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Anti-Melanoma Activity of Apoptogenic Carbonyl Scavengers

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Apoptogenic Carbonyl Scavengers

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(d) Nonstandard Abbreviations: ADMC, N-acetyl-DMC; AG, aminoguanidine; AGEs, advanced glycation end products; ANT, adenine nucleotide translocator; AV, annexinV; BHA, N-tert.butylhydroxylamine; DMBG, 1,1-dimethylbiguanide hydrochloride; DMC, 3,3-dimethyl-D-cysteine; DMCM, DMC methylester; DMCSS, DMC-disulfide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; ESI, electrospray ionization; *L*-DMC, 3,3-dimethyl-*L*-cysteine; MEC, 3-methyl-3-ethyl-*D*-cysteine; MG, methylglyoxal; MPT, mitochondrial permeability transition; NAC, N□-acetyl-*L*-cysteine; PI, propidium iodide; RCS, reactive carbonyl species; SAR, structure activity relationship; SMDMC, S-methyl-DMC; and PG, phenylglyoxal.

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ABSTRACT. Therapeutic induction of apoptosis is an important goal of anti-cancer drug design. Cellular carbonyl stress mediated by endogenous reactive carbonyl species (RCS) such as glyoxal and methylglyoxal (MG) affects proliferative signaling and metastasis of human tumor cells. Recent research suggests that RCS produced constitutively during increased tumor cell glycolysis may be anti-apoptotic survival factors and thus represent a novel molecular target for anti-cancer intervention. Here, we demonstrate the tumor cell specific apoptogenicity of carbonyl scavengers, which act by covalently trapping RCS, against human (A375, G361, LOX) and murine (B16) melanoma cell lines. A structure activity relationship study identified nucleophilic carbonyl scavenger pharmacophores as the functional determinants of apoptogenic anti-melanoma activity of structurally diverse agents such as 3,3-dimethyl-D-cysteine and aminoguanidine. Earlier work has demonstrated that covalent adduction of protein-arginine residues in the mitochondrial permeability transition (MPT) pore and heat shock protein 27 by intracellular MG, produced in tumor cell glycolysis, inhibits mitochondrial apoptosis and enhances cancer cell survival. Indeed, in various melanoma cell lines, carbonyl scavenger induced apoptosis was antagonized by pretreatment with the membrane-permeable RCS phenylglyoxal (PG). Carbonyl scavenger induced apoptosis was associated with early loss of mitochondrial transmembrane potential, and cyclosporin A antagonized the effects of carbonyl scavengers, suggesting a causative role of MPT pore opening in carbonyl scavenger apoptogenicity. Consistent with RCS-inhibition of mitochondrial apoptosis in melanoma cells, staurosporine-induced apoptosis also

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was suppressed by PG pretreatment. Our results suggest that carbonyl scavengers acting as direct molecular antagonists of RCS are promising apoptogenic prototype agents for anti-melanoma drug design.

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INTRODUCTION

Carbonyl stress is an important mechanism of tissue deterioration in several pathological conditions like diabetes, atherosclerosis, M. Alzheimer, and general aging (Baynes and Thorpe, 2000; Ulrich and Cerami, 2001; Wondrak et al., 2002). Recently, cellular carbonyl stress mediated by endogenous reactive carbonyl species (RCS) such as glyoxal, methylglyoxal (MG), and malondialdehyde has been implicated in proliferative signaling and metastasis in many human malignancies (Taguchi et al., 2000; Kuniyasu et al., 2002), particularly melanoma (Sander et al., 2003; Abe et al., 2004; Wondrak et al., 2005). Cellular carbonyl stress results in protein damage referred to as 'glycation' by spontaneous chemical reaction between RCS, such as reducing sugars and more reactive dicarbonyl compounds, with protein-bound arginine and lysine residues (Thornalley, 2005). RCS-derived protein modifications called advanced glycation endproducts (AGEs) formed by chemical reactions between RCS and tissue proteins are abundant in melanoma tissue, and AGEs are potent ligands of RAGE (receptor for advanced glycation endproducts), a membrane receptor involved in proliferation, invasion, and metastasis of melanoma cells (Huttunen et al., 2002; Abe et al., 2004).

In addition to the established role of AGE-RAGE signaling in many human malignancies, increasing evidence supports the hypothesis that RCS, originating from increased tumor cell glycolysis and mitochondrial oxidative stress, are small molecule anti-apoptotic effectors (Sakamoto et al., 2002; Speer et al., 2003; Johans et al., 2005). The □-dicarbonyl MG forms from glycolytic triose

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phosphates by spontaneous phosphate elimination (Thornalley, 1995), and intracellular MG levels are elevated under conditions of increased glycolytic flux such as hyperglycemia (Shinohara et al., 1998) and aerobic glycolysis associated with malignant transformation (Kawase et al., 1996). MG is a potent glycating agent leading to the posttranslational modification of protein-arginine and lysine residues with formation of AGEs such as arg-pyrimidine (Shipanova et al., 1997), N-carboxyethyllysine (Ahmed et al., 1997), and the hydroimidazolone MG-H1 (Thornalley, 2005). Recent evidence suggests that covalent modification of arginine residues by MG targets specific proteins involved in apoptosis, such as mitochondrial permeability transition (MPT) pore proteins (Johans et al., 2005) and heat shock protein 27 (Hsp 27) (Sakamoto et al., 2002). MG-modification of MPT pore proteins interferes with pore opening, inhibiting mitochondrial swelling, loss of transmembrane potential, and subsequent release of proapototic factors such as cytochrome C and AIF in response to apoptotic stimuli such as high Ca^{2+} and ganglioside GD3 (Speer et al., 2003; Johans et al., 2005). In various human cancer cell lines posttranslational MG-mediated arg-pyrimidine formation occurs at a single arginine residue of Hsp 27 with induction of Hsp27 oligomerization, essential for repression of cytochrome c-mediated apoptosome assembly (Bruey et al., 2000). Given the involvement of mitochondrial membrane permeabilization and subsequent apoptosome assembly in the activation of executioner caspases (Don and Hogg, 2004; Green and Kroemer, 2004), these findings suggest a role of RCS-mediated protein modification in anti-apoptotic survival signaling in cancer cells.

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We have developed a high throughput screen for the rapid identification of -dicarbonyl scavengers as potential therapeutic agents to exert cellular protection against carbonyl stress by covalent trapping of RCS (Wondrak et al., 2002). Effective -dicarbonyl scavengers identified in our screen contain two nucleophilic functional groups that trap -dicarbonyls such as MG and glyoxal by stable covalent adduction. Based on earlier reports of thiol-agent induced cancer cell apoptosis attributed to antioxidant activity (Havre et al., 2002), and intrigued by the emerging role of RCS originating from enhanced glycolytic flux as novel small molecule modulators of cancer cell proliferative control and anti-apoptotic survival signaling, we evaluated the anti-melanoma structure-activity-relationship (SAR) of various thiol- and non-thiol-carbonyl scavenger pharmacophores. Here, we provide experimental evidence that certain carbonyl scavengers may represent a novel class of anti-melanoma therapeutic agents.

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MATERIALS AND METHODS

Chemicals Most chemicals were from Sigma Chemical Co, St. Louis, MO. D,L-N-acetyl-beta-mercaptoisoleucine (N-acetyl-3-methyl-3-ethyl-L-cysteine) was from Aldrich, Milwaukee. Cyclosporin A was from Calbiochem-Novabiochem, San Diego, CA. Staurosporine was from Tocris, Ellisville, MO.

