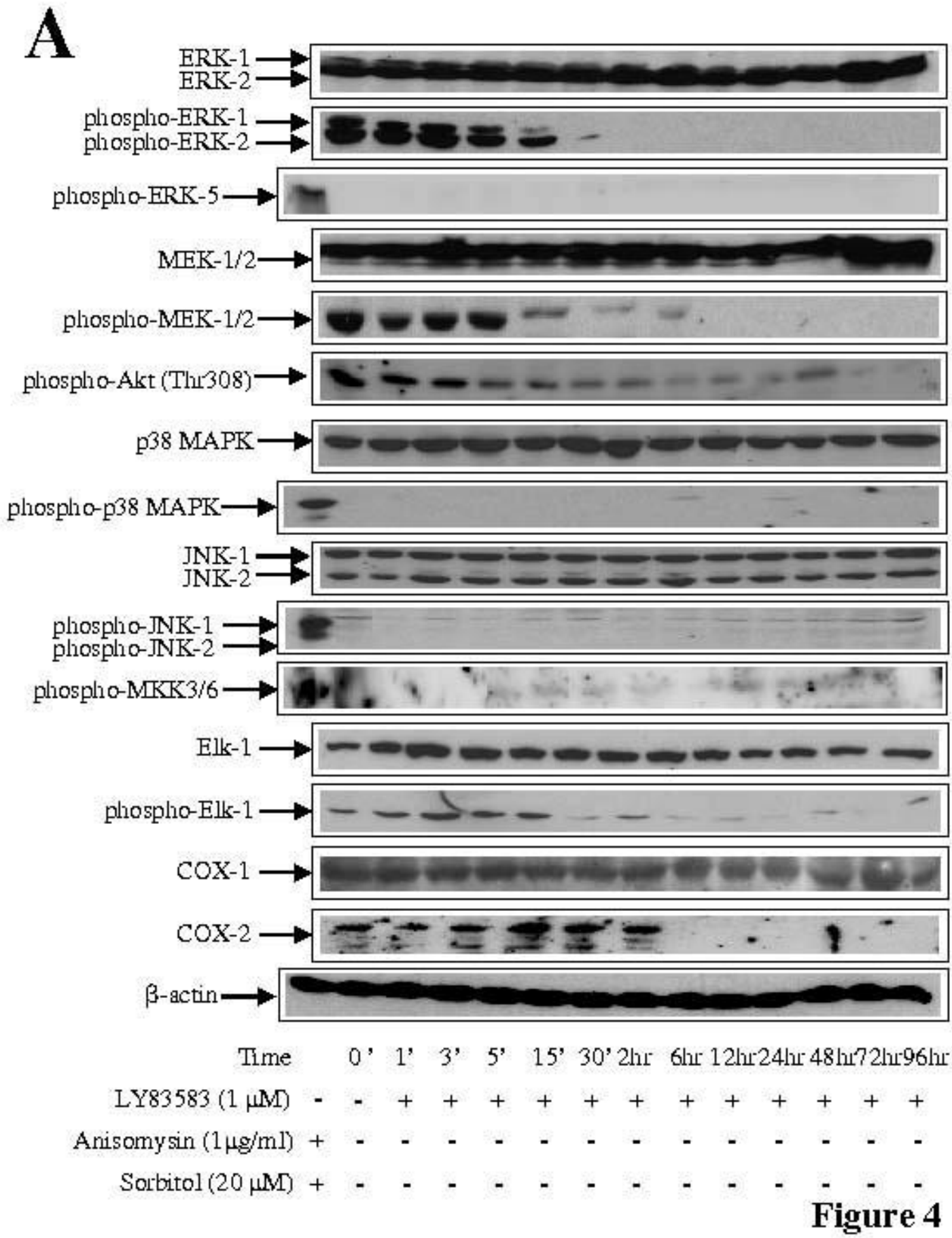

Figure 4

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B

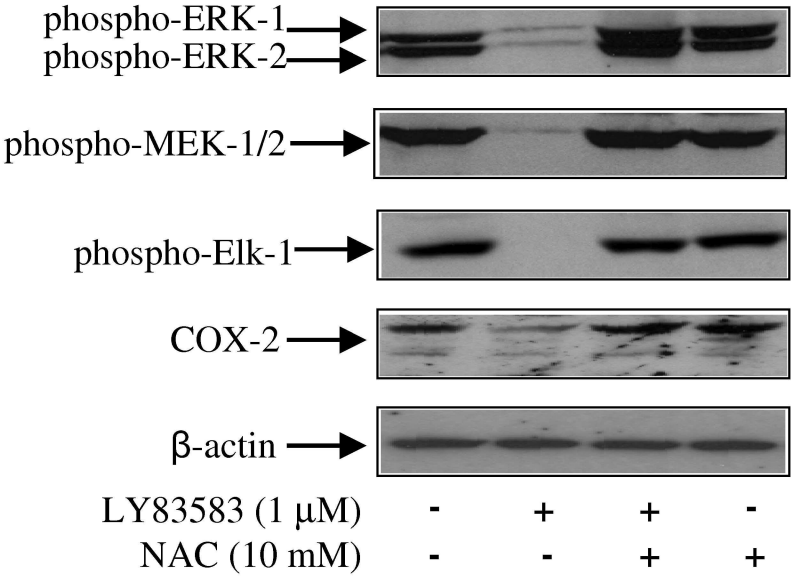

