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Formation and Uptake of Arylhydroxylamine-Haptenated Proteins in Human Dendritic Cells

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Abbreviations: APC- antigen presenting cell,; CDR – cutaneous drug reactions; DDS – dapsone; D-NOH – dapsone hydroxylamine; DC – dendritic cell; HLA – human leukocyte antigen; LC – Langerhans cell; MHC – major histocompatability complex; PBS – phosphate buffered saline; SMX – sulfamethoxazole; S-NOH – sulfamethoxazole hydroxylamine

ABSTRACT

Bioactivation of sulfonamides and the subsequent formation of haptenated proteins is believed to be a critical step in the development of hypersensitivity reactions to these drugs. Numerous lines of evidence suggest that the presence of such adducts in dendritic cells (DCs) migrating to draining lymph nodes is essential for the development of cutaneous reactions to xenobiotics. Our objective was to determine the ability of human DCs to form drug-protein covalent adducts when exposed to sulfamethoxazole (SMX), dapsone (DDS), or their arylhydroxylamine metabolites (S-NOH, D-NOH) and to take up pre-formed adduct. Naive and immature CD34+ KG-1 cells were incubated with SMX, DDS, or metabolites. Formation of haptenated proteins was probed using confocal microscopy and ELISA. Cells were also incubated with pre-formed adduct (Drug-BSA conjugate) and uptake determined using confocal microscopy. Both naive and immature KG-1 cells were able to bioactivate DDS forming drug-protein adducts, while cells showed very little protein haptenation when exposed to SMX. Exposure to S-NOH or D-NOH resulted in protein haptenation in both cell types. Both immature and naïve KG-1 cells were able to take up pre-formed haptenated proteins. Thus, DCs may acquire haptenated proteins associated with drugs via intracellular bioactivation, uptake of reactive metabolites, or uptake of adduct formed and released by adjacent cells (e.g., keratinocytes).

Introduction

Administration of sulfonamides or sulfones is associated with the development of cutaneous drug reactions (CDRs), which often complicate therapy. These reactions range from a mildly discomforting skin rash to potentially fatal toxic epidermal necrolysis(Svensson et al., 2001). Increasing evidence indicates that these reactions are immune-mediated(Hari et al., 2001; Roychowdhury and Svensson, 2005). Since drugs are generally small molecular weight compounds (and therefore, not recognized by the immune system) that are non-reactive in nature, it is believed they must undergo bioactivation to generate reactive metabolite(s) which can subsequently haptenate cellular proteins to trigger an immune response. While the liver is known to be the primary site for drug bioactivation, extra-hepatic drug metabolism leading to the generation of reactive metabolites is also suspected to play a potential role in the elicitation of these reactions(Sanderson et al., 2006). Recently, our laboratory has shown that different types of skin cells (i.e., keratinocytes or fibroblasts) are capable of bioactivating drugs leading to protein haptenation(Roychowdhury et al., 2005; Bhaiya et al., 2006).

Drug-protein adducts formed may be taken up by the antigen presenting cells (APCs) in the skin as neo-antigens, followed by processing and presentation to T-cells. In the skin, Langerhans cells (LC) are the primary APC, in addition to other skin dendritic cells (DCs). LC/DCs collect antigens in the skin, process them and transport them to the lymphoid organ in a synchronized fashion(Stoitzner et al., 2006). During this journey to draining lymph nodes, DCs undergo a maturation process that equips them to present processed antigen to T-cells in the draining lymph node. In humans, DCs

represent a heterogeneous population of APC that appear to arise from different distinct hematopoietic progenitor cells, such as $CD14^+$ monocytes or $CD34^+$ bone marrow cells. From the cellular perspective, these progenitor cells undergo two main stages of differentiation. The first stage involves differentiation of the progenitor cells to the immature or un-activated DC that show a high capacity for antigen uptake. In the second stage, immature DCs undergo a maturation process wherein they become more potent mediators of T-cell activation.

The ability of LC/DCs to bioactivate xenobiotics has not been carefully examined. Limited studies have demonstrated the presence of mRNA for different drug metabolizing enzymes in LC or monocyte-derived DCs, including different cytochrome P-450s (Saeki et al., 2002) and myeloperoxidase (Strobl et al., 1993). These data indicate that DCs may also play a direct role in drug metabolism leading to drug-protein adduct formation, which may be subsequently processed and presented to T-cells. Based on these observations, we hypothesized that DCs can also bioactivate chemically inert drugs to reactive metabolites with subsequent protein haptenation.

Human donor-derived DCs have a short life-span and exhibit diverse phenotypic as well as functional variability (Bracho et al., 2003; O´Neill and Wilson, 2004). To circumvent these problems, we have used immortalized myelogenous $CD34^+$ KG-1 cells for studying the bioactivation of drugs in a LC/DC-like cellular model(Koeffler and Golde, 1978; Hulette et al., 2001). Our results demonstrate that KG-1 cells are able to bioactivate drugs leading to protein haptenation. Moreover, the differentiation status of these cells (immature versus mature) does not appear to substantially impact the process of protein adduction.

Methods

Materials. Arylhydroxylamine metabolites of DDS and SMX were synthesized as described (Vyas et al., 2005) and determined by HPLC to be > 97% pure. Rabbit anti-sera was raised against SMX- and DDS-keyhole limpet hemocyanine conjugates and specificity assessed as described previously (Reilly et al., 2000). Rat tail collagen (type-I) was obtained from Sigma (St. Louis, MO). KG-1 cells were obtained from Dr. David Kusner (University of Iowa, USA). Microtiter ELISA plates (96 well) were obtained from Rainin Instruments (Woburn, MA). Goat-anti-rabbit IgG conjugated with Alexa fluor488, goat anti-mouse IgG conjugated with Alexa fluor568 and goat anti-rabbit antibody conjugated with alkaline phosphatase were purchased from Molecular Probes (Eugene, OR). Anti-HLA DR antibody was purchased from eBioscience (San Diego, CA). Bradford assay reagent was purchased from Pierce Chemical Company (Rockford, IL). Immunomount was obtained from Vector Laboratories (Burlingame, CA). All other chemicals were purchased from Sigma (St. Louis, MO) or FISHER Scientific (Chicago, IL).

Cell Culture. The human dendritic cell line, KG-1 cells, were cultured as described previously (Herrmann et al., 2005), with minor modifications. Cells ($2x10^5$/ml) were grown as suspension culture, in L-glutamine-supplemented minimum essential media (MEM) containing 20% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Media was changed every second day. Cells were maintained at concentration $2x10^5$/ml to $1x10^6$/ml. Cells were tested for HLA-DR staining, a marker for KG-1 cells, using flow cytometric analysis.

Flow cytometry. Cultured cells were washed, centrifuged at 550 x g for 10 min, suspended at $1x10^5$ cells in 200 µl of cold Dulbecco's phosphate buffered saline and incubated with FITC-conjugated HLA-DR antibody or its isotypic control for 30 min at room temperature. Cells were washed thrice and resuspended in 800 µl Dulbecco's Phosphate buffered saline. Cells were passed through a nylon mesh to eliminate cell-aggregates, and staining was analyzed with a FACS scanner (Becton). A minimum of 10^4 cells were analyzed three times for each sample.