Chemical Synthesis *3,3-Dimethyl-D-cysteinemethylester (DMCM)*: To a stirred solution of $SOCl_2$ (18 mL) in MeOH (75 mL) prepared and maintained at –10 ^{o}C was added 3,3-dimethyl-D-cysteine (15 g, 100.5 mmol). Stirring was continued and the mixture was allowed to reach room temperature. The reaction mixture was then refluxed for 60 h and solvent was evaporated to yield the crude product (15.2 g, 93.3 mmol, 93%). The product was then dissolved in methanol and crystallized by addition of diethylether. The crystalline product was collected and dried under vacuum to yield the pure product (7.6 g, 47 mmol, 46%) that was characterized by mass spectrometry {(ESI^+): m/z calculated for $C_6H_{14}O_2NS$: 164.1 $[M+H]^+$, observed 164.1} and ^{1}H-NMR {(250 MHz, $CDCl_3$): □ 1.57 (3H, s), 1.68 (3H, s), 2.95 (1H, s), 3.86 (3H, s), 4.22 (1H, s), 8.70 (2H,br s).

3-Methyl-3-ethyl-D,L-cysteine (MEC): The synthesis of 3-methyl-3-ethyl-D,L-cysteine was performed by acid hydrolysis of a commercially available precursor molecule (D,L-N-acetyl-beta-mercaptoisoleucine) following a standard procedure for acidic removal of an N-acetyl group (Miller, 1949). The hydrolytic removal of the acetyl group was confirmed by mass spectrometry. MS (ESI^+) m/z calculated for $C_6H_{14}O_2NS$: 164.1 $[M+H]^+$, observed 163.8.

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Cell culture The established cell line of human epidermal keratinocytes (HaCaT cells), a gift from Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), and human dermal fibroblasts (CF-3 cells), a gift from Dr. Robert Dell'Orco (Noble Center for Biomedical Research, Oklahoma City, USA) were cultured in DMEM containing 10% bovine calf serum (BCS). Adult human skin keratinocytes (Cascade Biologics, Portland, OR) were cultured using Epilife™ basal medium with HKGS supplement from the same supplier. Human A431 squamous cell carcinoma cells were from ATCC (Manassas, VA, USA) and cultured in DMEM, 10% BCS with high glucose (30 mM). G-361 human melanoma cells from ATCC (Manassas, VA, USA) were cultured in McCoy's 5a medium containing 10% BCS. A375, LOX and B16 murine melanoma cells from ATCC (Manassas, VA, USA) were cultured in RPMI medium containing 10% BCS and 2 mM L-glutamine.

Reaction kinetics of □-dicarbonyl scavenging. Potency of carbonyl scavenging by selected test compounds was determined quantitatively by establishing the second order rate constant of phenylglyoxal trapping as published before (Wondrak et al., 2002).

Apoptosis analysis. Induction of cell death was confirmed by annexin-V-FITC/propidium iodide (PI) dual staining of cells followed by flow cytometric analysis. Cells (200,000) were seeded on 35 mm dishes and received photosensitization 24 hours later. Cells were harvested at various time points

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after treatment and cell staining was performed using an apoptosis detection kit according to the manufacturer's specifications (APO-AF, Sigma, St. Louis, MO).

Mitochondrial Transmembrane Potential. Mitochondrial transmembrane potential (□□m) was assessed using the potentiometric dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) following a published procedure (Ocker et al., 2003). At high □□m, JC-1 forms red-fluorescent 'J-aggregates' distinct from the green-fluorescent monomer observed at low □□m. J-aggregate formation increases linearly with □□m over the range of 30-180 mV. In brief, cells were trypsinized, washed in PBS, resuspended in 300 µL PBS containing 5 µg/ml JC-1 for 15 min at 37°C and 5% CO_2 in the dark, then washed twice in PBS and resuspended in 300 µL PBS. Bivariate analysis was performed by flow cytometry with excitation at 488 nm, and mitochondrial function was assessed as JC-1 green (depolarized mitochondria, detector FL-1) or red (polarized mitochondria, detector FL-2) fluorescence.

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RESULTS

Apoptogenicity of the carbonyl scavenger DMC in melanoma cells. Accumulating evidence for a causative involvement of carbonyl stress in melanoma progression lead us to examine the anti-melanoma activity of carbonyl scavengers that we had previously identified as potent inhibitors of cellular carbonyl stress (Wondrak et al., 2002). 3,3-Dimethyl-D-cysteine (DMC), containing a recently identified scavenger pharmacophore, α-amino-β-mercapto-β,β-dialkyl-ethane, was studied first. Our data demonstrate melanoma cell-selective apoptogenicity of *DMC* as summarized in **Fig. 1**. DMC treatment induced strong cytotoxicity in human (G-361, A375, LOX) and murine (B16) melanoma cells with over 60% of cells undergoing apoptosis within 24 hours as assessed by annexin-V-propidium iodide staining followed by flow cytometry. In contrast, no appreciable induction of apoptosis was observed when primary human skin fibroblasts (CF3) and epidermal keratinocytes (data not shown) were treated with *D*-DMC.

Structure activity relationship (SAR) of apoptogenicity. Based on our earlier studies on screening and identification of α-dicarbonyl scavengers (Wondrak et al., 2002), a SAR analysis was performed to validate the involvement of carbonyl scavenging as a potential molecular mechanism of DMC apoptogenicity observed in melanoma cells. Chemical structures of test compounds are shown in **Fig. 2**. Induction of G361 melanoma cell apoptosis by various DMC structural analogs and structurally unrelated carbonyl scavengers was examined as

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detailed in **Fig. 3**. In addition, in order to compare carbonyl scavenger potency of selected test compounds, reaction kinetics of α-dicarbonyl trapping [second order rate constants; k_{2nd} ± SD ($M^{-1}sec^{-1}$)] were determined as published earlier (Wondrak et al., 2002). The absolute configuration was not critical for apoptogenic activity since DMC (k_{2nd}: 24.9 ± 4.2 $M^{-1}sec^{-1}$; the D-isomer used in all further experiments) and its L-isomer (*L*-DMC) were equally apoptogenic (data not shown). Since DMC also is a powerful thiol antioxidant and antioxidants can exert apoptogenic effects on malignant cells (Havre et al., 2002), structural analogues of *DMC* that retained antioxidant activity, but displayed diminished or no carbonyl scavenger activity, were tested for apoptogenicity on melanoma cells. No significant apoptogenic activity was observed with N-acetyl-*DMC* (ADMC; k_{2nd}: 5.2 x 10^{-3} ± 2.9 x 10^{-3} $M^{-1}sec^{-1}$) and the closely related thiol antioxidant Nα-acetyl-*L*-cysteine (NAC). As shown earlier, both ADMC and NAC are devoid of potent carbonyl scavenger activity due to inactivation by acetyl-substitution of the α-amino group known to be essential for carbonyl scavenging via thiazolidine ring formation (Wondrak et al., 2002). Consistent with an absolute structural requirement for the thiol-substituent, complete loss of apoptogenic activity was observed with *DMC*-disulfide (DMCSS). Additionally, the S-methylated derivative S-methyl-*DMC* (SMDMC) was devoid of apoptogenic activity (data not shown). As demonstrated previously, *L*-Cysteine (CYS; k_{2nd}: 0.63 ± 0.06 $M^{-1}sec^{-1}$) and *L*-cysteine-O-methylester (CYSM) are only weak carbonyl scavengers due to the absence of dialkyl substituents adjacent to the thiol group ('geminal dialkyl effect' (Jung and Gervay, 1991)) important for

