Excretory/secretory products of the carcinogenic liver fluke are endocytosed by human cholangiocytes and drive cell proliferation and IL-6 production

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Abstract

Liver fluke infection caused by *Opisthorchis viverrini* remains a major public health problem in many parts of Asia including Thailand, Lao PDR, Vietnam and Cambodia, where there is a strikingly high incidence of cholangiocarcinoma (CCA - hepatic cancer of the bile duct epithelium). Among other factors, uptake of *O. viverrini* excretory/secretory products (*Ov*ES) by biliary epithelial cells has been postulated to be responsible for chronic inflammation and proliferation of cholangiocytes, but the mechanisms by which cells internalize *Ov*ES are still unknown. Herein we incubated normal human cholangiocytes (H69), human cholangiocarcinoma cells (KKU-100, KKU-M156) and human colon cancer (Caco-2) cells with *Ov*ES and analysed the effects of different endocytic inhibitors to address the mechanism of cellular uptake of ES proteins. *Ov*ES was internalized preferentially by liver cell lines, and most efficiently/rapidly by H69 cells. There was no evidence for trafficking of ES proteins to cholangiocyte organelles, and most of the fluorescence was detected in the cytoplasm. Pretreatment with clathrin inhibitors significantly reduced the uptake of *Ov*ES products, particularly by H69 cells. *Ov*ES induced proliferation of liver cells (H69 and CCA lines) but not intestinal (Caco-2) cells, and proliferation was blocked using inhibitors of the classical endocytic pathways (clathrin and caveolae). *Ov*ES drove IL-6 secretion by H69 cells but not Caco-2 cells, and cytokine secretion was significantly reduced by endocytosis inhibitors. This the first study to address the endocytosis of helminths ES proteins by host epithelial cells and sheds light on the pathways by which this parasite causes one of the most devastating forms of cancer in SE Asia.

Keywords: *Opisthorchis viverrini*, endocytosis, carcinogenesis, excretory/secretory products, IL-6, cholangiocyte.

1. Introduction

Opisthorchiasis caused by infection with the carcinogenic liver fluke, *Opisthorchis viverrini*, remains an important health problem in Laos, Cambodia, Vietnam and Thailand, where >10 million people are infected (Sithithaworn et al., 2012). Humans acquire the infection by eating raw or undercooked cyprinoid fish, which act as the secondary intermediate host containing infective metacercariae (Kaewpitoon et al., 2008; Sripa et al., 2011). Adult worms inhabit the biliary system of the mammalian host where they can survive for many years (Kaewpitoon et al., 2008). Chronic *O. viverrini* infection is associated with several hepatobiliary diseases including cholangitis, biliary hyperplasia, periductal fibrosis and cholangiocarcinoma (CCA), a fatal type of bile duct cancer (Sripa et al., 2012a; Sripa et al., 2007). Indeed, the north-east region of Thailand where *O. viverrini* is endemic has the highest worldwide incidence of CCA (Sripa and Pairojkul, 2008).

O. viverrini-induced biliary pathology includes mechanical damage caused by the parasite's physical attachment to and grazing on the biliary epithelium, the release of excretory/secretory products (*Ov*ES), and immunopathology (Sripa, 2003; Sripa et al., 2012a). Indeed, immune-mediated pathogenesis in response to liver fluke infection is a major driving force in the onset of biliary disease, including CCA (Sripa et al., 2012a; Sripa et al., 2007). *Ov*ES released from the tegument and excretory openings of the fluke are highly immunogenic (Choi et al., 2003; Sripa and Kaewkes, 2000a, b; Wongratanacheewin et al., 1988). Sripa and Kaewkes (Sripa and Kaewkes, 2000a) observed an intense inflammatory response in areas of the biliary epithelium where parasite antigens were present, particularly those tissues in contact with the fluke. Moreover, numerous studies have provided evidence, albeit with low-resolution microscopy, that fluke antigens are detected inside cholangiocytes lining the biliary epithelium in *O. viverrini*-infected hamsters (Pinlaor et al., 2009; Smout et

al., 2009; Sripa and Kaewkes, 2000a). Until now, the mechanism by which liver fluke antigens are internalised by cholangiocytes remained elusive.

Endocytosis is a major pathway for cell-cell communication and internalisation of extracellular proteins in eukaryotic organisms (Doherty and McMahon, 2009). Two major pathways have been described: (i) clathrin mediated endocytosis whereby molecules are uptaken via clathrin-coated vesicles, and (ii) non-clathrin mediated endocytosis (or caveolae-mediated endocytosis) which requires cholesterol/sphingolipid-rich caveolae in membrane invagination and internalization (Le Roy and Wrana, 2005). The clathrin pathway controls targeting of signaling molecules to specialized membrane compartments (Ceresa and Schmid, 2000), while the caveolae mediated pathway acts as a regulator of cell signaling (Lipkowitz, 2003). In this study we used state-of-the-art microscopy techniques to investigate the internalisation mechanisms of *Ov*ES by human cholangiocytes and its function, particularly in cell proliferation and inflammatory cytokine IL-6 production. This may lead to better understanding of the pathogenesis of *O. viverrini*-induced hepatobiliary pathology and CCA.

2. Materials and methods

2.1 Chemicals

Bovine serum albumin and all cellular process inhibitors [protease inhibitor E64, chlorpromazine (CPZ, a cationic amphiphilic inhibitor acts on clathrin-coated pits), sucrose (hypertonic media that interferes with clathrin and adaptor protein interactions), bafilomycine A1 and filipin] were purchased from Sigma. ER-Tracker™Red, BODIPY TR C5-ceramide, LysoTracker, mouse anti-Rad5, Hoechst, propidium iodide, Alexa Fluor 488 and carboxylic acid succinimidyl ester were obtained from Molecular Probes.

92 *2.2 Parasites, animal infections and ethics approvals*

93 *O. viverrini* metacercariae were obtained from naturally infected cyprinoid fish
94 captured from an endemic area in Khon Kaen province, Northeast Thailand, as described
95 previously (Ninlawan et al., 2010). Briefly, fish were digested with pepsin-HCl, and after
96 several washes with normal saline, metacercariae were collected, identified under a dissecting
97 microscope and used to infect hamsters. Adult *O. viverrini* worms were obtained from the
98 liver, gallbladders and extrahepatic bile ducts of hamsters infected for 3 months. All the
99 hamsters used for this study were maintained at the animal facility, Faculty of Medicine,
100 Khon Kaen University, and the protocols used for animal experimentation were approved by
101 the Animal Ethics Committee of Khon Kaen University based on the Ethics of Animal
102 Experimentation of the National Research Council of Thailand.

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104 *2.3 Preparation of parasite ES products*

105 *Ov*ES was prepared as previously described with minor modifications (Sripa and
106 Kaewkes, 2000a, b). Briefly, fresh worms were cultured in RPMI-1640 containing antibiotic
107 and the protease inhibitor E64. Worms were maintained *in vitro* at 37°C and supernatants
108 containing the *Ov*ES were collected twice each day for up to 7 days and centrifuged at 2,090
109 *g* for 10 min to remove the eggs. The clarified supernatants were pooled, dialyzed in PBS,
110 concentrated and absorbed with Triton-X114 to remove residual lipopolysaccharide (LPS)
111 (Aida and Pabst, 1990), followed by Bio-Beads SM2 (Bio-Rad) to remove Triton-X114.
112 Finally, *Ov*ES was filtered through a 0.2 μm membrane and then aliquoted and stored at -
113 80°C. The LPS concentration was determined by Limulus amoebacyte assay (less than 100
114 ng/ml had no effect on cell proliferation and cytokine production).

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2.4 Fluorescent labeling of OvES proteins.