Cell differentiation. KG-1 cells were harvested by centrifugation, re-suspended in complete culture media at a concentration of $2x10^5$ cells/ml in a 25 cm^2 culture flask.

Immature DC-like cells: To induce immature DC-like cell differentiation, recombinant human interleukin-4 (rh-IL-4, 100 ng/ml) and recombinant human-granulocyte macrophage colony stimulatory factor (rhGM-CSF, 100 ng/ml) were added to the cultures. Cells were cultured for 5 days at 37^0C and 5% CO_2. Media was changed every third day with fresh cytokine cocktail supplemented media.

Mature DC-like cells: To induce mature DC-like cell differentiation, rh-IL-4 (100 ng/ml), rh-GM-CSF (100 ng/ml) and TNF-α (20 ng/ml) were added to the cultures. Cells were cultured for 5 days at 37^0C and 5% CO_2. Media was changed every third day with fresh cytokine cocktail supplemented media.

ELISA Analysis of Drug/metabolite-protein Adducts. Formation of haptenated proteins following SMX or DDS exposure was determined by cultivating KG-1 cells ($5x10^5$ cells/ml) for 3 h in 50 ml centrifuge tubes containing 5 ml of complete growth medium. Cells were then incubated with SMX or DDS (250 µM). After 3 h, tubes were

centrifuged at 550 x g for 10 min to pellet the cells. The supernatant containing the medium was drained off and the cell pellets were lysed in 1 ml of deionized water, using repeated cycles of freezing and thawing (three times) and ultrasonication to ensure complete lysis. The cell suspension was then thoroughly vortexed and centrifuged at 550 x g for 10 min and the pellet containing the cell debris was discarded. The supernatant containing cellular soluble proteins was collected for protein assay and subsequent ELISA.

ELISA analysis for detection of drug-protein adduct formation was performed as described previously (Reilly et al., 2000) with minor modifications. Following protein content measurement using the Bradford reagent kit, all samples were diluted to contain 100 μg/ml protein. An aliquot of 100 μl was adsorbed onto 96 well polystyrene microtiter plates for 16 h at 4^{o}C. Wells were washed three times using Tris- tri(hydroxymethyl)-aminomethane- casein buffer (0.5% casein, 0.9% NaCl, 0.01% Thimerosal, 10 mM Tris-HCl, pH 7.6) and then blocked with Tris-casein buffer for 1 h. After an additional wash, wells were incubated for 16 h at 4^{o}C with 100 μl of anti-SMX or anti-DDS rabbit serum (1:500 diluted with Tris-casein buffer) previously characterized in our laboratory (Reilly et al., 2000). Wells were subsequently washed four times with Tris-casein buffer and incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000 diluted in Tris-casein buffer) for 2 h at room temperature. After washing four times with Tris-casein buffer, antibody binding was detected with colorimetric alkaline phosphate substrate reagent. After 1 h of incubation at room temperature, optical density was measured at 405 nm using a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

To determine the effect of flavin monooxygenase (FMO) and peroxidase substrate/inhibitor on drug-protein covalent adduct formation following DDS exposure, KG-1 cells (2 X 10^6 cells) were incubated with DDS (250 µM) in presence or absence of the selective enzyme substrate/inhibitor for 3 h (as specified in Results). The FMO competitive substrate methimazole (MMZ 5 mM) was co-incubated with DDS while the peroxidase inhibitor 4-aminobenzoic acid hydrazide (ABH, 5 mM) was added 1 h before DDS exposure. Following incubation, tubes were centrifuged at 550 x g for 10 min to pellet the cells. Covalent adducts were determined as previously described (Vyas et al., 2005).

Determination of the cytotoxicity of enzyme inhibitors in KG-1 cells. To determine whether the concentrations of MMZ or ABH used in our studies were cytotoxic to KG-1, cells were exposed the agents and cytotoxicity determined using a cell impermeable DNA binding dye (YoYo - 1) as described previously (Vyas et al., 2005)

Immunocytochemistry. To detect protein haptenation in KG-1 cells of varying differentiation status, cells were cultured in the presence or absence of cytokines (GM-CSF and IL-4) for 5 days and then plated onto collagen-coated coverslips. Cells were allowed to attach to the collagen-coated (0.1 mg/ml) coverslips, placed in petri-dishes containing 2 ml of complete growth medium. After 24 h, cultures were subjected to different drug treatments for varying periods of time (specified in Results) followed by washing (three times) with phosphate buffered saline (PBS, 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4) and fixation for 20 min with 4% paraformaldehyde (PFA) in PBS. After fixation, cultures were washed three times with PBS followed by blocking for 60 min with Tris-casein buffer containing 0.3% Triton-X-100 and overnight incubation with

the anti-DDS or anti-SMX antisera (1:500 diluted in blocking buffer) at 4 ^{o}C. Coverslips were then washed with PBS, incubated for 3 h at 37 ^{o}C with the fluorochrome-conjugated secondary antibody (Alexa fluor-488 labeled goat-anti-rabbit IgG, 1:500 diluted in blocking buffer), and mounted on glass slides using Immunomount containing anti-fade reagent.

To differentiate between intracellular and cell surface drug-protein adducts, cells were treated with the native drug (DDS or SMX, 800 μM, 24 h) or its arylhydroxylamine metabolite (D-NOH or S-NOH, 100 μM, 3 h) followed by routine immunocytochemical procedure with the exception that the permeabilization step was deleted.

Fluorescence images were acquired with a Zeiss Laser Scanning Microscope (LSM 510, Zeiss Axiovert stand, Zeiss 63 x oil lens) using excitation at 488 nm. Emission was set to a long pass filter at 505 nm.

Statistical analysis. Data are presented as mean (SD). Data were analyzed using SIGMASTAT (USA). Statistical comparison between groups was made using ANOVA followed by the Holm-Sidak method for multiple pairwise comparisons. A value of $p<0.05$ was considered to be significant.

Results

Detection of protein haptenation in naïve KG-1 cells exposed to DDS or SMX. To detect drug-induced protein haptenation in naïve KG-1 cells, cells were exposed to DDS (250 μM, 3h) or its reactive metabolite D-NOH (25 μM, 3h), followed by washing, fixation and immunocytochemical detection using confocal microscopy. Formation of haptenated proteins were evident in both naïve and immature KG-1 cells exposed to either DDS or D-NOH (Fig. 1A, upper panel). Simultaneous DIC (Differential Integration coefficient) images were acquired from the same field to show the cellular morphology of the particular cells (Fig. 1A., lower panel). In contrast to DDS, SMX (250 μM, 3h) did not show any detectable protein haptenation in naïve KG-1 cells (Fig. 1B), while haptenated proteins were readily detected in cells exposed to S-NOH.