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efficient carbonyl trapping by thiazolidine ring formation (Wondrak et al., 2002), and did not display melanoma cell specific apoptogenicity (data not shown). In contrast, potent and cancer cell selective apoptogenicity was maintained upon structural modification of the carboxy-substituent (*DMC*-methylester [DMCM]; k_{2nd}: 28.8 ± 2.3 $M^{-1}sec^{-1}$, **Fig. 3B**) or the alkyl-substituent (3-methyl-3-ethyl-*D*-cysteine [MEC]; (k_{2nd}: 33.1 ± 1.5 $M^{-1}sec^{-1}$, **Fig. 3C**) of *DMC*. Remarkably, DMCM displayed an approximately 5 to 10-fold increased potency over DMC against melanoma cells (data not shown) and other cancer derived skin cell lines such as A431 human squamous carcinoma cells (**Fig 3B**), and MEC displayed enhanced apoptogenicity towards A375 melanoma cells (**Fig 3C**). Both ester modification and alkyl substitution in DMCM and MEC would be expected to increase lipophilicity and thiol-group nucleophilicity (Winterbourn and Metodiewa, 1999) with retention of the geminal dialkyl effect, and increased potency therefore could result from higher membrane penetration and/or intracellular carbonyl scavenger activity. To further test the hypothesis that apoptogenic anti-melanoma potential of our test compounds is dependent on carbonyl scavenger activity, structurally unrelated non-thiol carbonyl scavengers were examined for apoptogenicity against melanoma cells as shown in **Fig. 3A and D**. Indeed, pronounced induction of apoptosis was observed when various human melanoma cell lines were exposed to the hydrazine-type prototype carbonyl scavenger aminoguanidine (AG; k_{2nd}: 0.4 ± 0.02 $M^{-1}sec^{-1}$) (Thornalley et al., 2000), although higher doses were needed than for DMC-induced apoptosis. As observed earlier with DMC, AG treatment did not induce apoptosis in CF3 human skin fibroblasts

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(data not shown). Importantly, melanoma cell apoptosis was also observed upon exposure to other bifunctional nucleophilic carbonyl scavengers, such as semicarbazide hydrochloride (SC; k_{2nd}: 5.3 x 10^{-2} ± 0.3 x 10^{-2} $M^{-1}sec^{-1}$) (Lehman and Ortwerth, 2001) and, to a lesser extent, the oral antidiabetic 1,1-dimethylbiguanide hydrochloride (DMBG) (Ruggiero-Lopez et al., 1999) as shown in **Fig. 3D**. Interestingly, appreciable anti-melanoma activity was also observed with the nucleophilic hydroxylamine-derivative N-tert-butylhydroxylamine (BHA), a proposed carbonyl scavenger (Hipkiss et al., 2002), previously shown to extend proliferative life span of human fibroblasts (Atamna et al., 2000).

This SAR study demonstrates that (i) activity as carbonyl scavenger (DMC, *L*-DMC, DMCM, MEC, AG, SC, DMBG), but not as thiol-antioxidant (NAC, ADMC) is a determinant of antimelanoma activity, that (ii) synthesis of simple derivatives of the lead compound DMC resulted in enhanced apoptogenic potency towards malignant cells, and that (III) carbonyl scavenger induction of apoptosis may not be limited to melanoma cells as suggested by the results obtained with human A431 squamous carcinoma cells.

RCS- and carbonyl scavenger-modulation of melanoma cell apoptosis. The apoptogenicity of carbonyl scavengers on human melanoma cell lines suggests that covalent trapping of RCS may be part of the apoptogenic mechanism of action observed with these agents. Recently, MG modulation of regulatory mechanisms of apoptosis such as MPT pore opening and Hsp27 oligomerization

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have been demonstrated as part of an emerging cancer cell survival pathway that interferes with apoptosis by glycolytic formation of RCS (Speer et al., 2003; Johans et al., 2005; Wondrak et al., 2005). The possibility of a functional antagonism between carbonyl scavengers and RCS-mediated cell survival was further examined by inducing carbonyl scavenger-triggered apoptosis after external addition of the RCS phenylglyoxal (PG) as summarized in **Fig. 4**. PG is a membrane-permeable RCS that irreversibly inhibits MPT pore opening in isolated mitochondria (Speer et al., 2003), thereby potentially mimicking the effects of elevated intracellular MG. Indeed, PG pretreatment antagonized carbonyl scavenger induced G361 melanoma cell apoptosis (**Fig 4A**) and also suppressed loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) normally associated with MPT pore opening (**Fig 4B**). The antagonistic action of RCS (PG) and carbonyl scavenger (*DMC*) on cancer cell viability and $\Delta\Psi_m$ supports the hypothesis that apoptogenic carbonyl scavengers may target endogenous RCS as potential anti-apoptotic modulators (Speer et al., 2003).

Suppression of staurosporine-induced apoptosis by RCS-pretreatment. To test whether exogenous carbonyl stress could interfere with induction of apoptosis by agents that induce mitochondrial pathways of apoptosis independent of carbonyl scavenger activity (Tafani et al., 2001; Duan et al., 2003), the effect of exogenous RCS on staurosporine-induced apoptosis was examined in G361 human melanoma cells. Cells were pretreated with PG (5 mM, 30 min) followed by 24h continuous exposure to staurosporine [200 nM]. Pronounced protection against staurosporine-induction of apoptosis was

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observed in melanoma cells pretreated with PG (**Fig. 5A**). Forward/sideward scatter analyses also demonstrated that PG pretreatment suppressed changes in cell size (shrinkage) and granularity characteristic of apoptotic cells (**Fig. 5B**). The results of this experiment suggest that membrane permeable RCS can effectively interfere with induction of apoptosis by staurosporine, an apoptogenic kinase inhibitor that does not act as a carbonyl scavenger. Thus, RCS-suppression of apoptotic pathways may occur with various inducers of mitochondrial apoptosis, and carbonyl scavengers could overcome this RCS-inhibition of apoptosis in cancer cells.

Suppression of carbonyl scavenger-induced apoptogenicity by the MPT pore inhibitor cyclosporine A. To further elucidate a potential role of $\Delta\Psi_m$ breakdown during carbonyl scavenger-induced apoptosis the time course of induction of apoptosis and loss of $\Delta\Psi_m$ upon continuous exposure to *DMC* were compared as shown in **Fig. 6A**. Appearance of cells in early apoptosis (AV-positive, PI-negative; lower right quadrant) after 12 h and progression into later stages of cell death (AV-positive, PI-positive; upper right quadrant) occurred in close synchrony with loss of $\Delta\Psi_m$ suggesting an involvement of $\Delta\Psi_m$ breakdown in *DMC*-induced apoptosis. To provide a more stringent test for an involvement of MPT pore opening in carbonyl scavenger-induced apoptosis, *DMC*-induction of melanoma cell apoptosis was examined in the presence of a specific inhibitor of MPT pore opening, cyclosporine A (CysA) as shown in **Fig. 6B**. Cys A inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has

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been widely used as a sensitive molecular probe for the involvement of MPT pore opening in cell death pathways (Eriksson et al., 1997). Indeed, CysA pretreatment exerted significant cell protection against induction of apoptosis by *DMC*. CysA suppression of apoptosis provides strong evidence that MPT pore opening is involved in carbonyl scavenger apoptogenicity observed in human melanoma cells.

DISCUSSION

Recently, we have examined □-dicarbonyl scavengers as a novel class of compounds for therapeutic intervention in cellular carbonyl stress (Roberts et al., 2002; Wondrak et al., 2002). Here we report the apoptogenic activity of various carbonyl scavenger pharmacophores, such as □-amino-□,□-dialkyl-□-mercapto-ethane (Wondrak et al., 2002), hydrazines (Edelstein and Brownlee, 1992; Thornalley et al., 2000), hydrazides (Lehman and Ortwerth, 2001), and guanidines (Beisswenger et al., 1999; Ruggiero-Lopez et al., 1999), against various human and murine melanoma cell lines. Based on earlier reports of thiol-agent induced cancer cell apoptosis that was attributed to antioxidant activity (Havre et al., 2002) and our initial observation that prototype carbonyl scavengers such as AG and *DMC* induced apoptosis in human and murine melanoma cell lines, but not in untransformed primary human skin fibroblasts (**Figs.1** and **3**), a detailed SAR study of *DMC* anti-melanoma activity was performed (**Figs. 2** and **3**). Molecular reactivity as a bifunctional nucleophilic carbonyl scavenger was revealed as the structural determinant of apoptogenic

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antimelanoma activity of various test compounds (*DMC*, *L*-DMC, DMCM, MEC, AG, SC, DMBG). Apoptogenicity of various carbonyl scavengers decreased in the order of established rate constants of carbonyl trapping (Wondrak et al., 2002), suppression of AGE-fluorescence formation (Ruggiero-Lopez et al., 1999), and inhibition of [^{14}C]lysine-protein crosslinking (Lehman and Ortwerth, 2001) (*DMC* >> AG ≈ SC > DMBG) demonstrating a correlation between carbonyl scavenger potency and apoptogenic activity. Importantly, simple derivatization of the prototype agent *DMC* by esterification, expected to enhance thiol- and amino-group nucleophilicity and thereby carbonyl scavenger potency (Winterbourn and Metodiewa, 1999), potentiated anti-melanoma activity of the test compound suggesting the feasibility of future lead refinement with generation of more potent derivatives active in the micromolar range. Nevertheless, a favorable toxicity profile of DMC, an FDA-approved drug in clinical use as a copper ion chelator for the management of Wilson's disease (cuprimine ®, Merck), allows the long term administration of daily dosages of up two 2 g demonstrating that high tissue concentrations of a prototype carbonyl scavenger can be achieved without inducing systemic adverse effects.

