ASSAY DEVELOPMENT FOR THE PHARMACOKINETIC STUDY OF A NOVEL NON-MELANOMA SKIN CANCER DRUG

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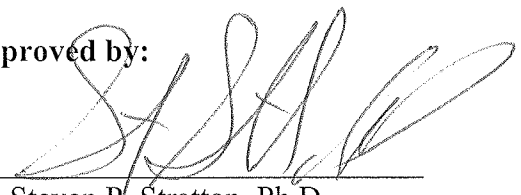

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Abstract

Non-melanoma skin cancer (NMSC) currently accounts for about half of all cancer diagnoses (American Cancer Society, 2008). There is a pressing need for a topical treatment as an alternative to surgical removal. Here we examined a novel drug that therapeutically attacks cancer cells specifically by targeting the unique metabolic state of tumors. High glycolytic flux in tumors (Warburg Effect) creates more intermediates of glycolysis than the cell normally handles. Methylglyoxal, a highly reactive di-carbonyl species, is one of the main byproducts of glycolysis (Thornalley, 1996). Methylglyoxal has recently been identified in an alternate pathway as an inducing factor of cell survival. Under elevated, sub-toxic concentrations, methylglyoxal reacts with two proteins important in cell apoptosis: a portion of the multi-protein Mitochondrial Permeability Transition Pore, and Heat-Shock Protein 27 (Speer et. al., 2003; Sakamoto et. al., 2002). Dialkylcysteine (DAC) is able to scavenge methylglyoxal and has been shown to selectively induce apoptosis in cancer cells (Wondrak et. al., 2006). To analyze topical DAC treatment, a GC-MS assay capable of detecting the drug in *ex vivo* hairless mouse skin and aqueous solvent was developed. DAC can be esterified to create derivatives of differing hydrophobicity; ester derivatives will display different diffusion properties through skin based on the alkyl chain length. A quantitative GC-MS assay will allow a pharmacokinetic study that will elucidate the DAC derivatives most suitable for topical treatment.

Introduction

Non-melanoma skin cancer (NMSC)—including the common Basal Cell and Squamous Cell Carcinomas—is the most common type of malignancy in the United States, accounting for about half of all cancer diagnoses (American Cancer Society, 2008). While the mortality rate associated with NMSC is lower than other types of cancer, its prevalence presents a major health and economic concern (Stratton, 2001). Currently, NMSC is most commonly treated by surgically removing the cancerous lesion. While this approach is typically effective in removing the primary tumor, it is costly and is accompanied by cosmetic draw-backs (Zbar and Canady, 2008). Several topical treatments, including chemotherapeutic 5-fluorouracil and Imiquamod, offer an adjunct to surgical removal, but are accompanied by undesirable side effects including intense inflammation, pain, erythema, and ulceration (Barrera and Herrera, 2007). Therefore, there is a pressing need for a topical therapeutic that targets tumor cells more directly with maximal efficacy and fewer side effects.

One approach to therapeutically attack cancer cells specifically involves targeting the unique metabolic state of tumors. Tumor cells exhibit an increased rate of anoxic metabolism through glycolysis, and a correspondingly decreased rate of oxidative phosphorylation in mitochondria. This phenomenon, termed the Warburg Effect, occurs even when oxygen is prevalent (Pedersen, 2007). Increased glycolysis (glycolytic flux) creates more intermediates of glycolysis than the cell normally handles. Methylglyoxal (MG), a highly reactive di-carbonyl species (RCS), is one of the main byproducts of glycolysis (Thornalley, 1996). Methylglyoxal reacts with and modifies nucleic acids and proteins (specifically at arginine, lysine, and cysteine residues) to form Advanced Glycation End-Products (Lo et. al., 1994). MG is therefore usually very cytotoxic, and cells express a system of detoxifying enzymes (Glyoxalase I and II) that convert MG and other RCS into useful metabolites (Mannervik, 2008).

However, MG has recently been identified in an alternate pathway as an inducing factor of cell survival. Under elevated, sub-toxic concentrations, MG reacts with arginine residues in two proteins important in cell apoptosis—a portion of the multi-protein Mitochondrial Permeability Transition Pore (MPTP), and Heat-Shock Protein 27 (Hsp27) (Speer et. al., 2003; Sakamoto et. al., 2002). MG modification of the MPTP induces a closed-pore conformation, preventing the release of cytochrome C and other apoptotic signaling factors, leading ultimately to cell survival (Speer et. al., 2003); and MG modification of Hsp27 allows the protein—which normally functions in dimers or tetramers—to form oligomers which inhibit pro-apoptotic caspase release, also leading to cell survival (Sakamoto et. al., 2002). The role of MG as both a mutagen and a modulator of apoptosis make it a key target for therapeutic treatments. Trapping MG and other RCS would potentially prevent mutation and block the cancer cell-survival pathway; so-called "carbonyl scavenger" compounds that bind to and mitigate the effects of MG should therefore be useful in preventing tumor growth and metastasis, and in treating existing tumors (Figure 1).

Emerging data suggests that a novel chemotherapeutic derivative of the amino acid cysteine selectively induces apoptosis in cancer cells (Wondrak et. al., 2006). Previous studies have found the most effective "carbonyl scavenger" compounds to be organic molecules with a pharmacophore consisting of a carbonyl, an alpha amino, and a

beta thiol group (Wondrak et. al., 2006). The reactivity of such compounds with RCS is greatly increased when the beta carbon is substituted with two alkyl groups, due to the "geminal dialkyl effect" (Jung and Gervay, 1991). The nucleophilic amino of the pharmacophore attacks a carbonyl carbon of the RCS, leading to a thiazolidine ring formation and deactivation of the RCS anti-apoptotic functionality (Wondrak et. al., 2002). Dialkylated cysteine (DAC) exhibits this pharmacophore and can be readily synthesized. Previous studies have demonstrated the efficacy of DAC treatment *in vitro* on Melanoma and Non-Melanoma keratinocyte cell cultures (Wondrak et. al., 2006).