Figure 4

A

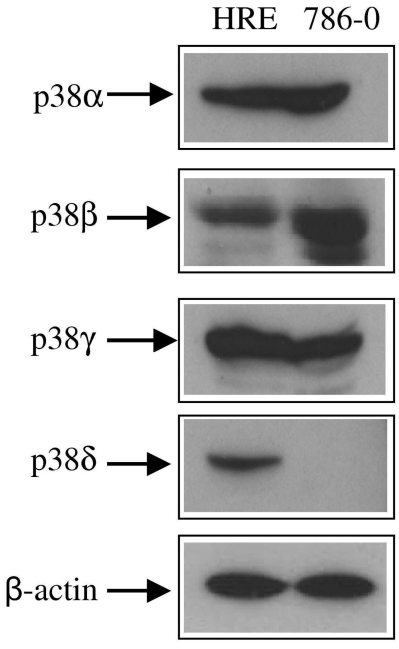

Figure 5

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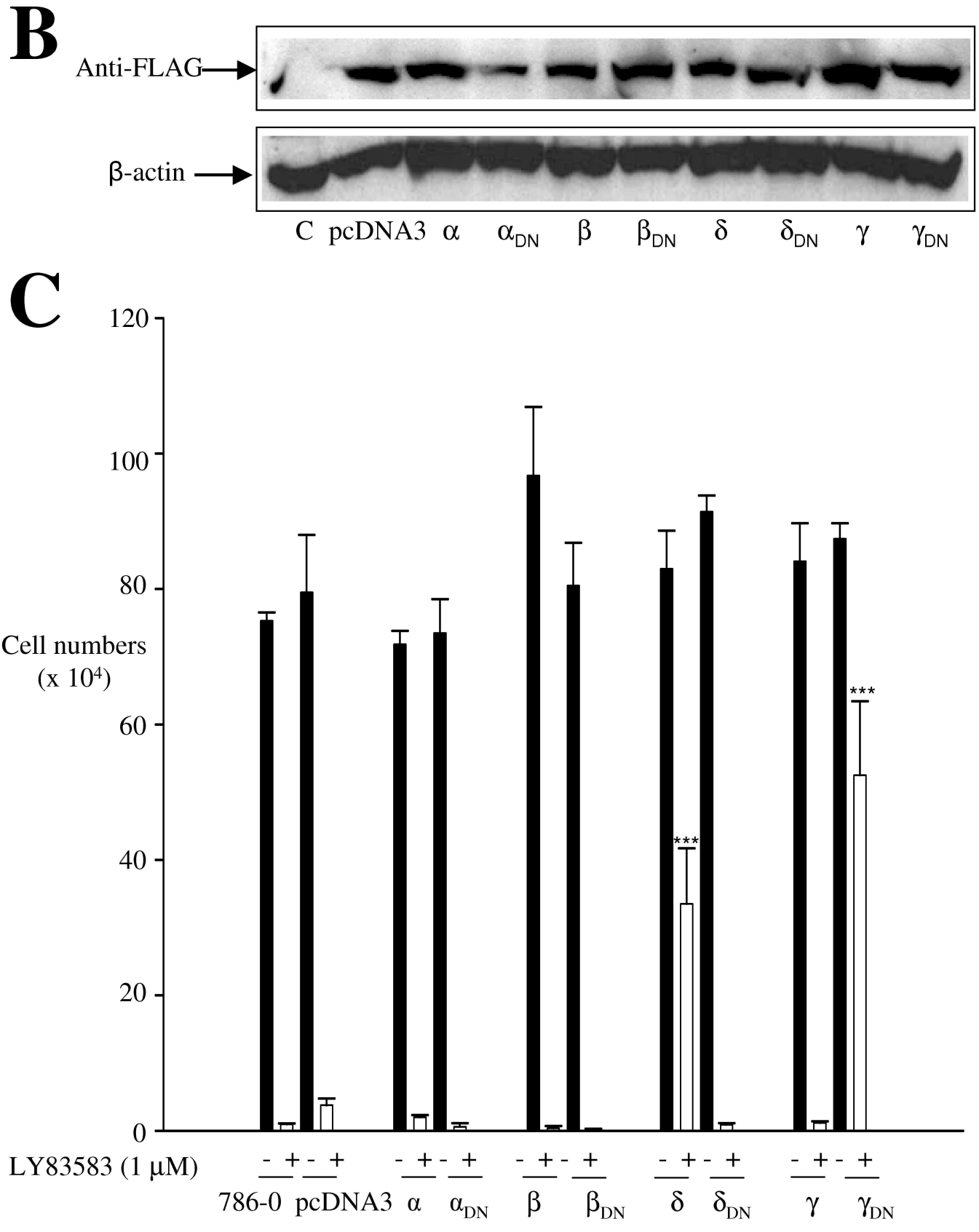