*Ov*ES proteins were labeled with Alexa Fluor 488 (Invitrogen) following the manufacturer's protocol. Briefly, 0.5 ml of 2 mg/ml *Ov*ES was gently mixed with 50 µl of 1.0 mg/ml Alexa Fluor 488 in DMSO for 1 h at room temperature (RT). A G10 gel filtration column (Amersham Biosciences) was used to isolate labeled proteins from unbound dye. The protein concentration was measured by Bradford assay (Bio-Rad), and labeled protein was kept at 4°C until required for cell culture.

2.5 Cell culture

Normal human cholangiocytes (H69) were maintained in Dulbecco's Modified Eagle Medium (DMEM/ HamF-12) (Gibco) supplemented with insulin, adenine, epinephrine, T3-T, epidermal growth factors (EGF) and hydrocortisone (Ninlawan et al., 2010). Human cholangiocarcinoma (KKU-100 and KKU-M156) were maintained in Ham-F12 (Gibco) and human colon cancer (Caco-2) cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing L-gutamine and non-essentails amino acids (Gibco) with at 37°C, 5% CO_2 with 10% fetal calf serum (Gibco) containing 100 U/ml penicillin and 100 µg/ml streptomycin solution (Gibco).

2.6 Internalization of OvES

To determine the uptake of *Ov*ES by cells, H69, KKU-100, KKU-M156 and Caco-2 cell lines were incubated with 10 µg/ml unlabeled *Ov*ES at 37ºC. The cells were collected at different time points (0, 15, 30, 45, 60 min), washed in PBS, fixed with cold 4% paraformaldehyde for 20 min at RT and permeabilised with 0.1% Triton X-100 for 15 min. The cells were then blocked for nonspecific binding with 3% BSA in PBS for 20 min and incubated with rabbit anti-ES antiserum (Sripa and Kaewkes, 2000a) diluted 1: 300 with 1% BSA in PBS for 1 h, followed by goat anti-rabbit-Alexa Fluor 488 (Invitrogen) for 1 h. Nuclei were stained with propidium iodide or Hoechst and samples were viewed under a

confocal microscope (Olympus-FV1000 or Zeiss, LSM 700) and a 3D structure illumination microscope (SIM) for providing orthogonal views using Zen 2009 software (©Carl Zeiss MicroImaging GmbH). The total intracellular fluorescence was quantified by manually drawing a region of interest around the cytoplasm and analyzed using FV10-ASW V.2.1 software (Olympus).

Internalization of *Ov*ES was also analyzed by flow cytometry. Briefly, *Ov*ES co-cultured cells were trypsinised, permealised and immunostained as above. After thorough washing with PBS the cells were examined using a flow cytometer (FC 500, Beckman Coulter, Inc., USA) and data was analyzed with FlowJo software (Tree Star, Inc., USA). For inhibition experiments, cells were pretreated with the following endocytosis inhibitors for 30 min prior to adding 10 µg/ml *Ov*ES: 5 µg/ml chlorpromazine (CPZ), 0.3 M sucrose, or 4 µg/ml filipin. All experiments were performed in triplicate and data are expressed as mean-S.E. Statistical differences were determined using one-way analysis of variance using GraphPad Prism™ V.5.3. $P<0.05$ was considered as significant for rejection of the null hypothesis.

2.7 IL-6 production

H69 and Caco-2 cells were seeded at 2,000 cells/well in complete media as described above for 24 h and starved for 12 h in media without serum prior to subsequent experiments. Cells were pretreated with endocytosis inhibitors (5 µg/ml CPZ, 4 µg/ml filipin and 1 nM bafilimycin A1) for 30 min, and subsequently cultured with 1.2 µg/ml *Ov*ES for 48 h. Cells incubated with and without *Ov*ES proteins and endocytosis inhibitors were used as controls. The culture media was collected and centrifuged at 929 *g* for 10 min to remove cell debris. Supernatant was then collected and IL-6 levels determined using a human IL-6 ELISA kit (R&D Systems) following the manufacturer's recommendations.

2.8 Subcellular localization of OvES in biliary cells

To determine *Ov*ES uptake and trafficking, H69, KKU-100 and KKU-M156 cells were pretreated with the following specific organelle-trackers: Endoplasmic reticulum (ER) - ER-Tracker™Red; Lysosomes – LysoTracker; Golgi - BODIPY TR C5-ceramide; early endosomes - anti-Rab5. Cells were stained with Hoechst dye for 30 min before incubating with Alexa Flour 488-conjugated *Ov*ES (10 µg/ml) for 2 h. After three rounds of washing, cells were fixed in cold 4% paraformaldehyde for 20 min at RT, mounted in 80% glycerol and viewed on a Nikon A1 confocal fluorescence microscope, equipped with a 60x (NA1.4 plan Apo) oil immersion objective. Z series images were collected in three channels (Ex 405 nm, Em 425-475 nm; Ex 488 nm, Em 500-550 nm; Ex 561 nm, Em 570-620 nm) with a step size of 200 nm.

2.9 Effect of OvES and endocytosis inhibitors on cell proliferation

To test the effect of *Ov*ES and various endocytosis inhibitors on cell proliferation, real-time monitoring of cell growth using an xCELLigence system (Roche) was employed. Briefly, cells were seeded (2,000 cells/well) in an E plate (Roche) for 24 h. Cells were treated with the clathrin-specific inhibitor CPZ (5 µg/ml CPZ) for 30 min and, subsequently, 1.2 µg/ml *Ov*ES was added and incubated for 48 h. Cells treated only with *Ov*ES (and not with inhibitors) were used as controls. Real-time cell growth was measured as cell index (CI) as previously described (Xing et al., 2005).

3. Results

3.1. Internalization of OvES by cancerous and non-cancerous biliary cell lines

To study the internalization of *Ov*ES into biliary cells, different cell lines (H69, KKU-100, KKU-M156 and Caco-2) were cultured with *Ov*ES and visualized at different time-points using confocal microscopy (FV100). Internalization rates of *Ov*ES proteins were

dependent on the cell type. For the first 15 min, *Ov*ES proteins were internalized by H69 and KKU-M156 cell lines and were attached to the surface membranes of KKU-100 cells, but, in contrast, significantly less fluorescence was detected on or inside of Caco-2 cells (Fig. 1A), suggesting that *Ov*ES internalization is cell type-dependent. To validate the internalization of *Ov*ES products by H69 cholangiocytes in 3 dimensions, we performed a 3D image analysis, with the orthogonal view of the Z-stack 3D-SIM imaging (Fig. 1B-D). Orthogonal views confirmed that labeled *Ov*ES was attached to the cholangiocyte plasma membrane within 15 min and detected in the cell cytoplasm after 60 min.

Using flow cytometry, we found that significantly greater quantities of *Ov*ES were detected inside cells of hepatic origin (H69, KKU-100 and KKU-156) compared to control colon-derived Caco-2 cells (Fig. 2). The maximum fluorescence intensity was detected in H69 cholangiocytes after 2 h of co-culture (Supplementary Fig. 1).

3.2 Ov*ES uptake by cholangiocytes is via clathrin-mediated endocytosis*

To investigate the pathway(s) implicated in *Ov*ES internalization by host cells, different endocytosis inhibitors were employed – sucrose, filipin and CPZ. Cells were treated with filipin, sucrose or CPZ for 30 min, following incubation with *Ov*ES at 37ºC for 2 h and imaged using a confocal microscope. CPZ and sucrose inhibited *Ov*ES protein internalization by cholangiocytes and cholangiocyte-derived cancer cells (H69, KKU-100 and KKU-M156) (Fig. 3). Treatment of H69 cells with CPZ, sucrose and filipin resulted in 52-64% decrease of *Ov*ES uptake (Fig. 4). In contrast, CPZ and sucrose were more effective at preventing uptake of *Ov*ES by KKU-100 and KKU-M156 CCA cell lines than was filipin (Fig. 4). Total fluorescence calculation also showed that CPZ and sucrose inhibited *Ov*ES internalization (Supplementary Fig. 2A-C). These results suggest that *Ov*ES proteins are internalized by cholangiocytes via clathrin- and caveolae-mediated endocytosis pathways.