To assess the localization of intracellular versus cell surface drug-protein adduction, cells were treated with the native drug (DDS or SMX, 800 μM, 24 h) or its arylhydroxylamine metabolite (D-NOH or S-NOH, 100 μM, 3 h) followed by routine immunocytochemical procedure without prior permeabilization of cells (meaning antisera could only access surface adducts). Under these conditions, negligible fluorescence was observed (data not shown), suggesting the absence of drug-protein adducts on the surface of these cells.

To confirm these observations, additional experiments were conducted employing ELISA to determine the formation of haptenated proteins in naïve KG-1 cells exposed to DDS or SMX. We observed significant protein haptenation when KG-1 cells were exposed to either DDS or D-NOH (Fig. 2A). As observed when protein haptenation was assessed via confocal microscopy, we found a relatively low level of haptenation in

cells exposed to SMX, but significant haptenation in those exposed to S-NOH (Fig. 2B). Moreover, DDS-treated naïve KG-1 cells showed much higher haptenation in comparison to the SMX-treated counterparts.

Detection of haptenated proteins in immature and mature KG-1 cells exposed to DDS. Because KG-1 cells can undergo differentiation to immature or mature DC-like cells which mimic DCs more closely, cells were subjected to differentiation-specific cytokine treatments followed by exposure to DDS or its reactive metabolite D-NOH. Following exposure to the cytokines, GM-CSF/IL-4 for 5 days *in vitro*, KG-1 cells showed rough and lobular cytoplasm with spikes, typical characteristics of the monocyte-derived immature DC. The presence of TNF-α, in addition to GM-CSF/IL-4, differentiated KG-1 cells into mature DC-like cells showing stellate morphology. As shown in Fig. 3, both immature and mature KG-1 cells exhibited comparable drug-protein adduct formation when exposed to DDS or D-NOH.

Role of peroxidase and flavin monooxygenase (FMO) on protein haptenation in naïve KG-1 cells. Since peroxidases and FMO have been shown to bioactivate DDS and SMX in human epidermal keratinocytes(Vyas et al., 2006b) leading to protein haptenation, we sought to determine the role of these enzymes in the DDS-specific protein haptenation in naïve KG-1 cells. For this purpose, naïve KG-1 cells were exposed to DDS in the presence or absence of a peroxidase inhibitor (aminobenzoic acid hydrazide, ABH) or a competitive substrate for FMO (methimazole, MMZ). MMZ was co-incubated with DDS while ABH was added 1 hr before the drug was added. Formation of haptenated proteins were quantitatively compared using ELISA. Both MMZ and ABH were able to markedly reduce (by >50%) the protein haptenation observed in

KG-1 cells exposed to DDS (Fig. 4A). As neither agent increased cell death above that seen with DDS alone (Fig. 4B), this reduction in bioactivation could not be attributed to cytotoxicity.

Uptake of preformed haptenated proteins by KG-1 cells. To compare the efficacy of naïve and immature cells to uptake preformed drug-protein conjugates, cells were exposed to drug-BSA conjugates for 10 and 60 min followed by washing, fixation and immunostaining with the corresponding drug-specific antisera. Our results demonstrated that both naïve and immature KG-1 cells were able to uptake the drug-BSA conjugates (Fig. 5 and 6). Visually, KG-1 cells exposed to DDS-BSA appear to show higher immunostaining than those were exposed to SMX-BSA. This could be a consequence of the higher sensitivity of the anti-DDS-antisera compared to the anti-SMX antisera or a true difference in drug-protein conjugate uptake. Moreover, exposure of DDS-BSA for 60 minutes resulted in higher immunostaining in both naïve and immature KG-1 cells compared to the cells exposed for 10 min. Neither naïve nor immature KG-1 cells exposed to SMX-BSA showed such time-dependent uptake of the drug-BSA conjugate.

Discussion

Sulfonamides and sulfones are known to elicit CDRs, although the exact mechanism is still unknown. Drug metabolism may play a pivotal role in the pathogenesis of these drug-induced immune reactions (Roychowdhury and Svensson, 2005; Sanderson et al., 2006). Bioactivation of these drugs to their reactive metabolites followed by binding to intracellular proteins has been suspected to play a key role in eliciting the immune response. In addition to liver microsomes, formation of these drug-protein covalent adducts have already been demonstrated in various non-hepatic cellular models that include keratinocytes (Roychowdhury et al., 2005), fibroblasts (Bhaiya et al., 2006), monocytes (Goebel et al., 1999) and a T-cell line (Manchanda et al., 2002).

DCs represent a unique group of cells with high phenotypic as well as functional diversity. DCs are believed to originate from $CD34^+$ hematopoietic stem cells and differentiate to circulating precursor cells that give rise to multiple cell types with distinct tissue localization and function(Reid et al., 1990). The primary function of DCs is to engulf particles and soluble compounds from their tissue environment, process these proteins to smaller peptides and present them to T-cells upon activation. Various stimuli, including local tissue damage, inflammation, stress or altered metabolic products can activate DCs. Activation of DCs is associated with various phenotypic as well as functional alterations which include reduction in phagocytic ability, induction of costimulatotry molecules, induction of cytokines and chemokines, and higher mobility(Ardavin et al., 2001). Following such changes, DCs become highly efficient in priming both helper and cytotoxic T-cells and are referred to as mature DCs.

LCs are the major APCs in skin(Jakob et al., 2001). LCs/DCs are known as the sentinels of skin. In addition to their role in antigen uptake, processing and presentation, these cells may play an important, yet to date unexplored, metabolic role. Limited data have shown metabolic activity in DCs and LCs(Sieben et al., 1999). The presence of either mRNA and/or protein of some metabolic enzymes (e.g., CYP450s or prostaglandin E2 synthase) have been found in monocyte-derived DCs or LCs (Norgauer et al., 2003), but no detailed study has been carried out to elucidate the role of these cells in drug metabolism.

$CD34^+$ KG-1 cells have been used as a cellular model for DCs in a variety of studies(St Louis et al., 1999; Ackerman and Cresswell, 2003; Berges et al., 2005). Our data show that KG-1 cells are able to metabolize DDS leading to protein haptenation (Figs. 1 and 2), while protein adduction in response to SMX exposure was very low. Exposure to the reactive metabolites of these drugs also resulted in adduction to cellular proteins, as evidenced from both our confocal images and ELISA data.

As KG-1 cells undergo differentiation upon exposure to different inflammatory stimuli mimicking immature or mature DC-like characteristics (Santiago-Schwarz et al., 1992; Romani et al., 1994), we determined whether the extent of protein adduction varies with the differentiation status of the KG-1 cells. Our results have clearly demonstrated that KG-1 cells of the three different differentiation states exhibit appreciable adduct formation when exposed to DDS or D-NOH. These data suggest there is not a substantial alteration in metabolic activity of KG-1 cells with progression of differentiation.

To elicit an immune response, processing of the antigen followed by presentation on the surface in conjunction with co-stimulatory molecules is an essential step. Since previous studies have shown that on transition from naive to immature stages KG-1 cells express co-stimulatory molecules on their surface, we anticipated that during this stage drug-protein adducts may also be processed and presented on the surface in the context of major histocompatibility complex (MHC). However, we found little evidence that these adducts are expressed on the surface of KG-1 cells at all three differentiation stages (data not shown).