Figure 5

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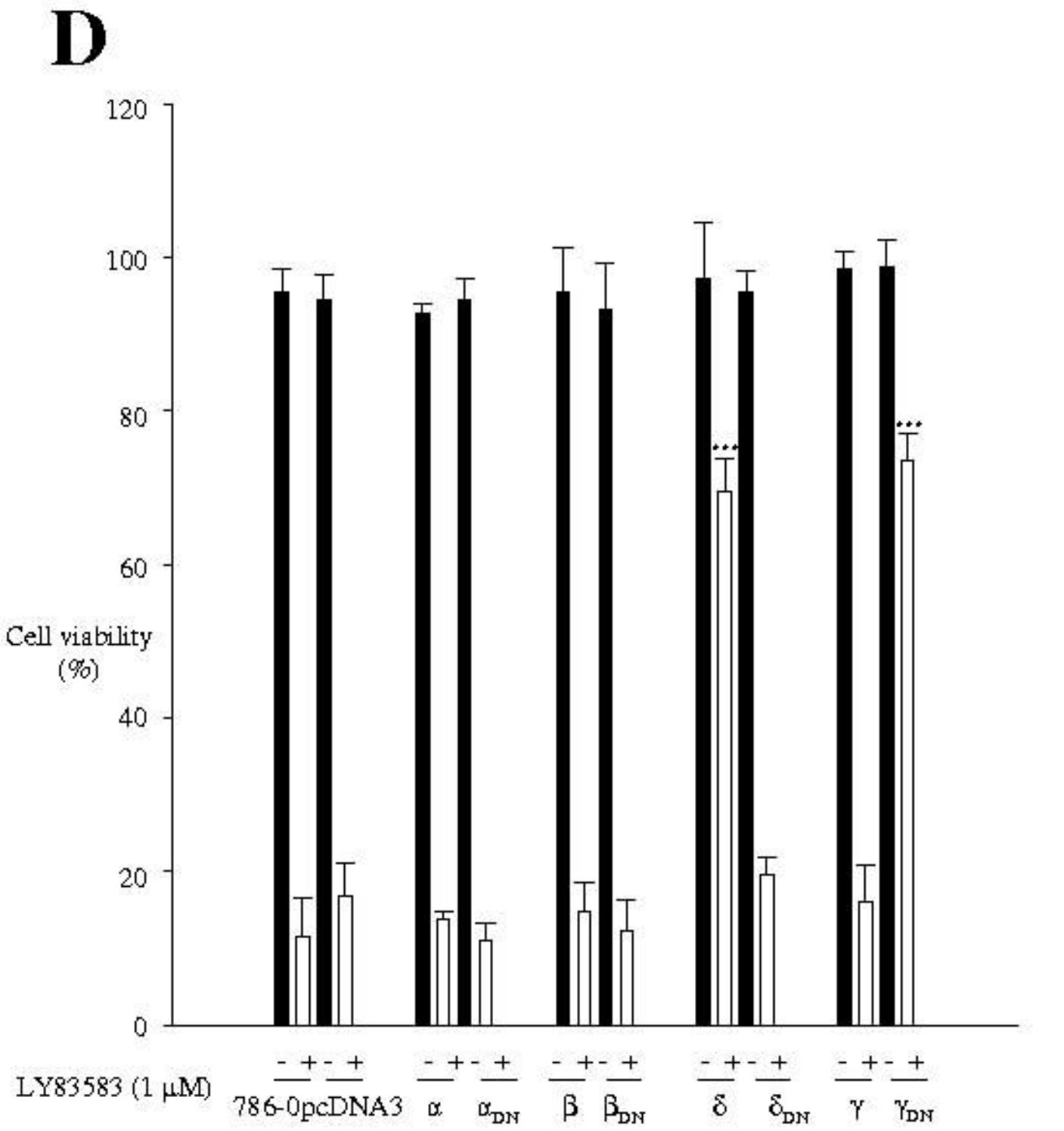