3.3. Cellular translocation of OvES proteins

To investigate *Ov*ES intracellular localization within cholangiocytes, Alexa Flour 488-conjugated *Ov*ES products were cultured with H69 cholangiocytes and different organelles were stained with specific fluorescent dyes. Using confocal microscopy, there was no evidence of *Ov*ES co-localization with Golgi, early endosomes, lysosomes, ER or nuclei, suggestive of a cytoplasmic location (Fig. 5).

3.4. Endocytosis of OvES proteins modulates cell proliferation and pro-inflammatory cytokine production.

To determine whether endocytosis of *Ov*ES proteins by cholangiocytes promoted cell proliferation we monitored cell proliferation in real-time using an xCelligence system (Xing et al., 2005). H69 cholangiocytes, KKU-100 and KKU-M156 CCA cell lines and the Caco-2 colon cancer cell line were incubated with 1.2 µg/ml *Ov*ES products in the presence or absence of CPZ and monitored for 48 h. ES products promoted proliferation of all three cholangiocyte-derived cell lines (H69 and the CCA lines) but not the colon-derived Caco-2. In the presence of CPZ, *Ov*ES stimulated proliferation of the two CCA lines within the first 48 hours of co-culture but thereafter significantly attenuated proliferation (Fig. 6A-C), while drug alone had no effect on cell growth (data not shown). Interestingly, *Ov*ES had no effect on proliferation of Caco-2 cells (Fig. 6D).

It has been previously described that interleukin (IL)-6 is a pro-inflammatory cytokine associated with advanced periductal fibrosis in *O. viverrini* infected patients (Sripa et al., 2009; Sripa et al., 2012b). IL-6 production from both cholangiocytes and colon cancer cells incubated with and without endocytosis inhibitors (CPZ, filipin and bafilomycin A1) before addition of *Ov*ES was measured. Addition of *Ov*ES promoted IL-6 production by cholangiocytes but had no effect on IL-6 production by Caco-2 cells (Fig. 7A). Moreover, all endocytosis inhibitors attenuated cholangiocyte IL-6 production by 69%, 55% and 53%,

242 respectively, with the greatest reduction occurring in the presence of the clathrin-mediated
243 endocytosis inhibitor, CPZ ($P< 0.001$; Fig. 7B).
244

245 ## 4. Discussion

246 The metabolic products secreted from the tegument and excretory openings of *O.*
247 *viverrini* are highly immunogenic and have diverse effects on host cells (Sripa, 2003).
248 Perhaps the most intriguing aspect of the interactions between *Ov*ES and human host
249 cholangiocytes is the active internalization of ES proteins, and the potential carcinogenic
250 ramifications of this process. We have shown previously that *Ov*ES proteins can be detected
251 inside biliary epithelial cells in the vicinity of *O. viverrini* in the bile ducts of infected
252 hamsters. Moreover, ES products were detected inside epithelial cells in the upper biliary tree
253 where adult flukes are too large to reach (Sripa and Kaewkes, 2000a). Since this initial
254 description, we and others raised antibodies to defined recombinant *Ov*ES products and
255 showed that individual ES products, including thioredoxin peroxidase (Laha et al., 2007),
256 granulin (Smout et al., 2009) and cathepsin F (Pinlaor et al., 2009) were internalized by
257 cholangiocytes. While other pathogens have been shown to produce proteins that are
258 internalized by host cells whereupon they induce toxic and functional changes, including pre-
259 cancerous events, this is the first evidence of the uptake by host cells of a secreted parasite
260 protein. The bacterium *Helicobacter pylori* secretes cag A, a virulence factor whose uptake
261 by epithelial cells can result in gastric cancer (Hatakeyama, 2004). Given that *Ov*ES products
262 induce severe inflammation by up-regulation of cholangiocyte TLR4 mRNA expression,
263 thereby promoting IL-6 and IL-8 production (Ninlawan et al., 2010), it is reasonable to
264 assume that internalized *Ov*ES products also promote a tumorigenic phenotype.

265 We show herein that *Ov*ES was internalized by cholangiocytes but not by the Caco-2
266 line of intestinal epithelial cells (IEC). IEC serve as a critical barrier to luminal bacteria and

food, and are an active participant in the intestinal innate immune response, responding to signals in both the luminal (apical) and lamina propria (basal) compartments (Hecht, 1999; Madara, 1997). While IEC are reported to be unresponsive to LPS, Caco-2 cells have very low levels of *tlr4* mRNA and weakly detectable *md-2* expression when compare to human dermal microvessel endothelial cells (Abreu et al., 2001). We suggest that the uptake of *Ov*ES by host cells might depend on the presence of a specific receptor, such as TLR4, that is highly expressed on cholangiocytes compared to intestinal epithelial cells.

To further address the mechanism of internalization of *Ov*ES products by different cell lines we analysed the role of the endocytic pathway. This pathway is the key process involved in the internalization of molecules from the cell surface to internal membrane compartments, and two major mechanisms are involved in this receptor-mediated endocytic pathway: clathrin-mediated endocytosis and the non-classical caveolae-mediated endocytosis (Le Roy and Wrana, 2005). Clathrin-mediated endocytosis requires clathrin and adaptor proteins such as AP-2 and Esp15 to form a coat pit (Keen, 1987), whereas the caveolae-mediated pathway depends on the balance between the caveolin-1 protein and lipid raft components such as cholesterol and glycosphingolipids (Sharma et al., 2004). By using different endocytosis blocking agents such as CPZ, sucrose and filipin, we showed that CPZ and sucrose significantly blocked the internalization of *Ov*ES in all hepatic cell types studied. CPZ is known to cause a loss of clathrin-coated pits at cell surfaces and is associated with accumulation of clathrin and AP-2 in the endosomal compartment (DiPaola et al., 1984; Sofer and Futerman, 1995), while sucrose serves as a hypertonic medium and induces abnormal clathrin polymerization into empty microcages (Heuser and Anderson, 1989). Despite the requirement of clathrin-mediated endocytosis for internalization of *Ov*ES products, filipin, a sterol-binging agent that disrupts structure and function of caveolae (Orlandi and Fishman, 1998; Schnitzer et al., 1994) also blocked the uptake of *Ov*ES by

normal cholangiocytes (H69), but not by CCA cell lines. These results imply that different *Ov*ES proteins use different pathways to enter host cells, although clathrin-mediated endocytosis clearly plays a major role in the overall process.