As our previous studies have shown the involvement of FMO and peroxidases in the bioactivation of DDS in keratinocytes (Vyas et al., 2006a; Vyas et al., 2006b), we sought to determine if these enzymes play a role in formation of DDS-protein adducts in KG-1 cells. Since there was no difference in DDS-protein adduct formation among KG-1 cells of various differentiated states, we utilized naïve cells to test this hypothesis. Our data demonstrate that DDS-protein haptenation was significantly reduced in naïve KG-1 cells in the presence of the peroxidase inhibitor ABH, as well as the competitive FMO substrate MMZ. These results suggest that, similar to our observations in keratinocytes, peroxidases and FMO play an important role in the bioactivation of DDS in KG-1 cells.

On transition from naïve to the immature stage, the endocytotic capacity of KG-1 cells are known to increase several fold (Berges et al., 2005). We, therefore, determined the localization of haptenated proteins formed intracellularly versus the localization when cells were exposed to pre-haptenated proteins. Our results demonstrate that both naïve and KG-1 cells were able to take up preformed drug-BSA adducts. When cells

were exposed to DDS-BSA, haptenated proteins were found in the cytosol. In the case of SMX-BSA, adducts were traced intracellularly as punctated vesicle like structures.

In summary, our data demonstrate that model human DCs are able to acquire drug-associated haptenated proteins through three potential mechanisms: 1) intracellular bioactivation of parent drug to reactive metabolites, 2) uptake of extracellular reactive metabolite, or 3) uptake of preformed haptenated proteins. Since it has been previously demonstrated that LCs can cross-present keratinocyte-derived antigens in a physiological environment(Stoitzner et al., 2006), our prior demonstration of the capacity of such cells to give rise to haptenate proteins suggests keratinocyte-LC exchange of antigens may play an important role in the mediation of CDRs.

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References

Ackerman AL and Cresswell P (2003) Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. *J Immunol* **170:**4178-4188.

Ardavin C, Martinez del Hoyo G, Martin P, Anjuere F, Arias CF, Marin AR, Ruiz S, Parrillas V and Hernandez H (2001) Origin and differentiation of dendritic cells. *Trends Immunol* **22:**691-700.

Berges C, Naujokat C, Tinapp S, Wieczorek H, Hoh A, Sadeghi M, Opelz G and Daniel V (2005) A cell line model for the differentiation of human dendritic cells. *Biochem Biophys Res Commun* **333:**896-907.

Bhaiya P, Roychowdhury S, Vyas PM, Doll MA, Hein DW and Svensson CK (2006b) Bioactivation, protein haptenation, and toxicity of sulfamethoxazole and dapsone in normal human dermal fibroblasts. *Toxicol Appl Pharmacol* **215:**158-167.

Bracho F, van de Ven C, Areman E, Hughes RM, Davenport V, Bradley MB, Cai JW and Cairo MS (2003) A comparison of ex vivo expanded DCs derived from cord blood and mobilized adult peripheral blood plastic-adherent mononuclear cells: decreased alloreactivity of cord blood DCs. *Cytotherapy* **5:**349-361.

Goebel C, Vogel C, Wulferink M, Mittmann S, Sachs B, Schraa S, Abel J, Degen G, Uetrecht J and Gleichmann E (1999) Procainamide, a drug causing lupus, induces prostaglandin H synthase-2 and formation of T cell-sensitizing drug metabolites in mouse macrophages. *Chem Res Toxicol* **12:**488-500.

Hari Y, Frutig-Schnyder K, Hurni M, Yawalkar N, Zanni MP, Schnyder B, Kappeler A, von Greyerz S, Braathen LR and Pichler WJ (2001) T cell involvement in cutaneous drug eruptions. *Clin Exp Allergy* **31:**1398-1408.

Hulette BC, Rowden G, Ryan CA, Lawson CM, Dawes SM, Ridder GM and Gerberick GF (2001) Cytokine induction of a human acute myelogenous leukemia cell line (KG-1) to a CD1a+ dendritic cell phenotype. *Arch Dermatol Res* **293:**147-158.

Jakob T, Ring J and Udey MC (2001) Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol* **108:**688-696.

Koeffler HP and Golde DW (1978) Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity. *Science* **200:**1153-1154.

Manchanda T, Hess D, Dale L, Ferguson SG and Rieder MJ (2002) Haptenation of sulfonamide reactive metabolites to cellular proteins. *Mol Pharmacol* **62:**1011-1026.

Norgauer J, Ibig Y, Gmeiner D, Herouy Y and Fiebich BL (2003) Prostaglandin E2 synthesis in human monocyte-derived dendritic cells. *Int J Mol Med* **12:**83-86.

O´ Neill HC and Wilson HL (2004) Limitations with in vitro production of dendritic cells using cytokines. *J Leukoc Biol* **75:**600-603.

Reid CD, Fryer PR, Clifford C, Kirk A, Tikerpae J and Knight SC (1990) Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. *Blood* **76:**1139-1149.

Reilly TP, Lash LH, Doll MA, Hein DW, Woster PM and Svensson CK (2000) A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. *J Invest Dermatol* **114:**1164-1173.

Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM and Schuler G (1994) Proliferating dendritic cell progenitors in human blood. *J Exp Med* **180:**83-93.

Roychowdhury S and Svensson CK (2005) Mechanisms of drug-induced delayed-type hypersensitivity reactions in the skin. *Aaps J* **7:**E834-846.

Roychowdhury S, Vyas PM, Reilly TP, Gaspari AA and Svensson CK (2005) Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. *J Pharmacol Exp Ther* **314:**43-52.

Saeki M, Saito Y, Nagano M, Teshima R, Ozawa S and Sawada J (2002) mRNA expression of multiple cytochrome p450 isozymes in four types of cultured skin cells. *Int Arch Allergy Immunol* **127:**333-336.

Sanderson JP, Naisbitt DJ and Park BK (2006) Role of bioactivation in drug-induced hypersensitivity reactions. *Aaps J* **8:**E55-64.

Santiago-Schwarz F, Belilos E, Diamond B and Carsons SE (1992) TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J Leukoc Biol* **52:**274-281.

Sieben S, Baron JM, Blomeke B and Merk HF (1999) Multiple cytochrome P450-isoenzymes mRNA are expressed in dendritic cells. *Int Arch Allergy Immunol* **118:**358-361.

St Louis DC, Woodcock JB, Franzoso G, Blair PJ, Carlson LM, Murillo M, Wells MR, Williams AJ, Smoot DS, Kaushal S, Grimes JL, Harlan DM, Chute JP, June CH, Siebenlist U and Lee KP (1999) Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. *J Immunol* **162:**3237-3248.

Stoitzner P, Tripp CH, Eberhart A, Price KM, Jung JY, Bursch L, Ronchese F and Romani N (2006) Langerhans cells cross-present antigen derived from skin. *Proc Natl Acad Sci U S A* **103:**7783-7788.

Strobl H, Takimoto M, Majdic O, Fritsch G, Scheinecker C, Hocker P and Knapp W (1993) Myeloperoxidase expression in CD34+ normal human hematopoietic cells. *Blood* **82:**2069-2078.