Our studies are in agreement with recent research indicating that RCS are important endogenous small molecule modulators of cellular protein targets involved in initiation and execution of cellular apoptosis. MG-modification of the MPT pore complex leads to suppression of pore opening and mitochondrial induction of apoptosis (Speer et al., 2003; Johans et al., 2005), and MG-modification of Hsp 27 enhances its interaction with cytochrome c preventing

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caspase activation (Bruey et al., 2000; Sakamoto et al., 2002). Evidence for covalent MG modification of an adenine nucleotide translocator (ANT)-bound arginine residue leading to an unknown adduct in isolated mitochondria and live cells has been presented (Speer et al., 2003). Moreover, MG adduction and activation of Hsp27 with arg-pyrimidine formation on the C-terminal Arg-188 residue has been demonstrated in various cancer cell lines (such as NCI-H23 lung cancer, U937 leukemia, PC3 prostate cancer), particularly under hyperglycemic conditions. These and other findings demonstrating MG-modification of potentially important targets involved in the regulation of cancer cell survival suggest that endogenous carbonyl stress is directly involved in modulating cell survival. This RCS-mediated survival pathway could be operative in cancer cells where increased RCS production from high glycoytic flux even under aerobic conditions (the 'Warburg effect' (Dang and Semenza, 1999)) and enhanced mitochondrial electron leakage and lipid peroxidation are known to occur (Wondrak et al., 2005). Thus, the bioenergetic differences between normal and transformed cells, that enable small-molecule inhibitors of apoptosis such as MG to accumulate at higher concentrations in cancer cells (Thornalley, 1995; Kawase et al., 1996; Sakamoto et al., 2002), could provide an explanation for the cancer cell-selective induction of apoptosis by carbonyl scavengers that may not be limited to melanoma cells as suggested by carbonyl scavenger induction of apoptosis in human A431 squamous carcinoma cells (**Fig. 3B**).

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Consistent with an antagonistic effect of cellular carbonyl stress and carbonyl scavenger treatment on melanoma cell viability, PG pretreatment protects melanoma cells from carbonyl scavenger-induced apoptosis (**Fig. 4**). Importantly, PG-protection against induction of apoptosis also was observed with treatment by staurosporine (**Fig. 5**), an apoptogenic kinase inhibitor and inducer of mitochondrial depolarization, swelling, outer membrane rupture, and cytochrome C release (Scarlett et al., 2000; Tafani et al., 2001; Duan et al., 2003). Earlier work has demonstrated that RCS including MG, glyoxal, and the synthetic cell-permeable MG analogue PG block MPT pore opening, transmembrane potential dissipation, and mitochondrial swelling induced by high Ca^{2+} and ganglioside GD3, known inducers of mitochondrial pathways of apoptosis by MPT pore opening (Eriksson et al., 1998; Johans et al., 2005). Consistent with involvement of MPT pore opening in carbonyl scavenger-induction of melanoma cell apoptosis, cyclosporine A treatment suppressed *DMC*-induced apoptosis (**Fig. 6B**), but carbonyl scavenger interference with RCS-modification of other crucial protein targets is likely to occur, since cyclosporine A rescue of *DMC*-treated cells was only partially effective.

The experimental evidence reported in this work supports a model of carbonyl scavenger-induction of melanoma cell apoptosis as presented in **Fig. 7**, which is consistent with earlier research on the potential suppression of cancer cell apoptosis by inhibition of MPT opening and caspase activation by endogenous RCS. In this model, RCS, e.g. MG from increased glycolytic flux during tumor cell hypoxia, hyperglycemia, and aerobic glycolysis, are endogenous small

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molecular inhibitors of apoptosis that potentially target proteins, such as the MPT pore protein ANT (Johans et al., 2005), the cytochrome C antagonist Hsp 27 (Sakamoto et al., 2002), and likely other unidentified molecular targets involved in apoptotic events. Potent carbonyl scavengers trap intracellular MG covalently potentially leading to MPT pore opening, apoptosome assembly and activation of executioner caspases. This model predicts that carbonyl scavengers overcome the apoptosis resistance characteristic of many tumor-derived cell lines that may partially result from covalent MG adduction of crucial protein targets.

Therapeutic induction of apoptosis is an important goal of anti-cancer drug design (Qin et al., 2005). The apoptogenic activity of carbonyl scavengers presented in this study raises the possibility that novel anti-melanoma agents may be based on molecular interference with endogenous carbonyl stress. Ongoing research aims at the identification of molecular targets modified by endogenous carbonyl stress and modulated by carbonyl scavenger intervention, particularly by proteomic identification of RCS-adducted proteins in melanoma cells. After successful target validation, a potential therapeutic application of more potent carbonyl scavenger agents necessitates lead optimization and efficacy studies in appropriate xenograft melanoma models.

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ACKNOWLEDGEMENTS

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Preferential induction of apoptosis by 3,3-dimethyl-D-cysteine (DMC) in malignant melanoma cell lines. Induction of apoptosis was examined in malignant human (G361, A375, LOX)] and murine (B16) melanoma cell lines and human skin fibroblasts (CF3) after continuous exposure (24 h) to 3,3-dimethyl-D-cysteine (*DMC*, 10 mM). The top panels represent untreated control (C) and the bottom panels DMC treated cells. Apoptosis was detected by flow cytometric analysis of annexin V-FITC/propidium iodide (PI) stained cells with early apoptotic and late apoptotic/necrotic cells located in the lower right (AV^+, PI^-) and upper right quadrant (AV^+, PI^+), respectively. One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV^-, PI^-, lower left quadrant) in percent of total gated cells (mean ± SD, n=3).

Figure 2: Test compounds used for structure-activity relationship studies on carbonyl scavenger apoptogenicity in human malignant cells. 3,3-dimethyl-D-cysteine (DMC) ; L-isomer of DMC (L-DMC); N-acetyl-DMC (ADMC); N^a-acetyl-L-cysteine (NAC); DMC-disulfide (DMCSS); S-methyl-DMC (SMDMC); L-Cysteine (CYS); L-cysteine-O-methylester (CYSM); DMC-methylester (DMCM); 3-methyl-3-ethyl-D-cysteine (MEC); aminoguanidine (AG); semicarbazide hydrochloride (SC, hydrazine carboxamide); 1,1-dimethylbiguanide hydrochloride (DMBG); N-tert-butylhydroxylamine (BHA).