We have shown that *Ov*ES products specifically induce production of IL-6 by human cholangiocytes, but not by human colon cancer epithelial cells, suggesting that cholangiocytes might be the primary source of IL-6 in the biliary epithelium of infected individuals. High levels of IL-6 have been associated with chronic periductal fibrosis and CCA in opisthorchiasis patients (Sripa et al., 2012b). Moreover, IL-6 has also been implicated in the maintenance of chronic inflammation that could lead to tumorigenesis (Schafer and Werner, 2008). Thuwajit et al. reported that *Ov*ES products may act as growth factors by inducing proliferation of a mouse fibroblast cell line (NIH-3T3) *in vitro* accompanied by increased expression of the TGF-β receptor (TGFβR) (Thuwajit et al., 2004). Interestingly, clathrin- and caveolae-mediated endocytosis can regulate TGFβR signaling and turnover (Di Guglielmo et al., 2003). In addition, different internalization pathways are associated with distinct intercellular fates. Several signaling receptors, including TGFβR, receptor tyrosine kinases (RTKs), GPCRs, NOTCH and WNT are involved in both clathrin-mediated endocytosis and non-clathrin endocytosis and influence the final signaling output (Le Roy and Wrana, 2005). Clathrin-mediated entry is associated with long-term signaling (Sadowski et al., 2009) whereas caveolae-mediated entry associated with Smad7-Smurf2 ubiquitin ligase complex is directly involved in degradation of the receptor by lysosome (Di Guglielmo et al., 2003). *Ov*ES products stimulate cell proliferation and signal transduction via clathrin- and caveolae-mediated endocytosis in normal cholangiocytes but only the clathrin pathway in CCA cells. The endocytosis pathway regulates the balance in the receptor systems acting as an effector or attenuator of the signal transduction (Lipkowitz, 2003). Thus, the internalization of *Ov*ES via clathrin-mediated endocytosis may steer receptors away from

a degradation fate, and enhance signaling from the plasma membrane, thereby increasing inflammatory cascade signaling and contributing to the immunopathogenesis of opisthorchiasis. However, more studies are required to assess this hypothesis.

We reveal here a role for the clathrin-mediated and caveolae endocytic pathways in the internalization of *Ov*ES by biliary epithelial cells, and subsequent stimulation of cell proliferation and the production of the pro-inflammatory cytokine IL-6. This is a key early step in pathogenesis of opisthorchiasis and CCA development, particularly seeing as inflammatory IL-6 can stimulate inflammation leading to free radical production causing oxidative DNA damage (Sripa et al., 2012). DNA damage can be from exogenous (i.e. nitrosamine in fermented dietary foods) or endogenous nitrosation from *O. viverrini* infection (Sripa et al., 2007). These biliary cells that have undergone DNA damage due to *Ov*ES exposure are induced to proliferate, and fixed genetic alterations ultimately occur. Accumulated genetic alterations can ensue with uncontrolled growth and subsequent malignancy. However, *Ov*ES also displays inhibitory effects on host cells, such as anti-apoptotic activity (Sripa et al. 2012). Further work is now required to characterize multiple pathways downstream of the process of parasite protein internalisation, as well as the individual fluke proteins involved and their cellular receptors. Recent reports have shown that parasitic helminths secrete extracellular vesicles (Buck et al., 2014; Marcilla et al., 2012), and future work should explore the possibility that *O. viverrini* secretes exosome-like vesicles that are internalized by host cells. A better understanding of the process of host cell-mediated internalization of liver fluke proteins will shed light on the immunopathogenesis of the infection and provide novel pathways to target in the development of vaccines against this carcinogenic infection.

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Contributions

All authors designed the study; S.C. performed experiments; S.C., B.S., J.S. and A.L. wrote and assembled the manuscript with input from all authors.

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Figure Legends

Fig. 1. Internalization of *O. viverrini* ES products (*Ov*ES) by biliary cells (H69, KKU-100, KKU-M156) and colon cancer cells (Caco-2) at 15, 30, 45 and 60 min post-incubation. Internalization of *Ov*ES (green fluorescence) by different cell types over time (A). Orthogonal views using Z-stack immunofluorescence microscopy showing 3-D internalization of *Ov*ES at 0 min (B), 15 min (C) and 120 min (D) in H69 cholangiocytes. Note that *Ov*ES is more readily internalized by biliary cells than by colonic epithelial cells. Nucleus was stained with propidium iodide (Red fluorescence).

Fig. 2. Mean intracellular fluorescence depicting the internalization of *O. viverrini* ES products (*Ov*ES) by normal cholangiocytes (H69), cholangiocarcinoma (KKU-100, KKU-M156) and colon cancer (Caco-2) cell lines over 2 h. Fluorescence intensity was measured by flow cytometry (A). Histogram is the average intensity of *Ov*ES internalization from 3 independent experiments ± SEM (B).

Fig. 3. Confocal fluorescence microscopy images showing internalization of *O. viverrini* ES products (*Ov*ES) in H69, KKU-100 and KKU-M156 biliary cells with and without endocytosis inhibitors. Cells treated with an inhibitor of the clathrin pathway (chlorpromazine, CPZ) showed reduced internalization of *Ov*ES (green fluorescence) in all cell types tested. Similarly, sucrose-treated cells showed substantially reduced internalization of *Ov*ES in the cholangiocarcinoma lines KKU-100 and KKU-M156 and partially reduced internalization in H69 cholangiocytes.

Fig. 4. Endocytosis pathway inhibitors block the uptake of *O. viverrini* ES products (*Ov*ES) by normal (H69) and cholangiocarcinoma (KKU-100 and KKU-M156) biliary cell lines using flow cytometry. Cells were pretreated with the endocytosis inhibitors chlorpromazine (CPZ), sucrose (clathrin-mediated inhibitor) and filipin (caveolae-mediated inhibitor) before incubation with *Ov*ES for 2h. Fluorescence intensity was measured by flow cytometry (upper panel). The histogram depicts the average of three independent experiments ± SEM (lower panel).

Fig. 5. Co-staining of H69 cholangiocytes for internalized *O. viverrini* ES products (*Ov*ES) and cell organelles revelaed an absence of co-localization. *Ov*ES is observed as punctate green fluorescence. Cells were co-stained for endoplasmic reticulum with ER-Tracker-red (A), Golgi with Golgi-tracker-red (B), early endosomes with Anti-Rab5-red (C) and lysosomes with LysoTracker-red (D). Nuclei were counterstained with Hoechst dye (blue).

Fig. 6. Real time cell proliferation of biliary (H69, KKU-100, KKU-M156) and colon (Caco-2) cells induced by *O. viverrini* ES products (*Ov*ES) in the presence or absence of chlorpromazine using an xCELLigence system. *Ov*ES stimulated proliferation of bilary cells (A-C) but not colon cells (D) compared to media control over 96 h of culture. CPZ significantly inhibited growth of all cell types. Growth of each cell type was normalized to its media control without *Ov*ES.

Fig. 7. IL-6 production. IL-6 production in normal human cholangiocytes (H69) and human colon cancer (Caco-2) cocultured with *Opisthorchis viverrini* excretory-secretory products

(*Ov*ES) in several dilutions for 48h (A). The biliary cells were strongly stimulated by *Ov*ES protein, but has no effected in colon cancer cell when compare with control cells. IL-6 production from normal human cholangiocyte cell (H69) with and without endocytosis inhibitors (chlorpromazine; CPZ, Filipin and Bafilomycine A1) before *Ov*ES stimulated for 48h using ELISA technique (B). *Ov*ES stimulated IL-6 production while all inhibitor of endocytosis suppressed the IL-6 secreted significantly. Histograms represent the average of three independent experiments ± SEM of the absorbance at 450 nm measured by Versamax microplate reader using SoftMax pro V.5 program (*** *P< 0.001*).

Supplementary Figures

Supplementary Fig. 1. The kinetics of internalization of *O. viverrini* ES products (*Ov*ES) at 0, 15, 30, 45, 60 min in normal cholangiocytes (H69), cholangiocarcinoma cell lines (KKU-100 and KKU-M156) and Caco-2 colon cancer cells. Histograms represent the average of three independent experiments ± SEM of the fluorescence intensity measured by flow cytometry.

Supplementary Fig. 2. Fluorescence intensity of internalization of *O. viverrini* ES products (*Ov*ES) by H69 cholagiocytes, KKU-100 and KKU-M156 cholangiocarcinoma cell with and without the endocytosis inhibitors cholorpromazine (CPZ) and sucrose. CPZ and sucrose have significant inhibitory effects on *Ov*ES internalization in all biliary cell types (A, B, C). Histograms represent the average of three independent experiments ± SEM of the fluorescence intensity measured by image analysis.