Svensson CK, Cowen EW and Gaspari AA (2001) Cutaneous drug reactions. *Pharmacol Rev* **53:**357-379.

Vyas PM, Roychowdhury S, Woster PM and Svensson CK (2005) Reactive oxygen species generation and its role in the differential cytotoxicity of the arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes. *Biochem Pharmacol* **70:**275-286.

Vyas PM, Roychowdhury S, Khan FD, Prisinzano TE, Lamba JK, Schuetz EG, Blaisdell J, Goldstein JA, Munson KL, Hines RN and Svensson C (2006a) Enzyme-mediated protein haptenation of dapsone and sulfamethoxaozle in human keratinocytes - 1. Expression and role of cytochromes P450. *J Pharmacol Exp Ther*. **319:**488-496.

Vyas PM, Roychowdhury S, Koukouritaki SB, Hines RN, Krueger SK, Williams DE, Nauseef WM and Svensson CK (2006b) Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes - 2. Expression and role of flavin-containing monooxygeanses and peroxidases. *J Pharmacol Exp Ther*. **319:**497-505.

Footnotes

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Legends for Figures

Figure 1. Detection of protein haptenation in naive KG-1 cells after incubation with parent drugs or their arylhydroxylamine metabolites. Naïve KG-1 cells were incubated either with (A) DDS (250 μM for 3 h)/ D-NOH (25 μM for 3 h) or (B) SMX (250 μM for 3 h)/S-NOH (25 μM for 3 h). At the end of the incubation, cells were fixed, permeabilized, immunostained, and imaged on a confocal microscope for DDS-specific covalent adduct formation. Top micrographs were obtained via immunofluorescent confocal microscopy, while bottom micrographs are the corresponding phase contrast for the same view field.

Figure 2. Assessment of protein haptenation via ELISA in naïve KG-1 cells exposed to drugs or their metabolites. Cells ($5x10^5$ cells/ml) were incubated in the presence or absence of 250 μM DDS/SMX or 25 μM DNOH/SNOH and DDS/SMX-protein adducts determined using ELISA. Results shown are the optical density (OD) mean (SD) from three separate incubations for each condition. DDS/SMX-BSA was added to cell free wells as a positive control, though not included in the statistical analysis. (A) Results for incubations using DDS or D-NOH, (B) results for incubations using SMX or S-NOH. *$p<0.05$ compared to control incubations, **$p<0.05$ compared to control and DDS incubations.

Figure 3. Protein haptenation in immature versus mature KG-1 cells exposed to DDS or D-NOH. KG-1 cells were exposed to different cytokine combinations to achieve specific cellular differentiation. Following differentiation, both immature (A) and mature

(B) cells were incubated with DDS (250 μM) or D-NOH 25 μM for 3 h while corresponding control groups were exposed to vehicle (dimethyl sulfoxide) only. At the end of the incubation cells were fixed, permeabilized, immunostained, and imaged on a confocal microscope for DDS-specific protein haptenation. Top row micrographs were obtained via confocal microscopy, while bottom row represents corresponding micrographs visualized via phase contrast.

Figure 4. Effect of methimazole (MMZ) and aminobenzoic acid hydrazide (ABH) on protein haptenation in naïve KG-1 cells exposed to DDS. **A.** Cells ($5x10^5$ cells/ml) were incubated with 250 μM DDS in the presence or absence of MMZ or ABH and DDS-protein adducts determined using ELISA. MMZ was co-incubated with DDS while the incubation with ABH started 1 hr before the addition of DDS. Results shown are the optical density (OD) mean (SD) from three separate incubations for each condition. *$p<0.05$ compared to cells exposed to DDS alone.

B. Cytotoxicity of DDS, in presence and absence of MMZ or ABH was examined in KG-1 cells using a cell impermeable nucleic acid binding dye (Yo-Yo-1). Results shown are the mean (SD) % cytotoxicity of 3 replicate incubations for each condition.

Figure 5. DDS-BSA conjugate uptake in naive (A) and immature (B) KG-1 cells. Both naïve and immature KG-1 cells were incubated with DDS-BSA (100 μg/ml) for 10 and 60 min. Controls were incubated with vehicle (PBS) only. At the end of the incubation cells were fixed, permeabilized, immunostained, and imaged on a confocal microscope for DDS-specific covalent adduct formation. Top row represents images

obtained via confocal microscopy. Bottom row represents corresponding micrographs of the same view field visualized via phase contrast.

Figure 6. SMX-BSA conjugate uptake in naive (A) and immature (B) KG-1 cells. Both naïve and immature KG-1 cells were incubated with SMX-BSA (100 μg/ml) for 10 and 60 min. Controls received the vehicle (PBS) only. At the end of the incubation cells were fixed, permeabilized, immunostained, and imaged on a confocal microscope for SMX-specific covalent adduct formation. Top row represents images obtained via confocal microscopy. Bottom row represents corresponding micrographs of the same view field visualized via phase contrast.