Figure 3: Structure activity relationship (SAR) of apoptogenicity. (A) Induction of apoptosis in G361 melanoma cells after continuous exposure (24 hr) to various DMC structural analogs and structurally unrelated carbonyl scavengers was examined using the following test compounds: C: untreated control; *DMC* (10 mM); DMCSS (10 mM); NAC (10 mM); ADMC (10 mM); AG (25 mM). (B) Induction of apoptosis in human squamous cell carcinoma A431 cells after continuous exposure (24 hr) to DMC (1 and 10 mM) and DMCM (1 and 10 mM). (C) Induction of apoptosis in human malignant melanoma A375 cells and foreskin fibroblasts after continuous exposure (24 hr) to MEC (5 mM). C: untreated control. (D) Induction of apoptosis in G361 melanoma cells after continuous exposure (48 hr) to various carbonyl scavengers (25 mM). Apoptosis

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was detected by flow cytometric analysis of annexinV-FITC/propidium iodide (PI) stained cells. One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV^-, PI^-, lower left quadrant) in percent of total gated cells (mean ± SD, n=3).

Figure 4: **Carbonyl scavenger-induced apoptosis and loss of mitochondrial depolarization: suppression by RCS pretreatment.** G361 melanoma cells were exposed to *DMC* (10 mM) as described above and induction of apoptosis (panel A) and loss of $\Delta\Psi_m$ (panel B) were monitored after 18 hours using annexinV-PI and JC-1 flow cytometric analysis, respectively. *DMC*-induced apoptosis and $\Delta\Psi_m$ were also examined after pretreatment of G361 cells with the RCS phenylglyoxal (PG, 5 mM, 15 min). One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV^-, PI^-, lower left quadrant) in percent of total gated cells (mean ± SD, n=3).

Figure 5: RCS-suppression of staurosporine-induced apoptosis in G361 melanoma cells. G361 melanoma cells were exposed to staurosporine (STS, 200 nM) and induction of apoptosis was monitored after 24 hours using annexinV-PI staining (panel A) and forward (FSC)/sideward scatter (SSC) analysis (panel B) by flow cytometry. STS-induced apoptosis was also examined after pretreatment of G361 cells with the RCS PG (5 mM, 15 min). One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV^-, PI^-, lower left quadrant) in percent of total gated cells (mean ± SD, n=3).

Figure 6: Carbonyl scavenger-induced melanoma cell apoptosis: partial suppression by pore modulation using cyclosporine A. (A) Time course of *DMC*-induced apoptosis and loss of $\Delta\Psi_m$ in G361 human melanoma cells using annexinV-PI and JC-1 flow cytometric analysis, respectively. (B) *DMC*-induction of melanoma cell apoptosis was examined by annexinV-PI flow cytometric analysis after pretreatment with a specific inhibitor of MPT pore opening, cyclosporine A (CysA, 5 μM, 60 min). One representative experiment of three

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similar repeats is shown. The numbers indicate viable cells (AV^-, PI^-, lower left quadrant) in percent of total gated cells (mean ± SD, n=3).

Figure 7: A model of carbonyl scavenger interference with modulation of survival signaling by endogenous carbonyl stress in cancer cells. RCS from increased glycolytic flux are emerging small molecular inhibitors of apoptosis in cancer cells that potentially target MPT pore proteins, the cytochrome C antagonist Hsp 27, and other, currently unidentified molecular targets involved in apoptotic events. See discussion for detailed explanation.

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Fig. 1

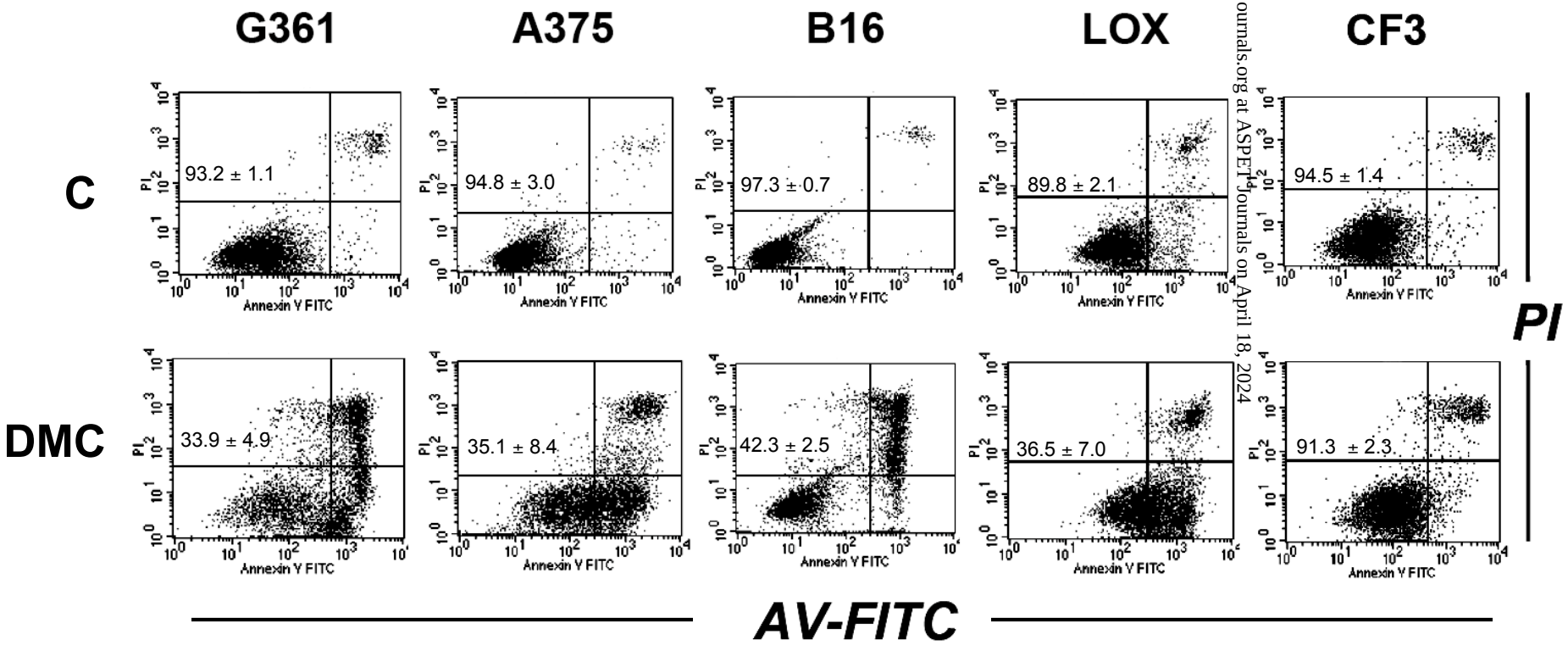

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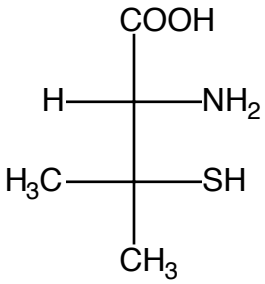
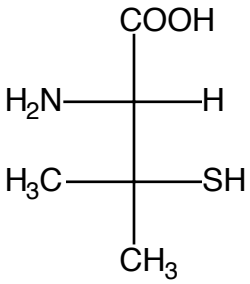
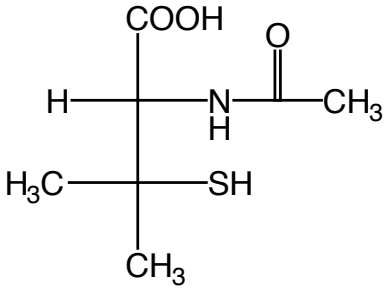
Fig. 2