Figure 1

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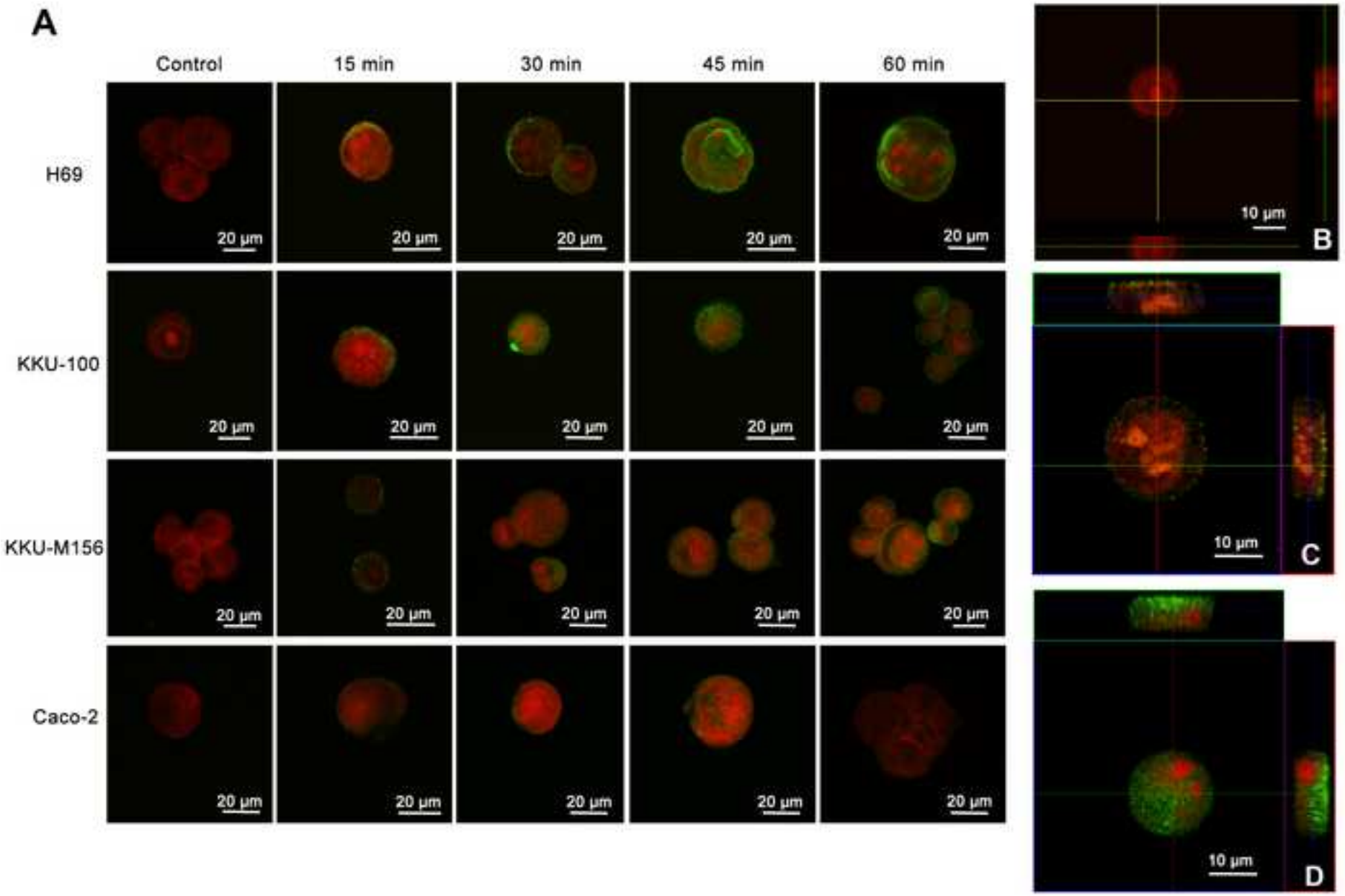

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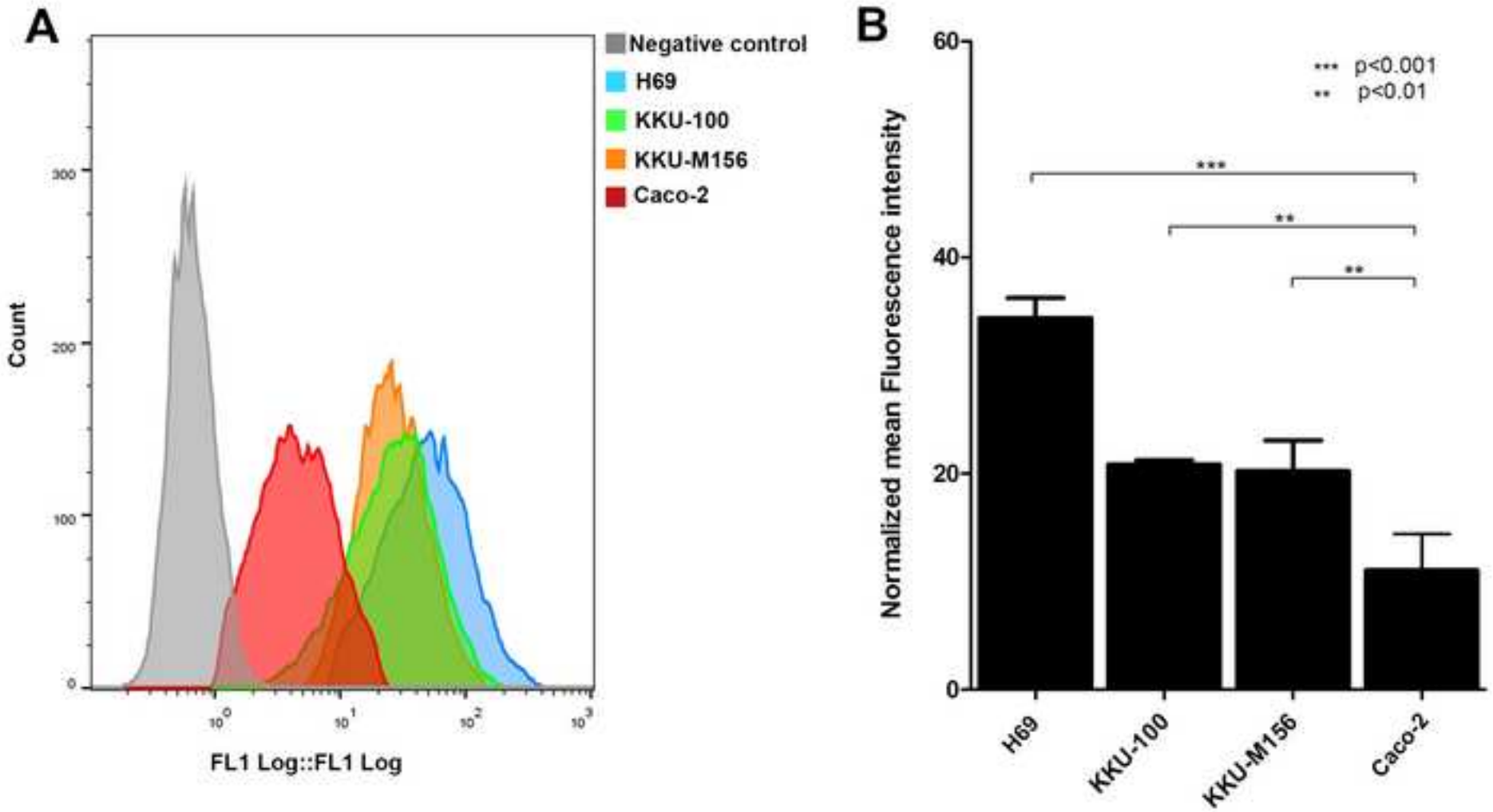