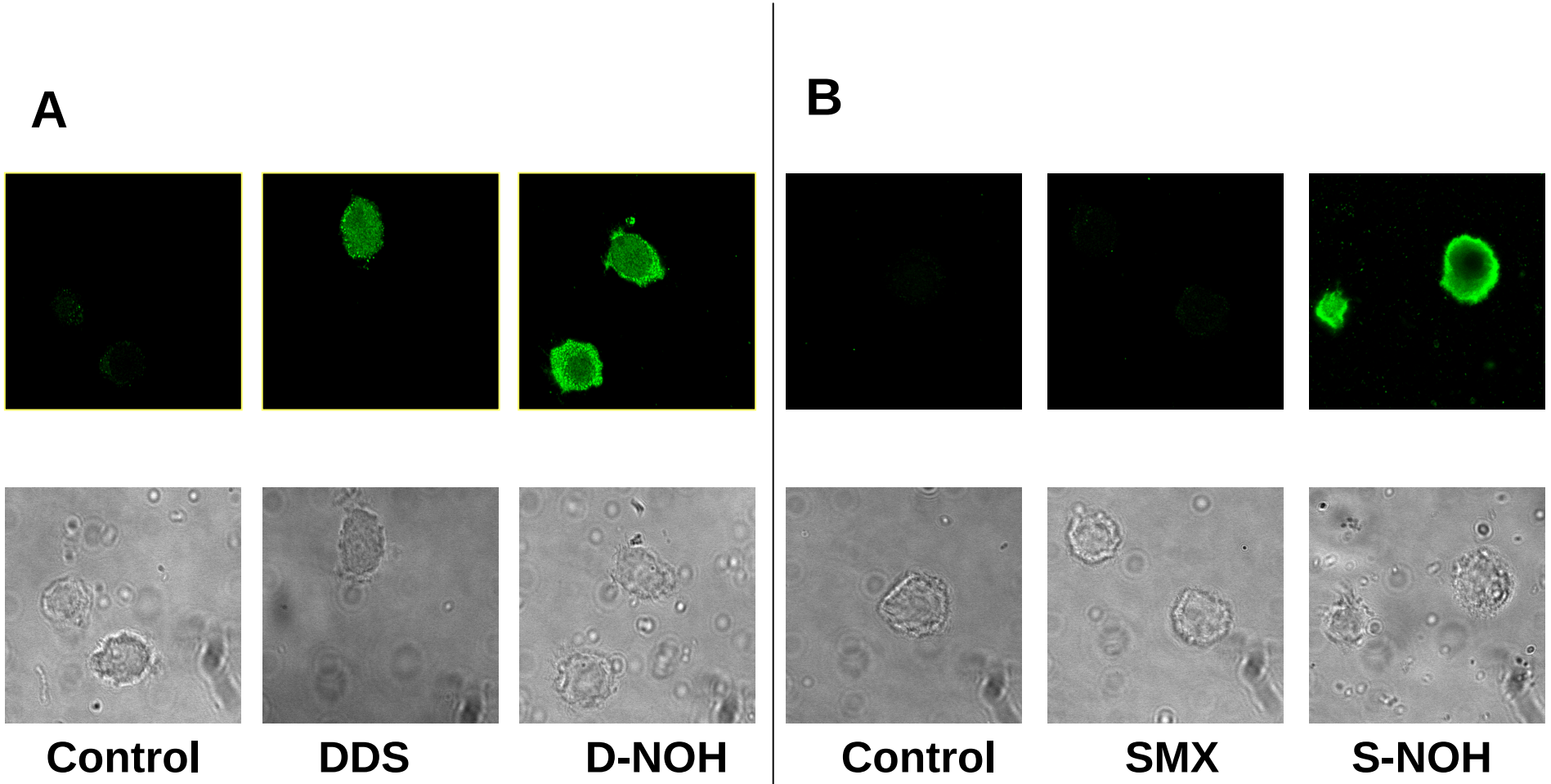

Figure 1

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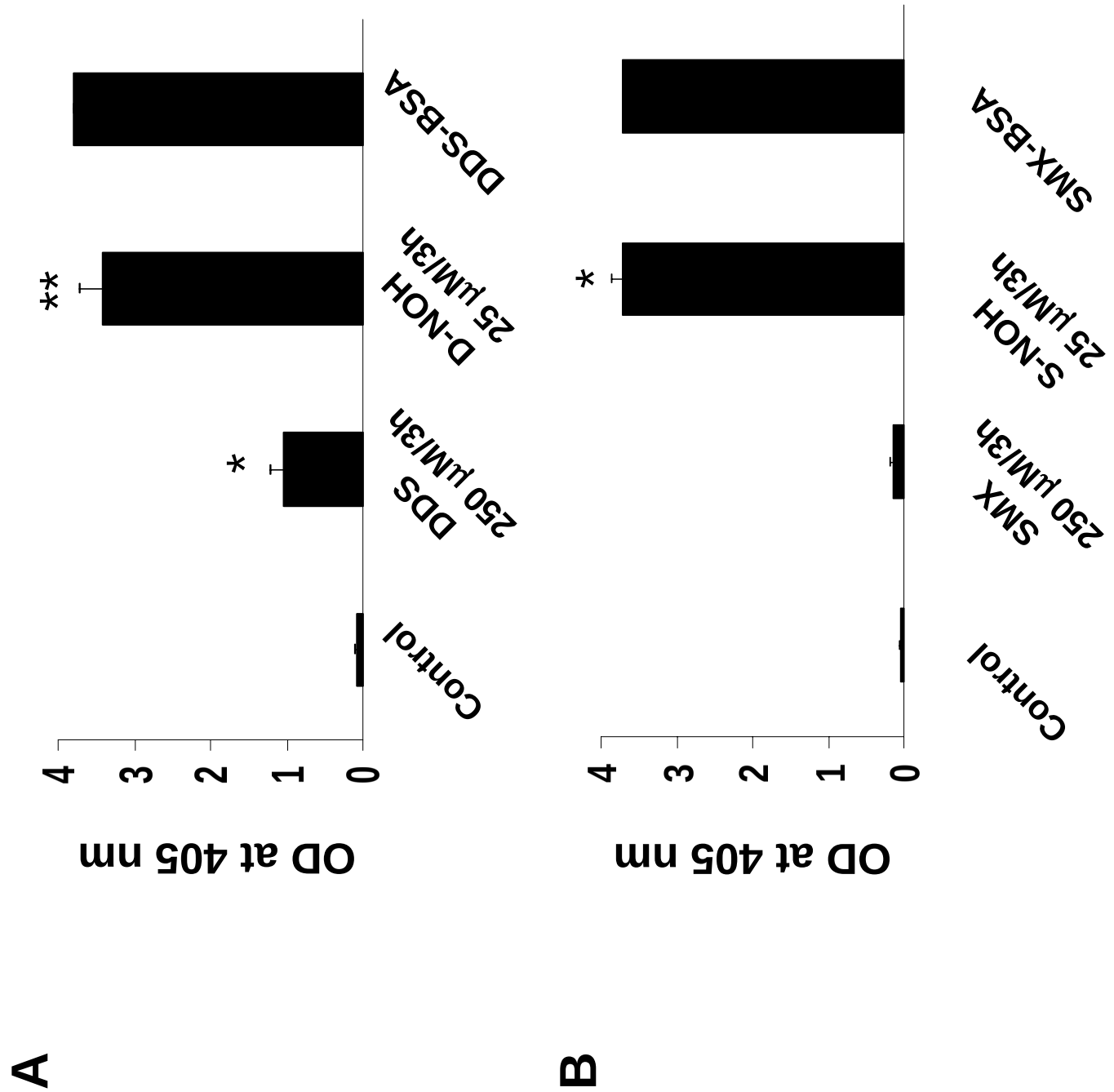


