Antimelanoma Activity of Apoptogenic Carbonyl Scavengers

Georg T. Wondrak, Myron K. Jacobson, and Elaine L. Jacobson

Department of Pharmacology and Toxicology, College of Pharmacy, Arizona Cancer Center, University of Arizona, Tucson, Arizona

Received August 30, 2005; accepted October 5, 2005

ABSTRACT
Therapeutic induction of apoptosis is an important goal of anticancer 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 antiapoptotic survival factors and thus represent a novel molecular target for anticancer intervention. Here, we demonstrate the tumor cell-specific apoptogenicity of carbonyl scavengers, which act by covalently trapping RCS, against human (A375, G361, and LOX) and murine (B16) melanoma cell lines. A structure-activity relationship study identified nucleophilic carbonyl scavenger pharmacophores as the functional determinants of apoptogenic antimalanoma activity of structurally diverse agents such as 3,3-dimethyl-α-cysteine and aminoguanidine. Previous 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 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, staurosponine-induced apoptosis also was suppressed by PG pretreatment. Our results suggest that carbonyl scavengers acting as direct molecular antagonists of RCS are promising apoptogenic prototype agents for antimelanoma drug design.

Carbonyl stress is an important mechanism of tissue deterioration in several pathological conditions such as diabetes, atherosclerosis, Alzheimer’s disease, 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 end products (AGEs) formed by chemical reactions between RCS and tissue proteins are abundant in melanoma tissue, and AGEs are potent ligands of receptor for advanced glycation end products, 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-receptor for advanced glycation end product 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 antiapoptotic effectors (Sakamoto et al., 2002; Speer et al., 2003; Johans et al., 2005). The α-dicarbonyl MG forms from glycolytic triose phosphates by spontaneous phosphate elimination.

ABBREVIATIONS: RCS, reactive carbonyl species; MG, methylglyoxal; AGE, advanced glycation end products; MPT, mitochondrial permeability transition; Hsp, heat shock protein; MEC, 3-methyl-3-ethyl-cysteine; BCS, bovine calf serum; FITC, fluorescein isothiocyanate; PI, propidium iodide; Δψm, mitochondrial transmembrane potential; JC-1, 5,5′,6,6′-tetrachloro-1′,1′,3′,3′-tetracyanobenzimidazolyl-carbocyanine iodide; PBS, phosphate-buffered saline; DMC, 3,3-dimethyl-α-cysteine; SAR, structure-activity relationship; ADMC, N-acetyl-3,3-dimethyl-α-cysteine; NAC, N′-acetyl-L-cysteine; DMCSS, 3,3-dimethyl-α-cysteine-dissulfide; AG, aminoguanidine; SC, semicarbazide hydrochloride; DMBG, 1,1-dimethylbiguanide hydrochloride; PG, phenylglyoxal; AV, Annexin V; DPCM, 3,3-dimethyl-α-cysteine-methylester.
tion (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 glycat ing agent leading to the post-translational modification of protein-arginine and lysine residues with formation of AGEs, such as arginine-pyrimidine (Shpanova et al., 1997), N-carboxyethyllysine (Ahmed et al., 1997), and the hydroximidazolone N\textsuperscript{4}(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (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 proapoptotic factors such as cytochrome c and apoptosis-inducing factor in response to apoptotic stimuli such as high Ca\textsuperscript{2+} and ganglioside GD3 (Speer et al., 2003; Johans et al., 2005). In various human cancer cell lines, post-translational MG-mediated arginine-pyrimidine formation occurs at a single arginine residue of Hsp 27 with induction of Hsp 27 oligomerization essential for repression of cytochrome c-mediated apoptosis assembly (Bruey et al., 2000). Given the involvement of mitochondrial membrane permeabilization and subsequent apoptosis 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 antiapoptotic survival signaling in cancer cells.

We have developed a high-throughput screen for the rapid identification of α-dicarbonyl scavengers as potential therapeutic agents to exert cellular protection against carbonyl stress by covalent trapping of RCS (Wondrak et al., 2002). Effective α-dicarbonyl scavengers identified in our screen contain two nucleophilic functional groups that trap α-dicarbonyls, such as MG and glyoxal, by stable covalent addition. Based on previous 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 antiapoptotic survival signaling, we evaluated the antimelanoma structural-activity relationship (SAR) of various thiol and nonthiol carbonyl scavenger pharmacophores. Here, we provide experimental evidence that certain carbonyl scavengers may represent a novel class of antimelanoma therapeutic agents.