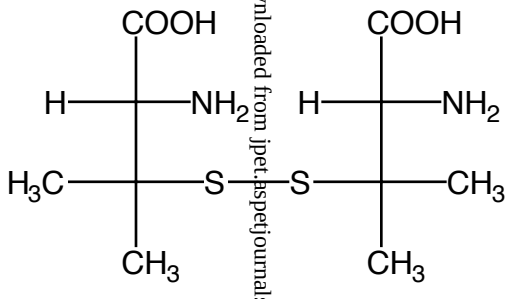
DMC



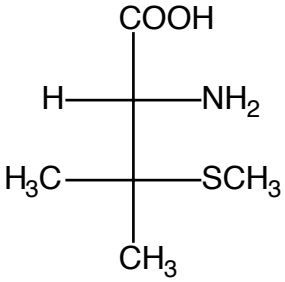
***L*-DMC**



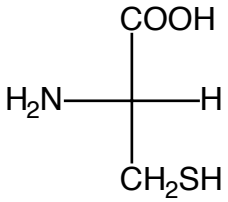
ADMC



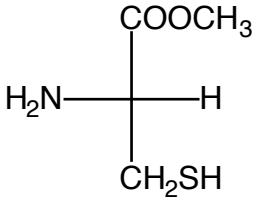
DMCSS



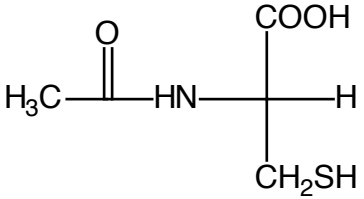
SMDMC



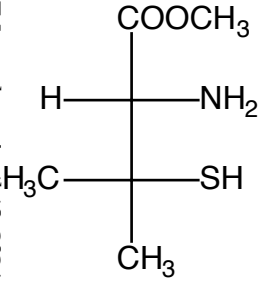
CYS



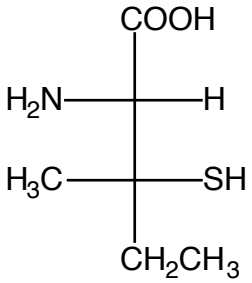
CYSM



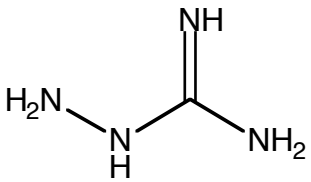
NAC



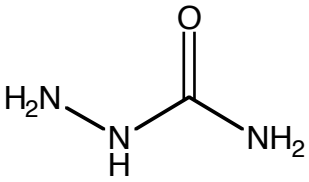
DMCM



MEC



AG



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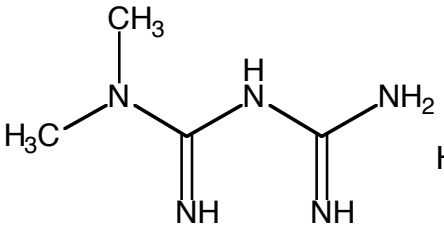
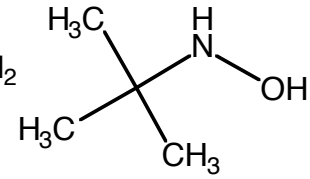