Figure 2

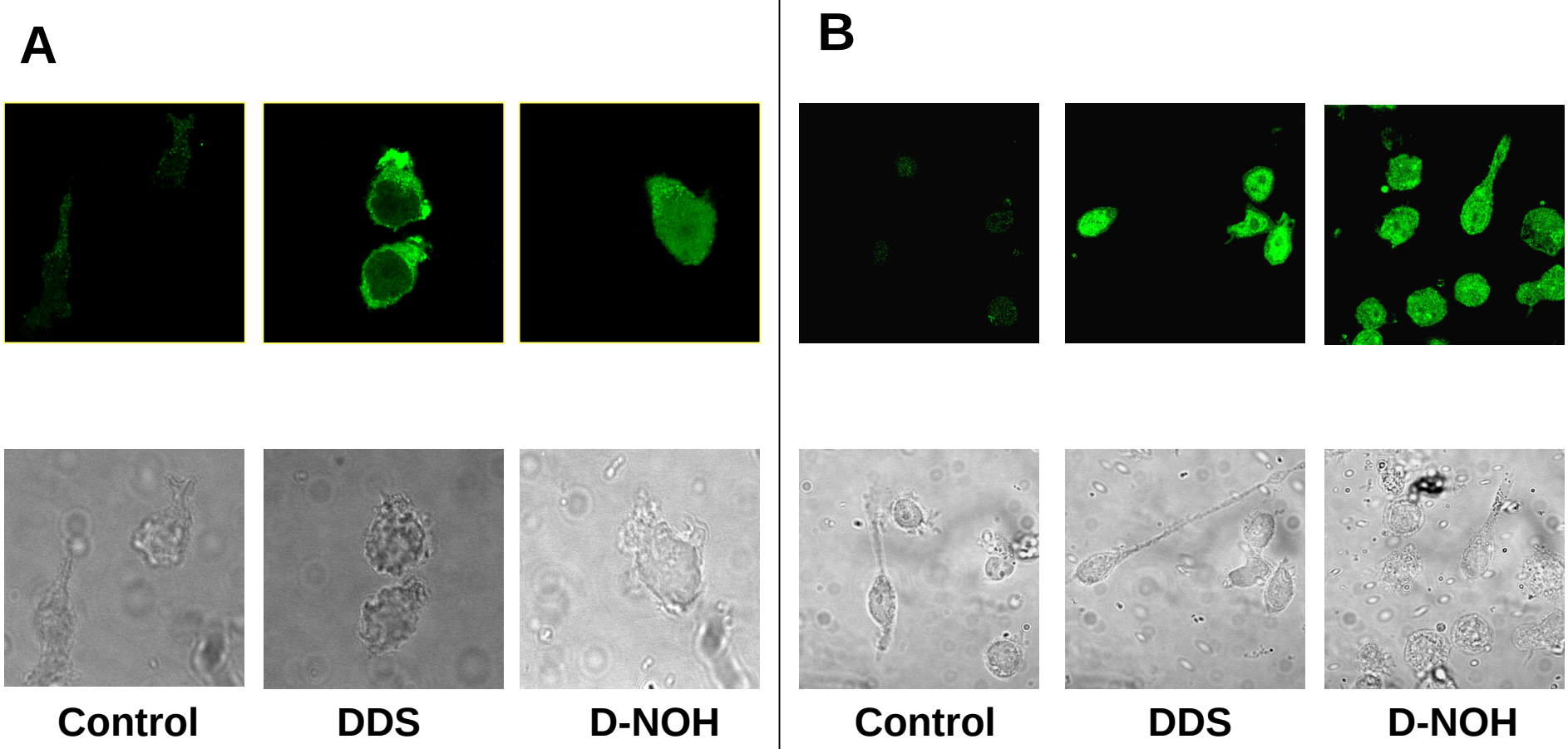
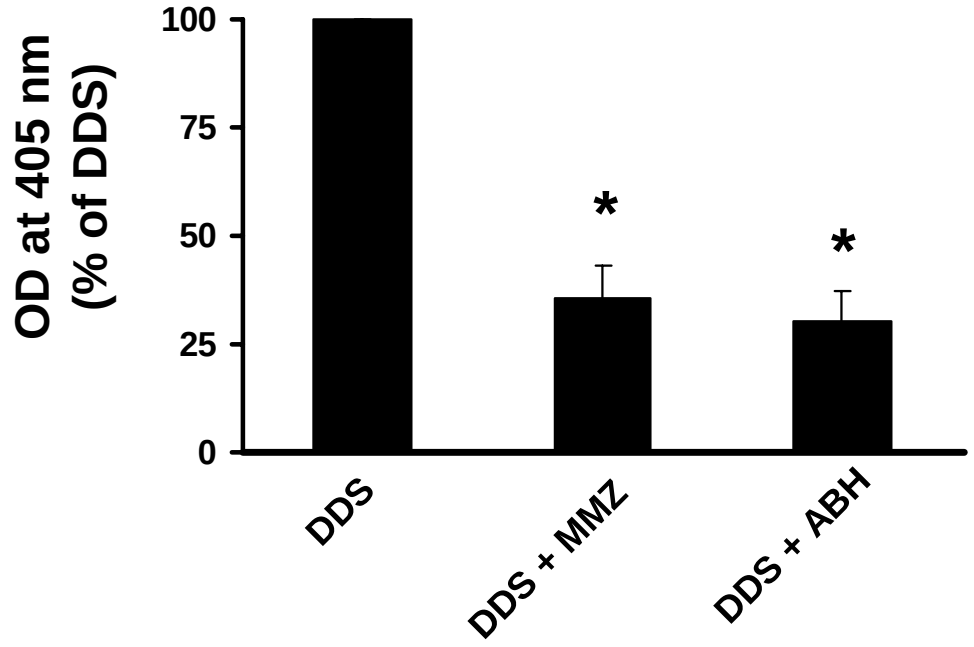


Figure 3

A



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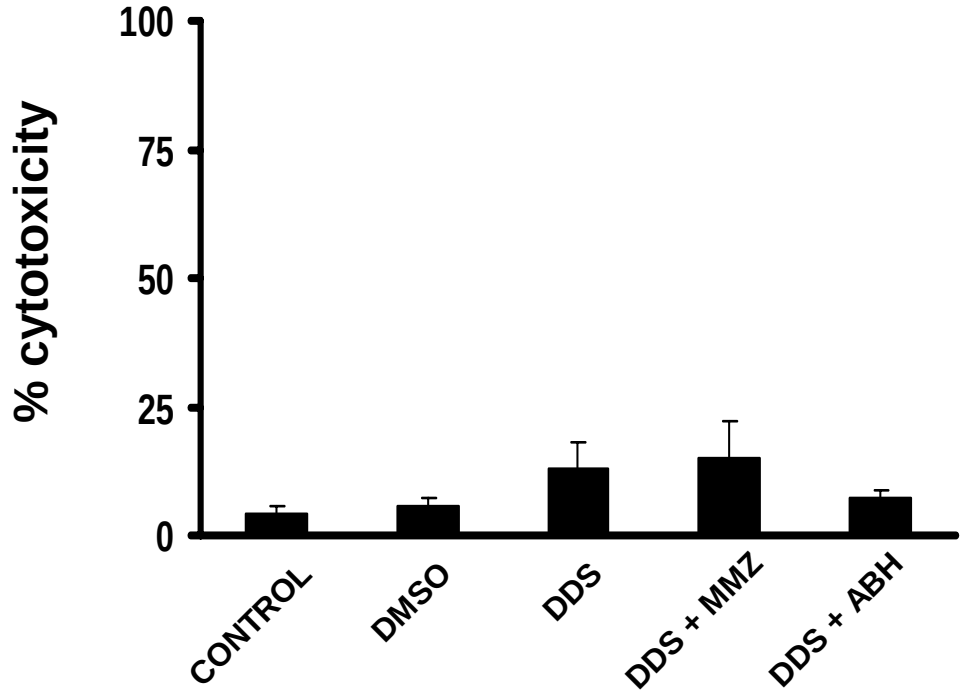