Figure 5

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E

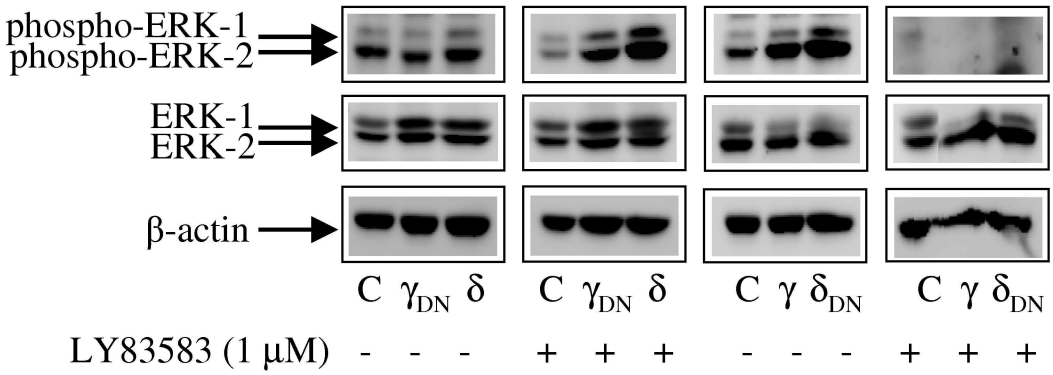

Figure 5

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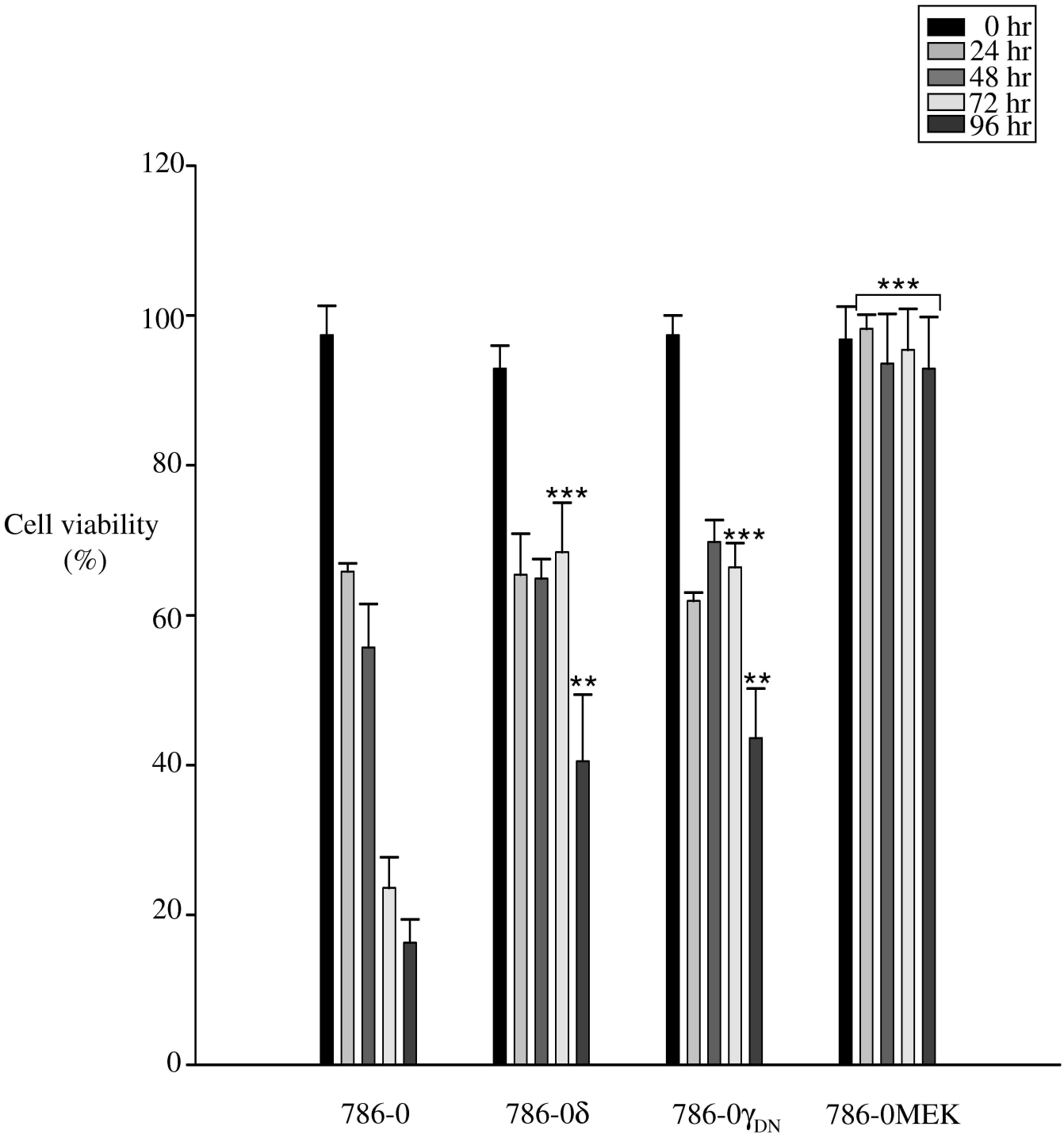