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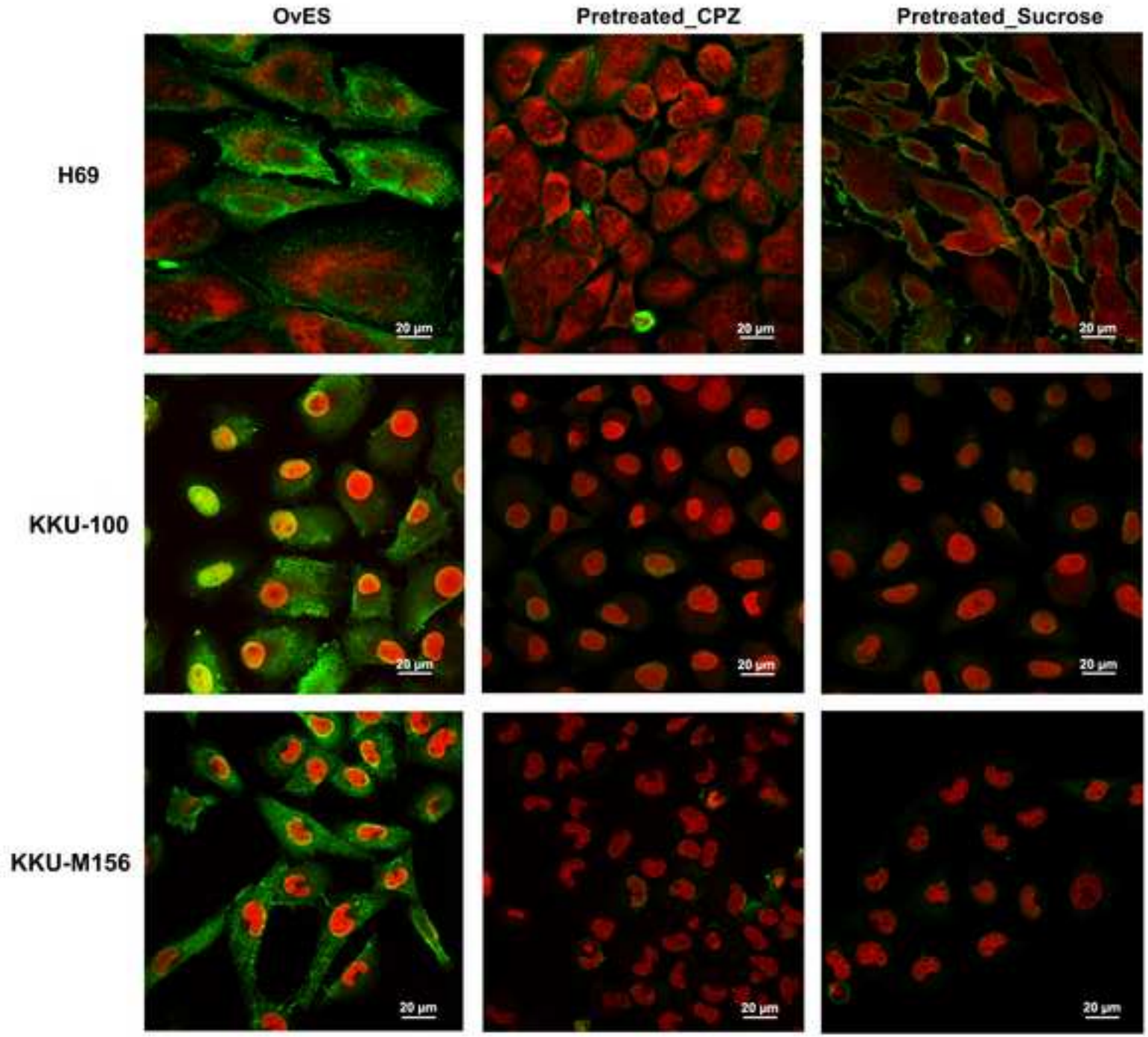

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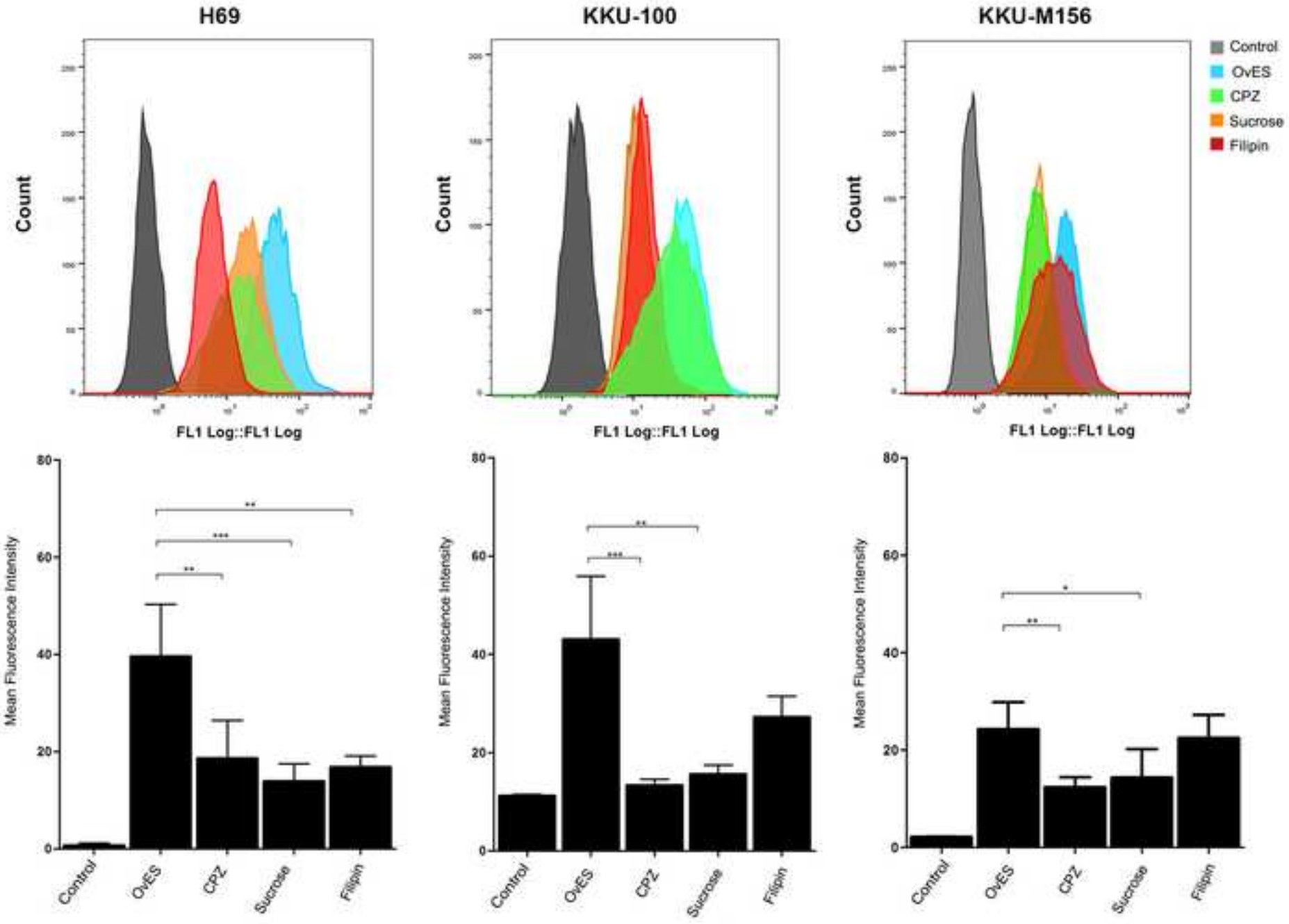