Materials and Methods

Chemicals

Most chemicals were from Sigma-Aldrich (St. Louis, MO). DL-α-N-Acetyl-β-mercaptoisoleucine (N-acetyl-3-methyl-3-ethyl-L-cysteine) was from Aldrich Chemical Co. (Milwaukee, WI). Cyclosporin A was from Calbiochem (San Diego, CA). Staurosporine was from Toeris Cookson, Inc. (Ellisville, MO).

Chemical Synthesis

3,3-Dimethyl-D-cysteinemethylester. 3,3-Dimethyl-D-cysteine (15 g; 100.5 mmol) was added to a stirred solution of SOCl\textsubscript{2} (18 ml) in MeOH (75 ml), prepared and maintained at \(-10^\circ\text{C}\). 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 diethyl ether. 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 ([electrospray ionization positive] \textit{m/z} calculated for C\textsubscript{4}H\textsubscript{12}O\textsubscript{4}N\textsubscript{3}: 164.1 [M + H]+, observed 164.1] and \textit{1H NMR} ([250 MHz, CDCl\textsubscript{3}]: δ 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-L-lysine. The synthesis of 3-methyl-3-ethyl-L-lysine (MEL) was performed by acid hydrolysis of a commercially available precursor molecule (DL-N-acetyl-β-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 (electrospray ionization positive) \textit{m/z} was calculated for C\textsubscript{14}H\textsubscript{32}O\textsubscript{12}N\textsubscript{5}: 164.1 [M + H]+, observed 163.8.

Cell Culture

The established cell line of human epidermal keratinocytes (HaCaT cells), a gift from Dr. Norbert Fusenig (German 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, OK), were cultured in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum (BCS). Adult human skin keratinocytes (Cascade Biologics, Portland, OR) were cultured using EpiLife basal medium with human keratinocyte growth supplement from the same supplier. Human A431 squamous cell carcinoma cells were from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium containing 10% BCS with high glucose (30 mM). G361 human melanoma cells from American Type Culture Collection were cultured in McCoy’s 5a medium containing 10% BCS. A375, LOX, and B16 murine melanoma cells from American Type Culture Collection were cultured in RPMI 1640 medium containing 10% BCS and 2 mM L-glutamine.

Reaction Kinetics of α-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 previously (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 h later. Cells were harvested at various time points after treatment, and cell staining was performed using an apoptosis detection kit according to the manufacturer’s specifications (APO-AF; Sigma-Aldrich).

Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential (ΔΨ\textsubscript{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 ΔΨ\textsubscript{m}, JC-1 forms red fluorescent “J-aggregates” distinct from the green fluorescent monomer observed at low ΔΨ\textsubscript{m}. J-aggregate formation increases linearly with ΔΨ\textsubscript{m} over the range of 30 to 180 mV. In brief, cells were trypanpsinized, washed in PBS, resuspended in 300 μl of PBS containing 5 μg/ml JC-1 for 15 min at 37°C and 5% CO\textsubscript{2} in the dark, and then washed twice in PBS and resuspended in 300 μl of PBS. Bivariate analysis was performed by flow cytometry with excitation at 488 nm, and mitochondrial function was assessed as JC-1 green (depolarized mitochondria, de-
Apoptogenic Carbonyl Scavengers

Results

Apoptogenicity of the Carbonyl Scavenger DMC in Melanoma Cells. Accumulating evidence for a causative involvement of carbonyl stress in melanoma progression led us to examine the anti-intelamela activity of carbonyl scavengers that we had previously identified as potent inhibitors of cellular carbonyl stress (Wondrak et al., 2002). 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 (G361, A375, and LOX) and murine (B16) melanoma cells with more than 60% of cells undergoing apoptosis within 24 h 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 β-DMC.