Figure 6

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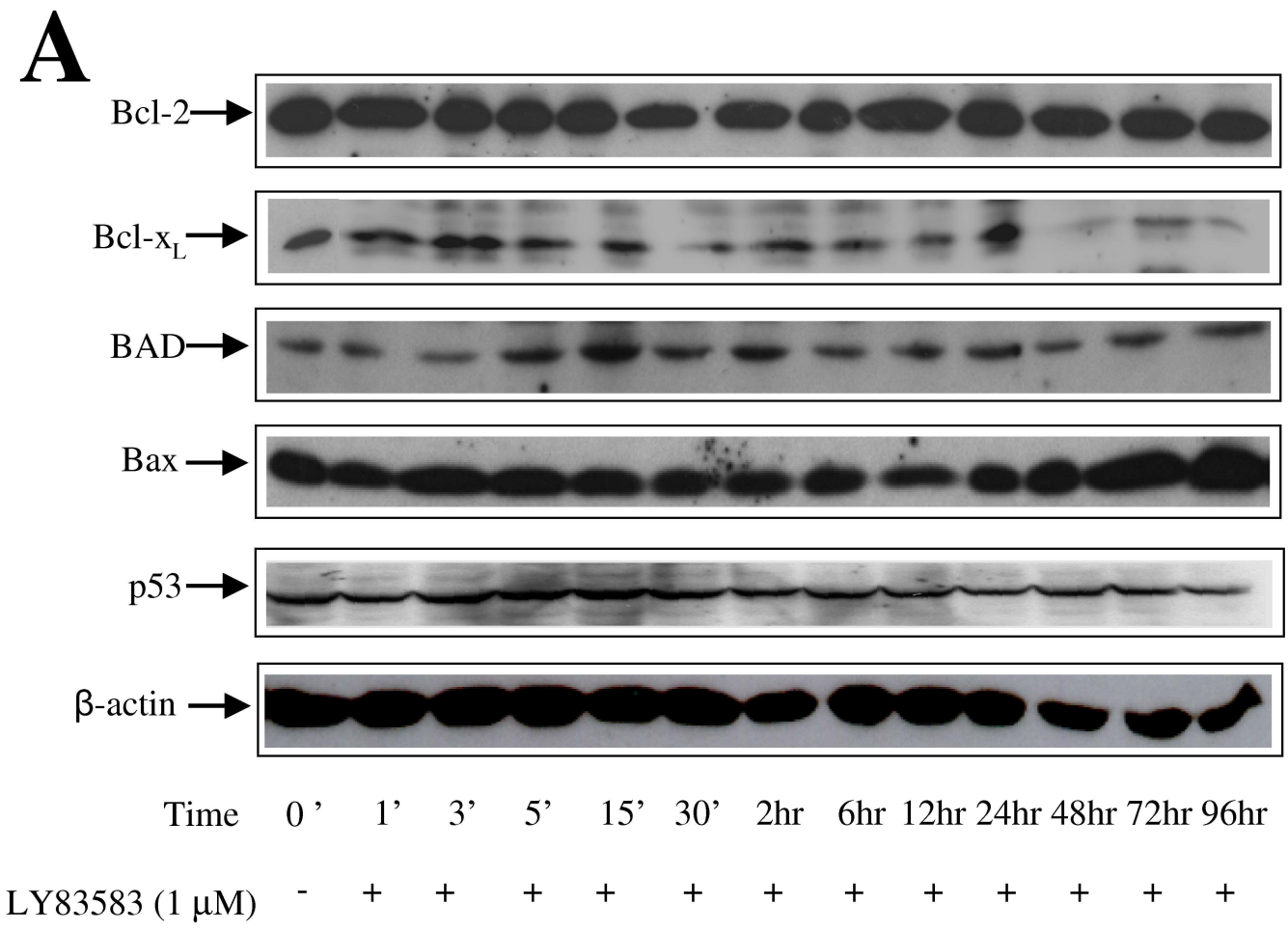