DMBG



BHA

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Fig. 3

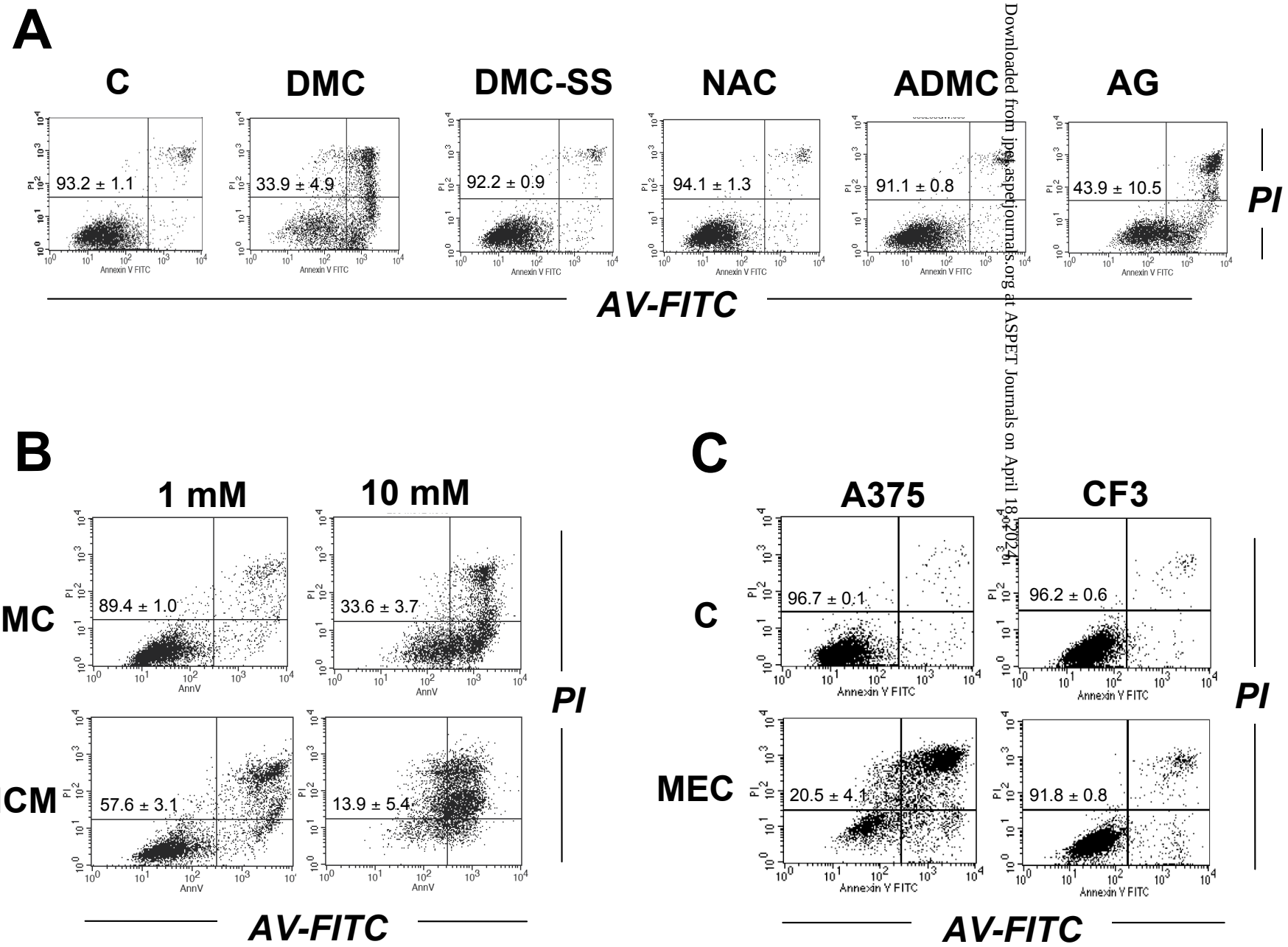

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Fig. 3

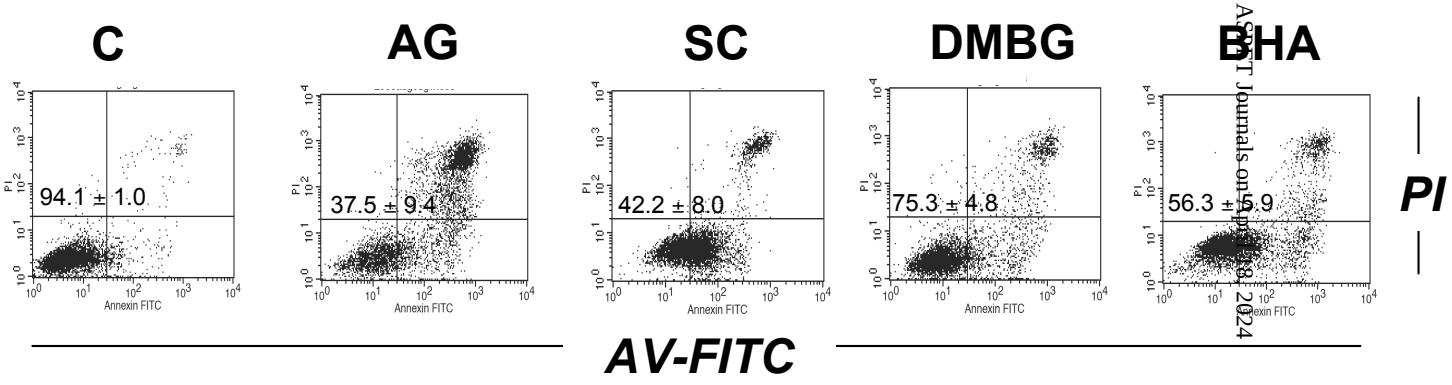

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Fig. 4

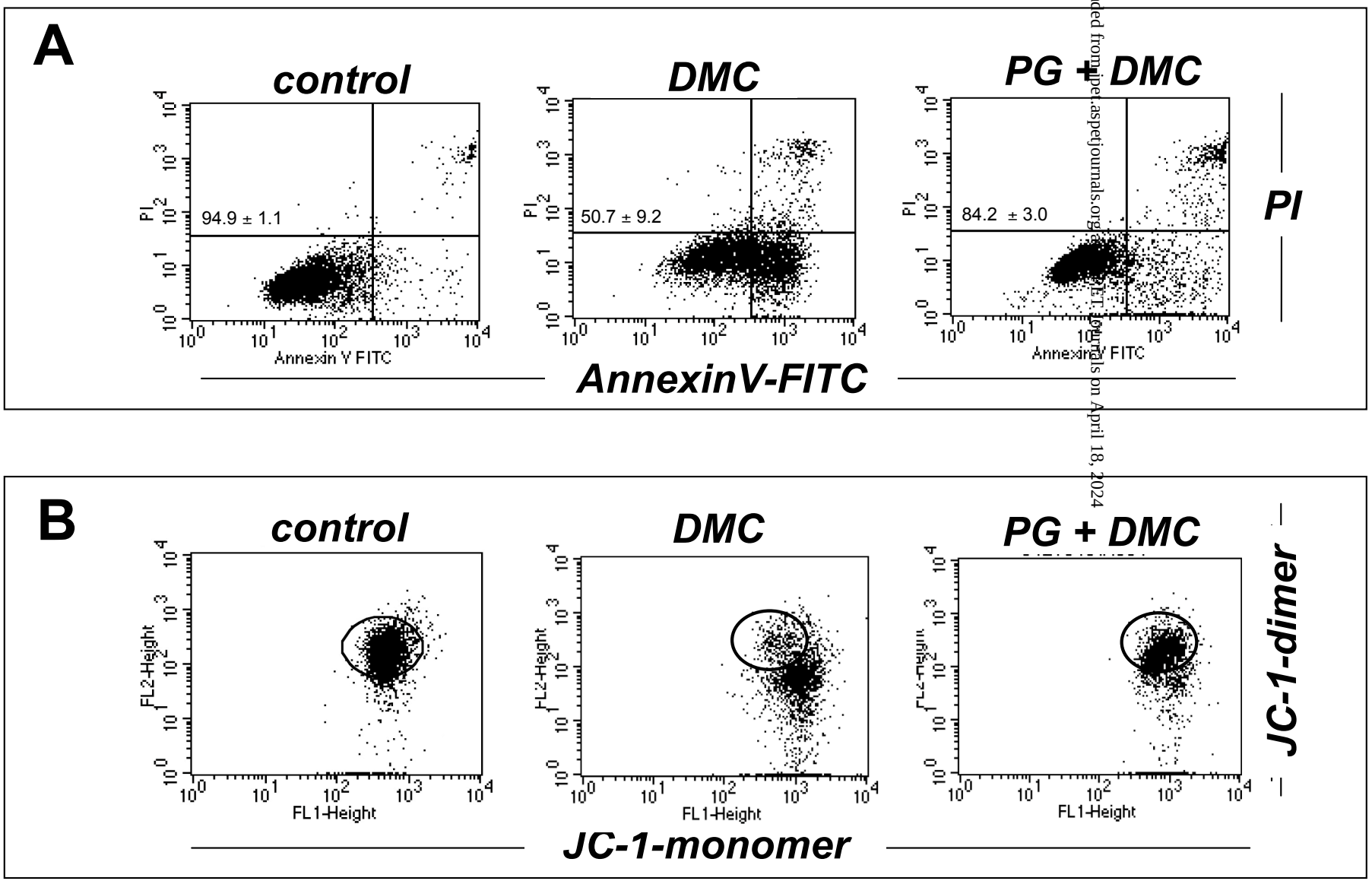