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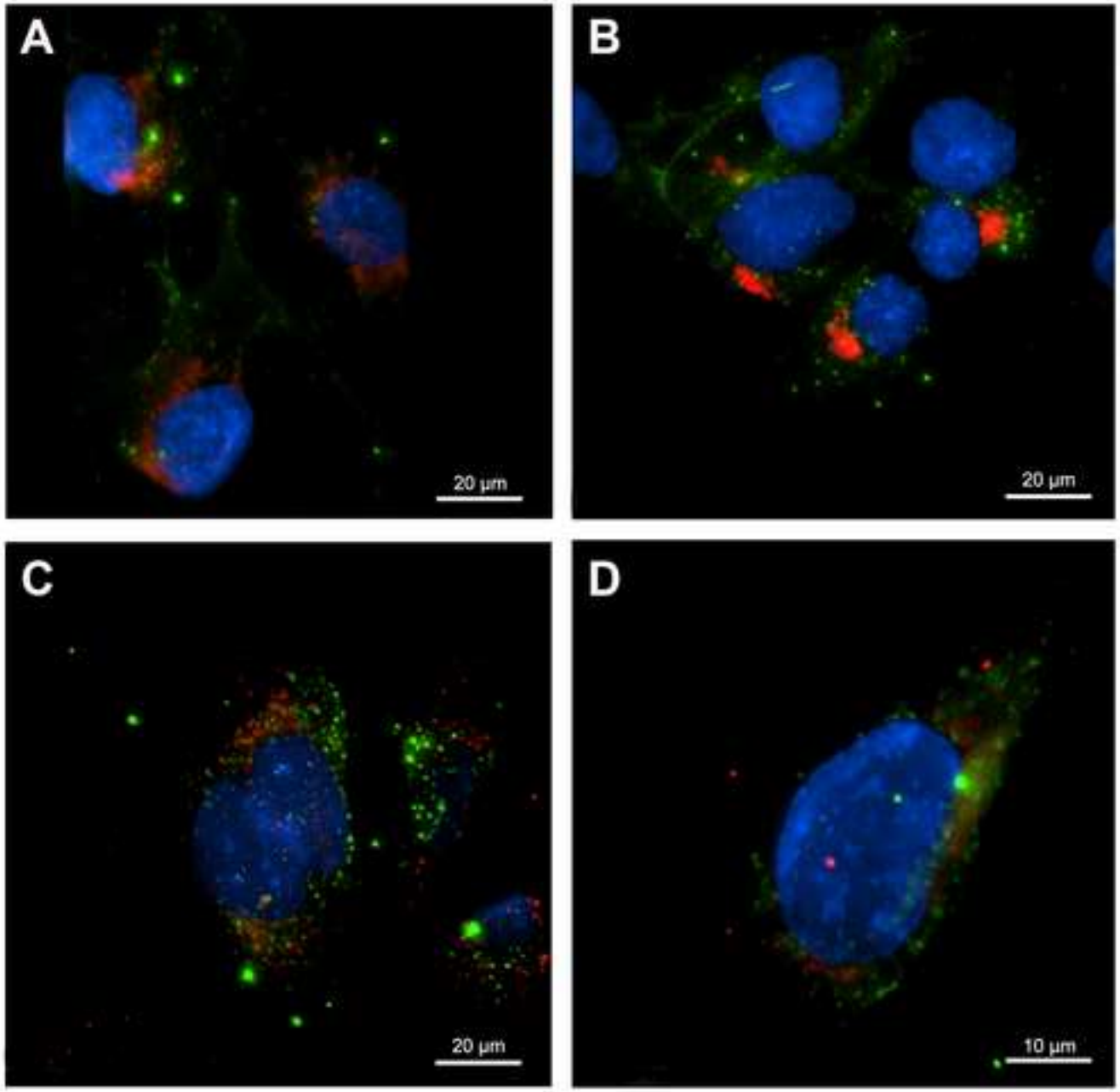

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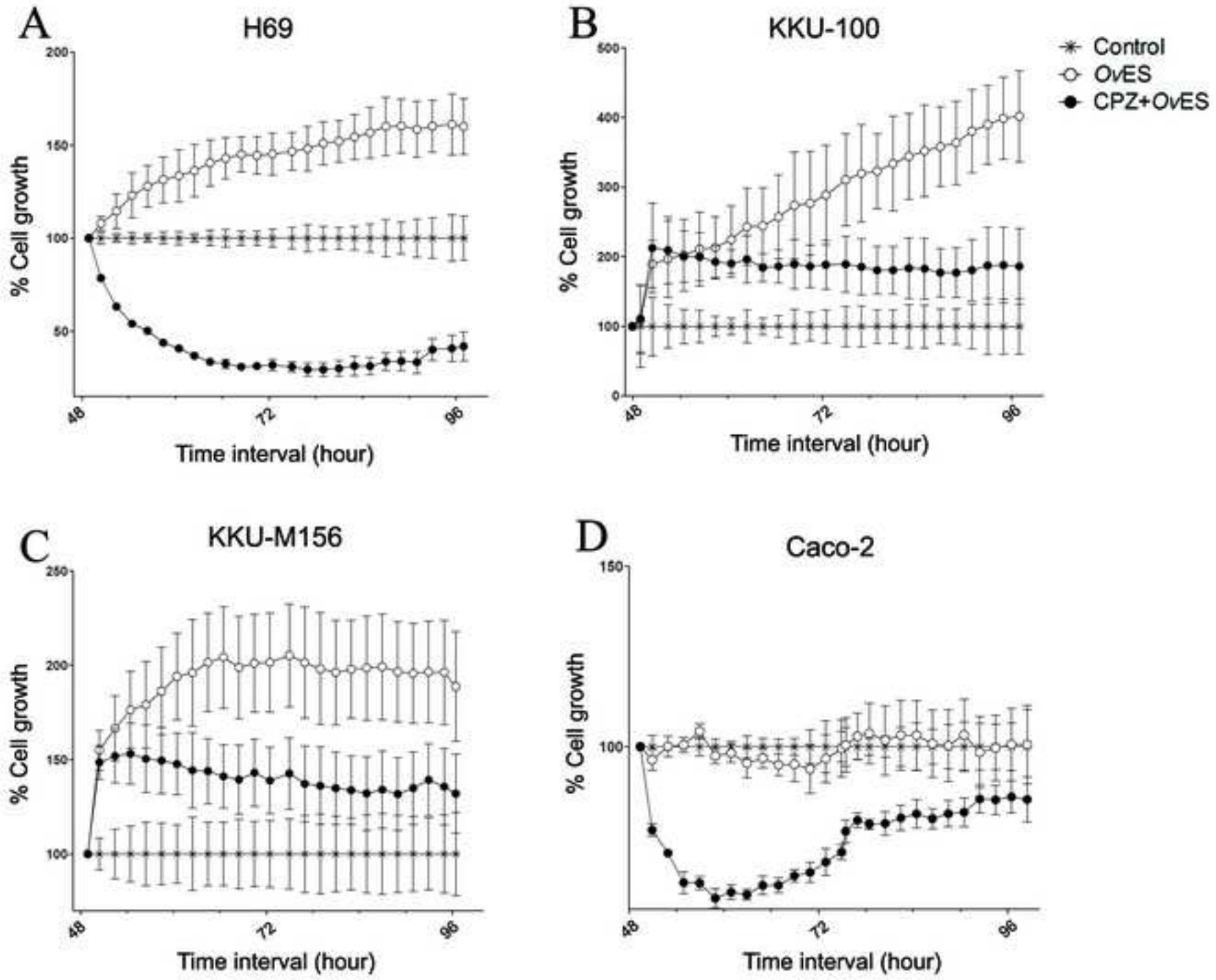