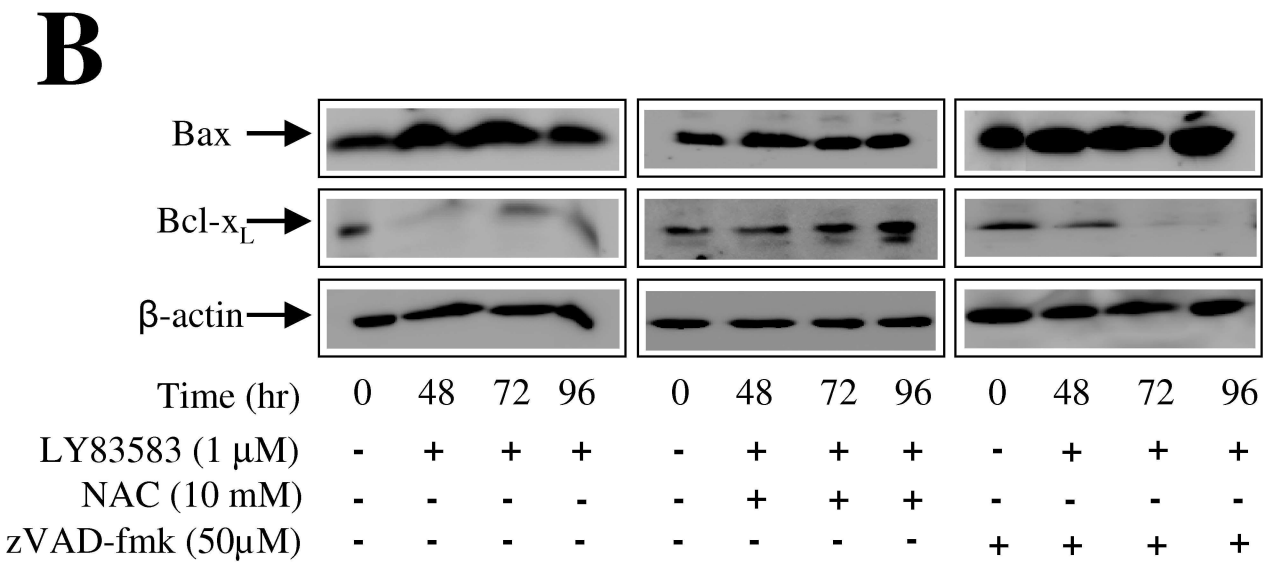

Figure 7

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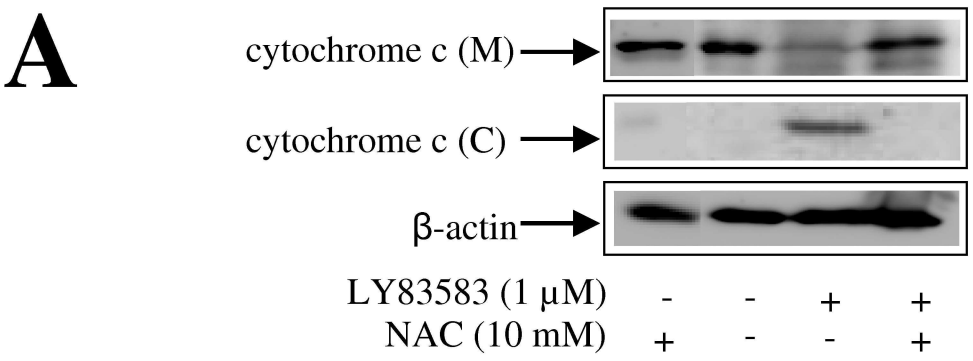