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Fig. 5

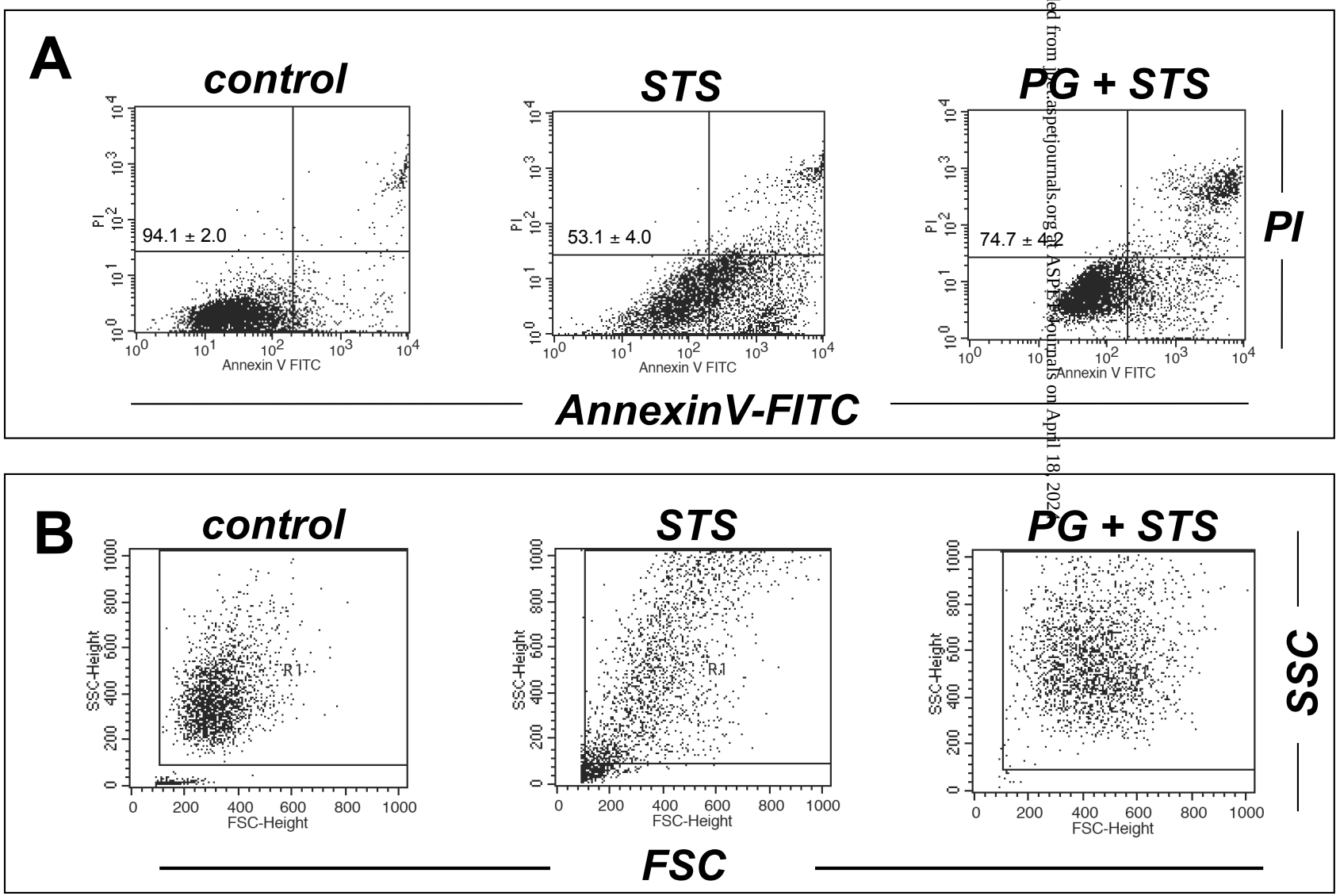

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Fig. 6

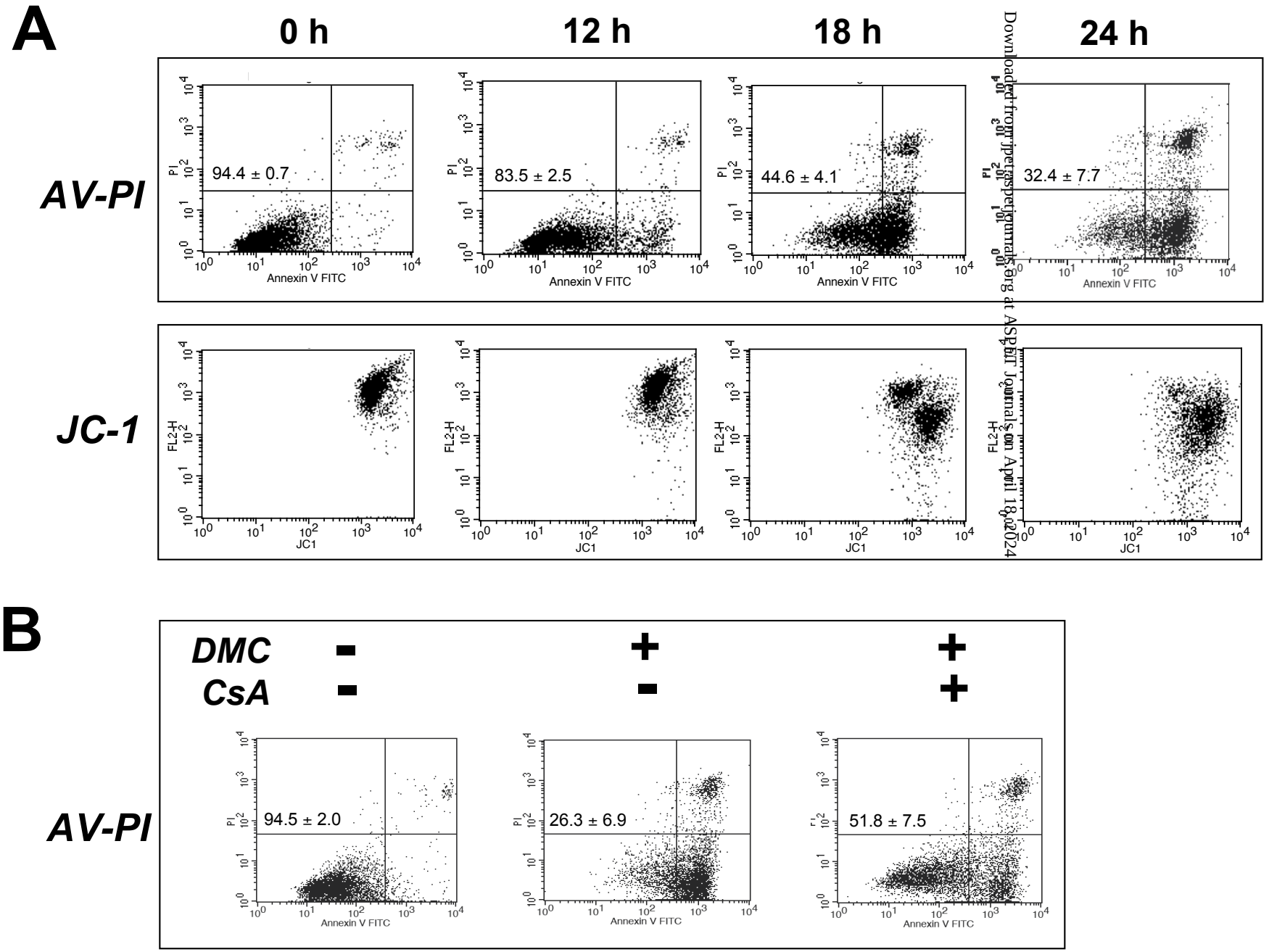

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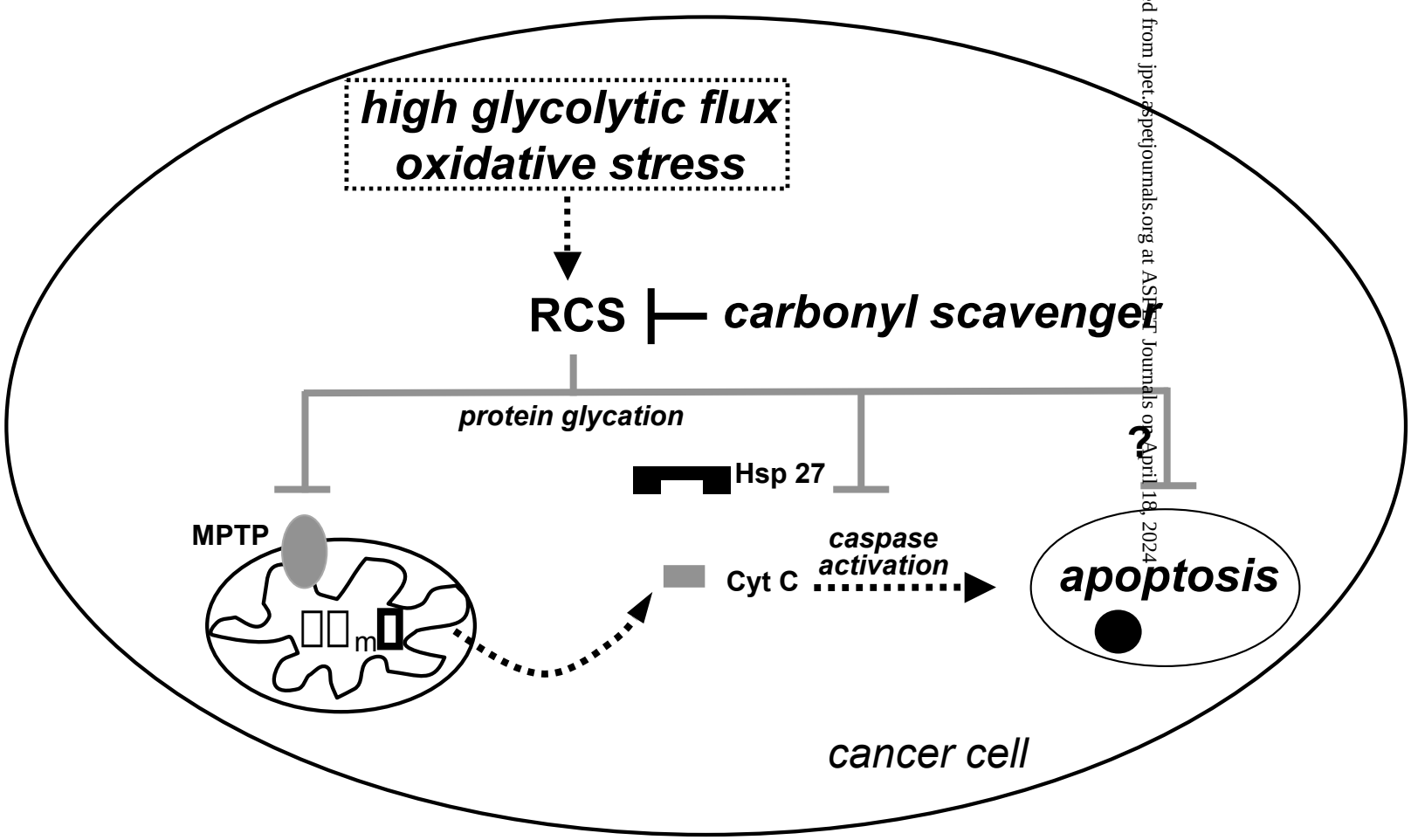
Fig. 7



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