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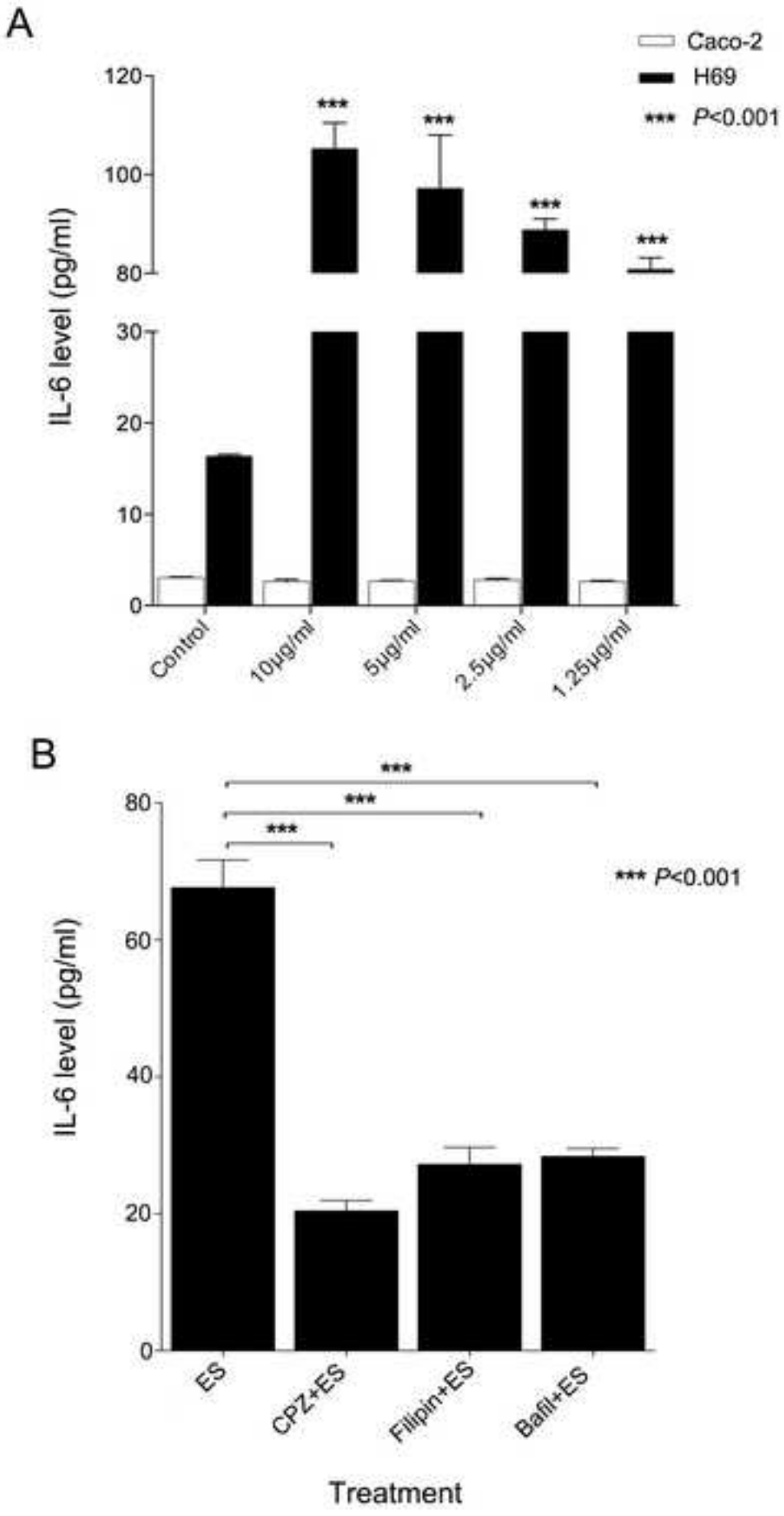

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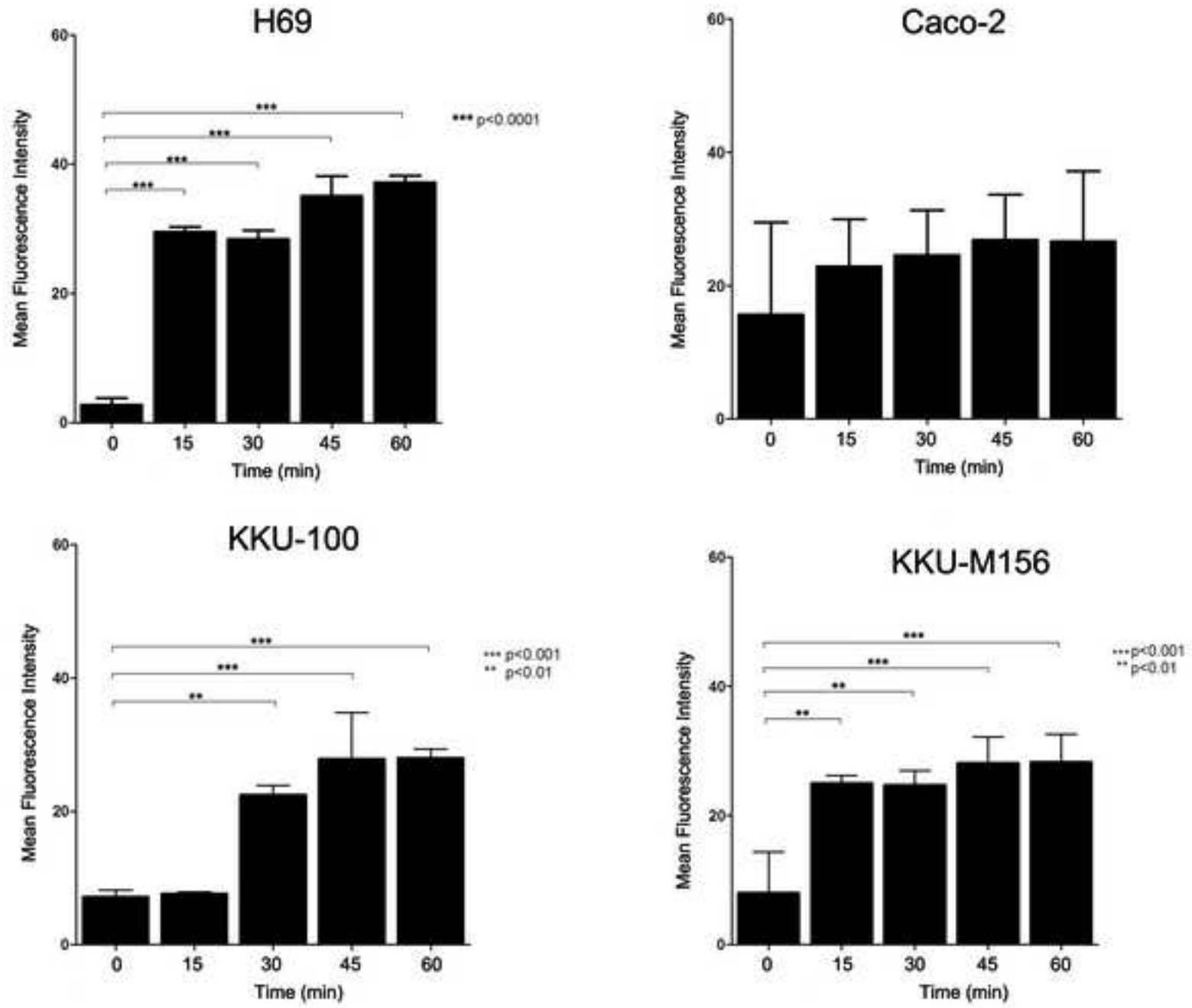

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