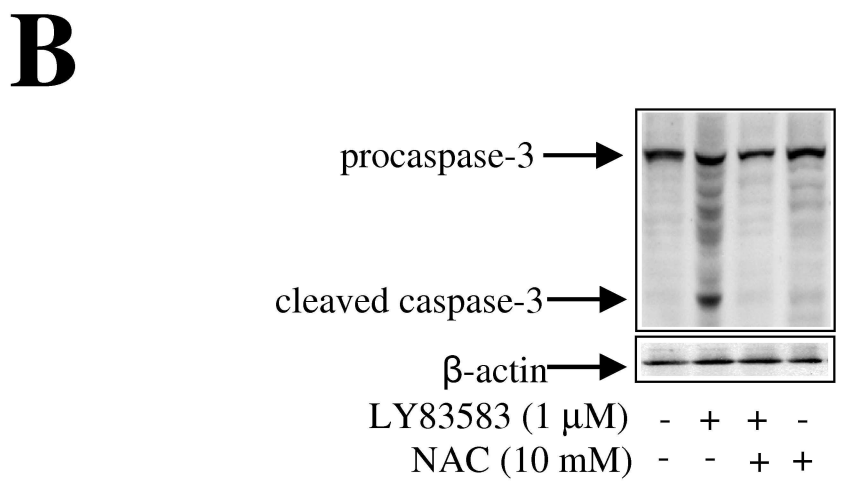




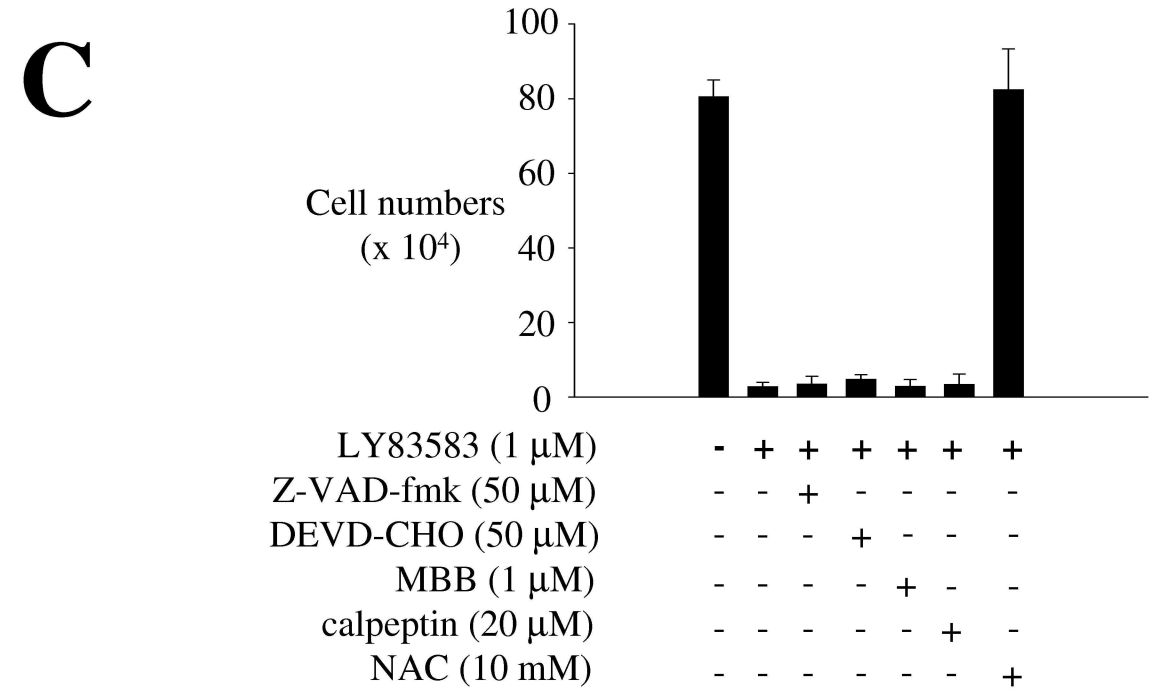
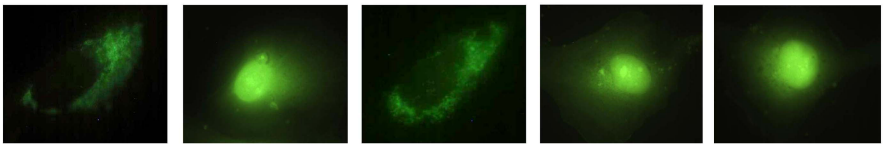
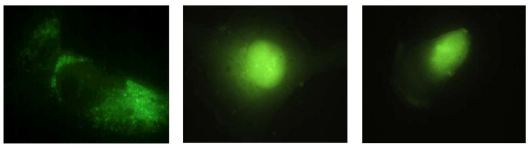