Figure 4

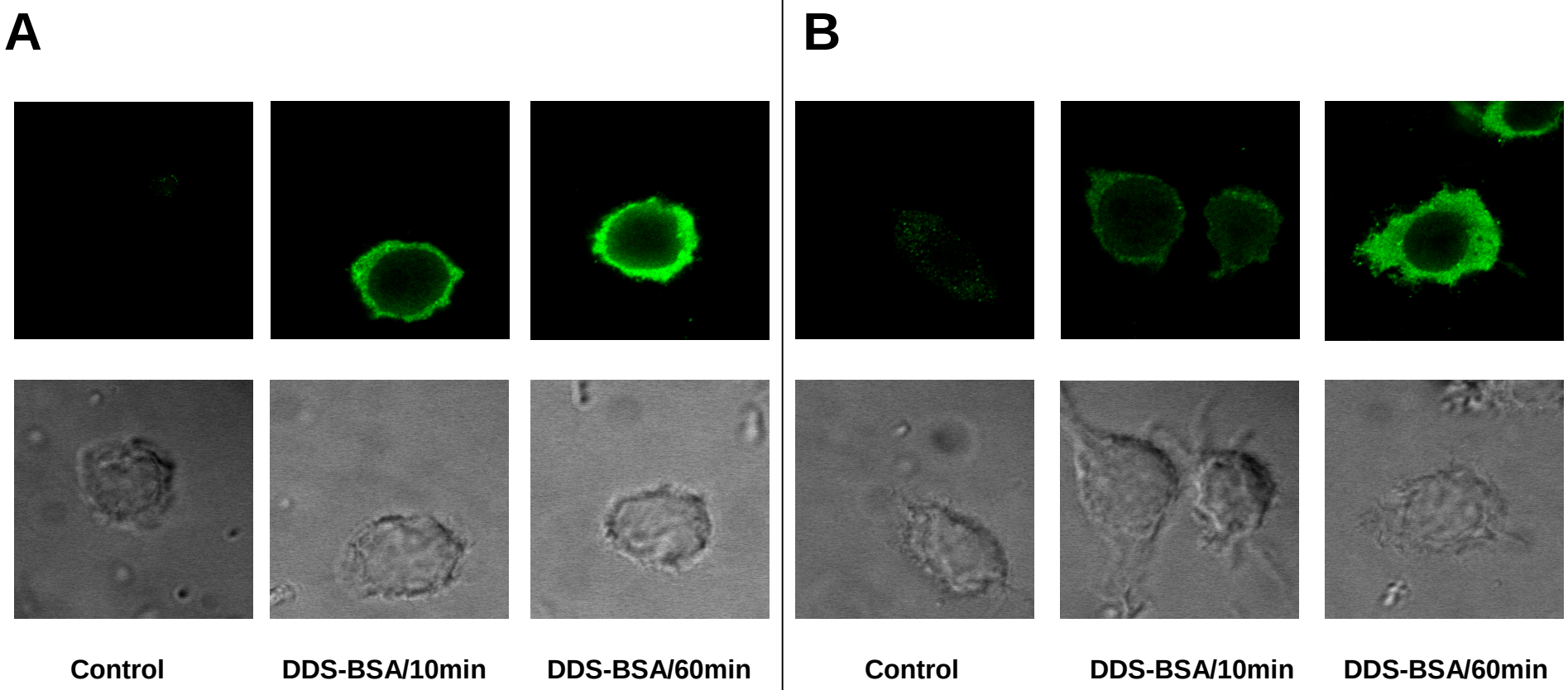

Figure 5

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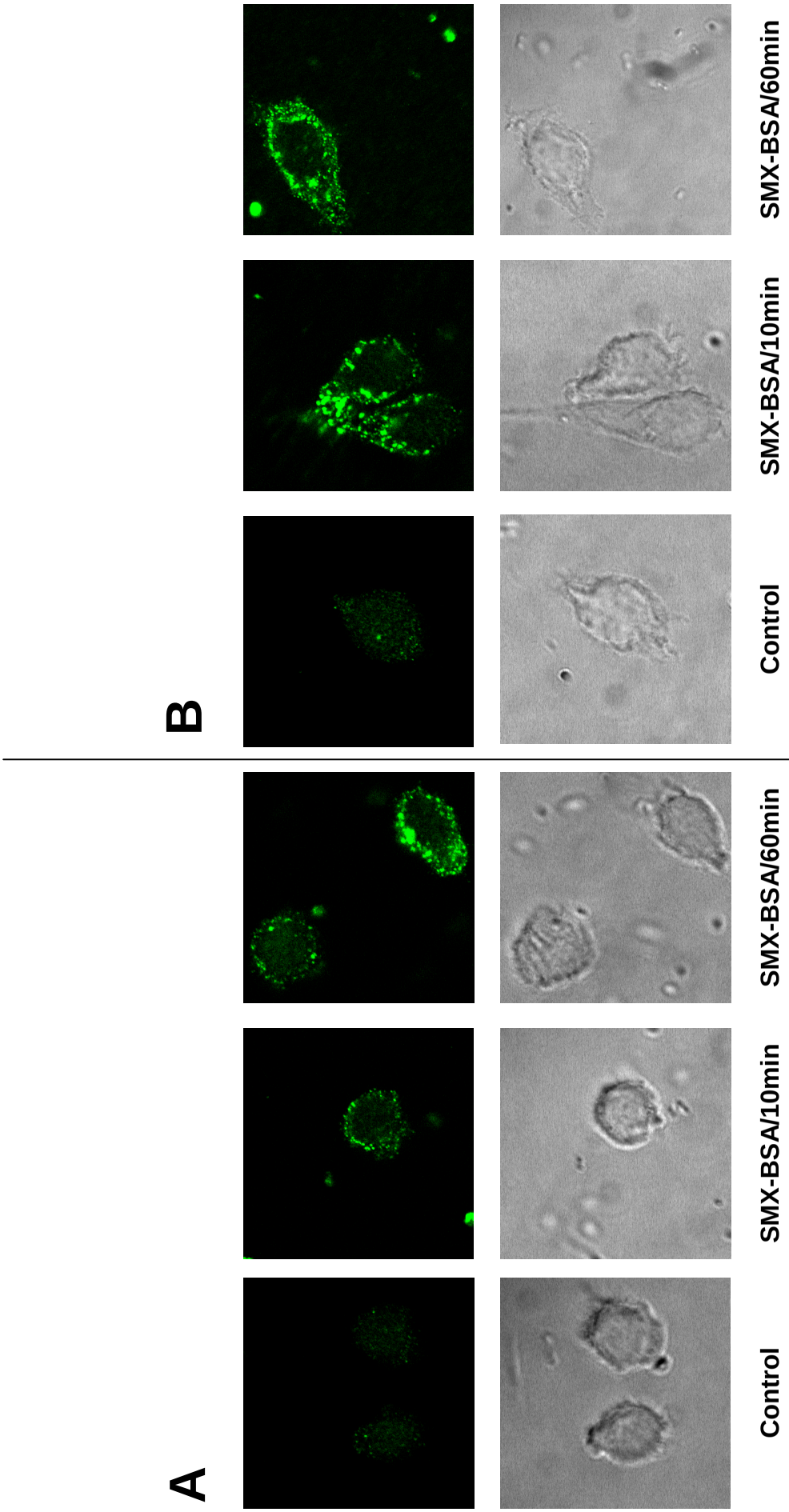

Figure 6