Structure-Activity Relationship of Apoptogenicity. Based on our previous studies on screening and identification of α-dicarbonyl scavengers (Wondrak et al., 2002), 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 detailed in Fig. 3. In addition, to compare carbonyl scavenger potency of selected test compounds, reaction kinetics of α-dicarbonyl trapping [second order rate constants; \( k_{2nd} \pm S.D. \) (molar\(^{-1}\) second\(^{-1}\))] were determined as published previously (Wondrak et al., 2002). The absolute configuration was not critical for apoptogenic activity since DMC \( (k_{2nd} = 24.9 \pm 4.2 \text{ M}^{-1} \text{s}^{-1}) \), the β-isomer used in all further experiments) and its L-isomer (L-DMC) were equally apoptogenic (data not shown). Because DMC also is a powerful thiol antioxidant and antioxidants can exert apoptogenic effects on malignant cells (Havre et al., 2002), structural analogs 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; \( k_{2nd} = 5.2 \times 10^{-3} \pm 2.9 \times 10^{-3} \text{ M}^{-1} \text{s}^{-1} \)) and the closely related thiol antioxidant N\(^{\prime}\)-acetyl-L-cysteine (NAC). As shown previously, both ADMC and NAC are devoid of potent carbonyl scavenger activity because of 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). In addition, the S-methylated derivative S-methyl-DMC was devoid of apoptogenic activity (data not shown). As demonstrated previously, L-cysteine \( (k_{2nd} = 0.63 \pm 0.06 \text{ M}^{-1} \text{s}^{-1}) \) and L-cysteine-O-methylester are only weak carbonyl scavengers because of the absence of dialkyl substituents adjacent to the thiol group (“geminal dialkyl effect”; Jung and Gervay, 1991), important for efficient carbonyl trapping by thiazolidine ring formation (Wondrak et al., 2002), and they 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 [DMCM; \( k_{2nd} = 28.8 \pm 2.3 \text{ M}^{-1} \text{s}^{-1} \); Fig. 3B] or the alkyl-substituent MEC \( (k_{2nd} = 33.1 \pm 1.5 \text{ M}^{-1} \text{s}^{-1} \); 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 en-

![Fig. 1. Preferential induction of apoptosis by DMC in malignant melanoma cell lines. Induction of apoptosis was examined in malignant human (G361, A375, and LOX) and murine (B16) melanoma cell lines and human skin fibroblasts (CF3) after continuous exposure (24 h) to DMC (10 mM). Top, untreated control (C). Bottom, DMC-treated cells. Apoptosis was detected by flow-cytometric analysis of Annexin V-FITC/PI-stained cells with early apoptotic and late apoptotic/necrotic cells located in the bottom right (AV\(^{-}\), PI\(^{+}\)) and top right quadrant (AV\(^{-}\), PI\(^{+}\)), respectively. One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV\(^{-}\), PI\(^{−}\), bottom left quadrant) in percentage of total gated cells (mean \( \pm S.D. \); n = 3).](image-url)
hanced apoptogenicity toward 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; therefore, increased potency could result from higher membrane penetration and/or intracellular carbonyl scavenger activity. To further test the hypothesis that apoptogenic antimelanoma potential of our test compounds is dependent on carbonyl scavenger activity, structurally unrelated nonthiol carbonyl scavengers were examined for apoptogenicity against melanoma cells as shown in Fig. 3, A 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; \( k_{2nd} = 0.02 \) M \( s^{-1} \)) (Thornalley et al., 2000), although higher doses were needed than for DMC-induced apoptosis. As observed above with DMC, AG treatment did not induce apoptosis in CF3 human skin fibroblasts (data not shown). It is noteworthy that melanoma cell apoptosis was also observed upon exposure to other bifunctional nucleophilic carbonyl scavengers, such as semicarbazide hydrochloride (SC; \( k_{2nd} = 5.3 \times 10^{-2} \pm 0.3 \times 10^{-2} \) M \( s^{-1} \)) (Lehman and Ortworht, 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 antimelanoma activity was also observed with the nucleophilic hydroxylamine derivative N-tert-butylhydroxylamine, 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 1) activity as carbonyl scavenger (DMC, L-DMC, DMCM, MEC, AG, SC, and DMBG) but not as thiol antioxidant (NAC and ADMC) is a determinant of antimelanoma activity; 2) synthesis of simple derivatives of the lead compound DMC resulted in enhanced apoptogenic potency toward malignant cells, and 3) 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 Hsp 27 oligomerization, 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 \( \Delta \psi_m \) 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 antiapoptotic modulators (Speer et al., 2003).

**Suppression of Staurosporine-Induced Apoptosis by RCS Pretreatment.** To test whether exogenous carbonyl stress could interfere with the 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 stauro-
sporine-induced apoptosis was examined in G361 human melanoma cells. Cells were pretreated with PG (5 mM; 30 min) followed by 24 h of continuous exposure to staurosporine (200 nM). Pronounced protection against staurosporine-induction of apoptosis was 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

Fig. 3. SAR of apoptogenicity. A, induction of apoptosis in G361 melanoma cells after continuous exposure (24 h) 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); and AG (25 mM). B, induction of apoptosis in human squamous cell carcinoma A431 cells after continuous exposure (24 h) 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 h) to MEC (5 mM). C, untreated control. D, induction of apoptosis in G361 melanoma cells after continuous exposure (48 h) to various carbonyl scavengers (25 mM). Apoptosis was detected by flow-cytometric analysis of Annexin V-FITC/PI-stained cells. One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV−, PI−; bottom left quadrant) in percentage of total gated cells (mean ± S.D.; n = 3).
apoptosis, and carbonyl scavengers could overcome this RCS inhibition of apoptosis in cancer cells.