Figure 8

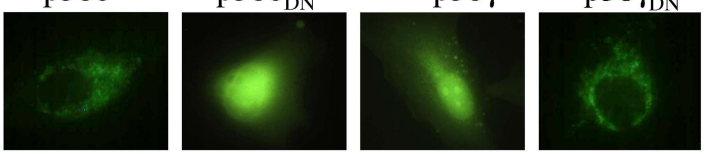
D



LY83583 (1 μM)	-	+	+	+	+
NAC (10 mM)	-	-	+	-	-
Z-VAD-fmk (50 μM)	-	-	-	+	-
calpeptin (20 μM)	-	-	-	-	+



LY83583 (1 μM)	-	+	+



LY83583 (1 μM)	+	+	+	+

Figure 8

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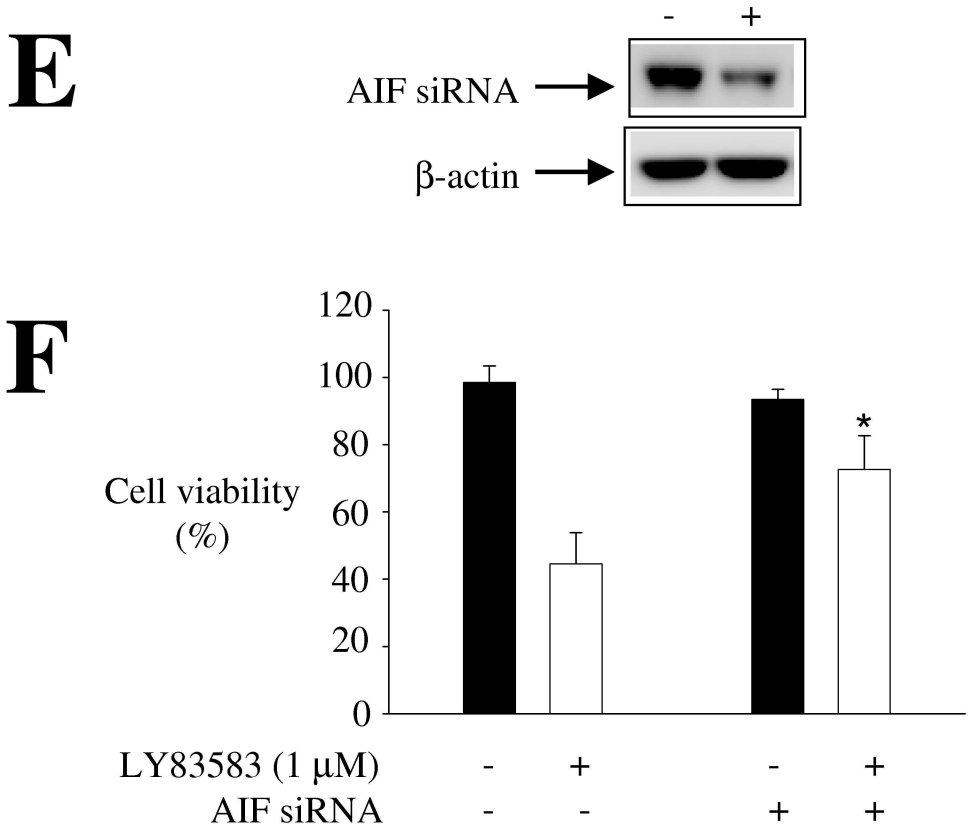

Figure 8