**Suppression of Carboxyl Scavenger-Induced Apoptogenicity by the MPT Pore Inhibitor Cyclosporine A.**

To further elucidate a potential role of Δψm breakdown during carbonyl scavenger-induced apoptosis, the time course of induction of apoptosis and loss of Δψm upon continuous exposure to DMC were compared as shown in Fig. 6A. Appearance of cells in early apoptosis (AV-positive, PI-negative; bottom right quadrant) after 12 h and progression into later stages of cell death (AV-positive, PI-positive; upper right quadrant) occurred in synchrony with the loss of Δψm, suggesting an involvement of Δψm 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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm breakdown in MPT-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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm 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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm 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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm 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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm 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. CysA inhibits MPT pore opening by interaction with the pore constituent cyclophilin D and has been widely used as a sensitive molecular probe for the involvement of Δψm breakdown in DMC-induced apoptosis.

**Discussion**

Recently, we have examined α-dicarbonyl scavengers as a novel class of compounds for therapeutic intervention in cellular carbonyl stress (Wondrak et al., 2002; Roberts et al., 2003). Here, we report the apoptogenic activity of various carbonyl scavenger pharmacophores, such as α-amino-β,β-dialkyl-β-mercaptopo-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 previous 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 antimelanoma activity was performed (Figs. 2 and 3). Molecular reactivity as a bifunctional nucleophilic carbonyl scavenger was revealed as the structural determinant of apoptogenic antimelanoma activity of
various test compounds (DMC, t-DMC, DMCM, MEC, AG, SC, and 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 [14C]lysine-protein cross-linking (Lehman and Ortwerth, 2001) (DMC > AG > SC > DMBG), demonstrating a correlation between carbonyl scavenger potency and apoptogenic activity. It is noteworthy that 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 antimelanoma 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, a Food and Drug Administration-approved drug in clinical use as a copper ion chelator for the management of Wilson’s disease (Cuprimine; Merck, Whitehouse Station, NJ), 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 caspase activation (Bruey et al., 2000; Sakamoto et al., 2002). Evidence for covalent MG modification of an adenine nucleotide translocator-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 Hsp 27 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, and 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 glycolytic 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

**Fig. 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 h using Annexin V–PI staining (A) and forward (FSC)/sideward scatter (SSC) analysis (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−; bottom left quadrant) in percentage of total gated cells (mean ± S.D.; n = 3).
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).

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). It is noteworthy that 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). Previous work has demonstrated that RCS, including MG, glyoxal, and the synthetic cell-permeable MG analog 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, because 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 previous 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 molecular inhibitors of apoptosis that potentially target proteins, such as the MPT pore protein adenine nucleotide translocator (Johans et al., 2005), the cytochrome c antagonist Hap 27 (Sakamoto et al., 2002), and probably other unidentified mo-

---

**Fig. 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 \(\Delta \psi \) in G361 human melanoma cells using Annexin V-PI and JC-1 flow-cytometric analysis, respectively. B, DMC induction of melanoma cell apoptosis was examined by Annexin V-PI flow-cytometric analysis after pretreatment with a specific inhibitor of MPT pore opening, CysA (5 \(\mu\)M; 60 min). One representative experiment of three similar repeats is shown. The numbers indicate viable cells (AV\(^{-}\), PI\(^{-}\); bottom left quadrant) in percentage of total gated cells (mean \(\pm\) S.D.; \(n = 3\)).
l cellular 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 addition of crucial protein targets. Therapeutic induction of apoptosis is an important goal of anticancer drug design (Qin et al., 2005). The apoptotic activity of carbonyl scavengers presented in this study raises the possibility that novel antianimal 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 protomic 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.

Acknowledgments
Flow cytometric analysis was performed at the Arizona Cancer Center flow cytometry laboratory. Chemical synthesis was performed at the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, University of Arizona, Tucson, AZ). Carbonyl scavenger kinetic data were determined with technical assistance by M. J. Kimzey.

References


Address correspondence to: Dr. Georg T. Wondrak, Arizona Cancer Center, University of Arizona, 1515 North Campbell Ave., Tucson, AZ 85724. E-mail: wondrak@pharmacy.arizona.edu