Dialkylated cysteine (DAC) provides the necessary apoptotic functionality, and can be modified through esterification and long-chain alkyl substitution to provide derivatives with suitable hydrophobic properties for diffusion through skin tissue. It should therefore be possible to develop a DAC derivative with suitable properties for use in a topically applied cream. The main considerations when identifying the optimal DAC derivative are: the ability of the compound to partition into the skin; and the amount of time the compound is retained in the skin.

A quantitative assay was developed to measure these parameters in a time-course study. The optimal alkyl chain length can be determined experimentally by conducting an *ex vivo* pharmacokinetic study of the corresponding DAC derivatives. A skin penetration system with hairless mouse skin as a barrier has been used in such pharmacokinetic studies and provides a good model for diffusion of a drug compound through full thickness skin (Dvorakova et. al., 1999). This method allows for pharmacokinetic analysis of dermal absorption and percutaneous flux of topically delivered drugs. Using Gas Chromatography – Mass Spectrometry, a highly sensitive method to measure DAC and DAC ester derivatives in skin has been developed.

Materials and Methods

2.1 Materials

DAC, DAC ethyl ester, and N-acetyl-D-penicillamine were kindly supplied by Dr. Georg Wondrak, Ph.D. (Arizona Cancer Center). Female SKH-1 hairless mice were obtained from Charles River Laboratories. The silylating agent MTBSTFA (N-tert-Butyldimethylsilyl-N-methyl-trifluoroacetamide) was obtained from Sigma-Aldrich (Fluka). All solvents used were HPLC grade.

2.2 Instrumentation and Conditions

A Gas Chromatography – Mass Spectrometry (GC-MS) system consisting of a Trace GC coupled to a DSQ Mass Spectrometer (Thermo Electron Co.) was used for sample analysis. Solution volumes of 1uL were injected via an AS3000 Auto-Sampler (Thermo Electron Co.) and run through a DB1 Capillary Column (30m length by 0.25mm internal diameter, J&W Scientific) with Helium as the carrier gas (1.5mL/min flow rate). The following GC temperature profile was used: 180^{o}C hold for 4 minutes; ramp to 280^{o}C over 20 minutes then hold for 2 minutes; ramp to 300^{o}C over 40 seconds and hold until 30 minutes total run time elapsed. Full scans in the positive ion mode were collected from 5.0 to 20.0 minutes using electron-impact ionization. To further increase sensitivity, a specific ion monitoring (SIM) program was used as follows:

Retention Time (min)	Peaks Analyzed (m/z)
5.0 – 10.0	115, 189, 216
10.0 – 13.0	189, 214, 362
13.0 – 15.0	115, 189, 302

All analyte solutions were prepared in silanized 1.5mL glass vials (Alltech). Vials were silanized prior to use by treatment with dichloromethylsilane, followed by washes with toluene and methanol.

2.3 Derivatization Optimization

Silyl derivatization of analytes by the addition of either trimethylsilyl (TMS) or *t*-butyldimethylsilyl (TBDMS) groups increases the volatility of the analyte and therefore the sensitivity of the GC-MS assay (Pierce, 2003-2004). Drug derivative samples were reacted with MTBSTFA (N-tert-Butyldimethylsilyl-N-methyl-trifluoroacetamide) to create volatile and stable *t*-butyl dimethylsilyl derivatives that could be analyzed with the DB-1 column. Many procedures of this commonly used derivatization are available; here, variations of three published conditions were tested on DAC (the parent drug compound). Standard solutions were prepared in silanized vials by diluting DAC from a 1ug/uL stock solution to a total volume of 50uL. Volumes of stock added were adjusted to create a final [DAC] = 300ng/uL in the injected sample. Blank solutions were prepared by adding 50uL H_2O to silanized vials. The solutions were then dried completely under a gentle nitrogen stream.

First, one blank solution and one DAC solution were derivatized with the following conditions: 100uL pyridine was added to re-dissolve the dried residue; derivatization was carried out by adding 60uL MTBSTFA and heating at 75°C for 30 minutes (Woo and Chang, 1993). Second, one blank solution and one DAC solution were re-dissolved in 50uL acetonitrile, followed by addition of 50uL MTBSTFA and 60 minutes heating at 80°C (Wood et. al., 2006). Finally, one blank solution and one DAC solution were re-dissolved in 50uL acetonitrile, followed by addition of 50uL MTBSTFA and 30 minutes heating at 60°C (Deng et. al., 2002). Derivatized samples were analyzed by GC-MS using the full mass spectrum method.

The original procedure outlined by Woo and Chang used triethylamine to raise the pH of the derivatization mix. The effect of triethylamine was examined on two sets of dried-down standard DAC solutions with concentrations of 0ng/ul, 148ng/ul, and 247ng/ul. One set was derivatized by re-dissolving in 100uL pyridine, adding 60uL MTBSTFA, and heating for 30 minutes at 60°C. The second set was derivatized under the same conditions with the addition of 8uL triethylamine to the reaction mix. Derivatized samples were analyzed by GC-MS in the full-scan mode.

2.4 Standard Solutions and Calibration Curve

Aqueous and organic stock solutions of DAC and DAC ethyl ester were prepared by dissolving solid drug compound in H_2O and pyridine to a final concentration of 1ug/uL. Aqueous standards were stored at 4°C for up to two weeks, and organic standards were stored at -20°C for up to two weeks. Standard solutions were diluted to obtain a concentration range of 20-200ng/ul.

From the aqueous DAC ethyl ester stock, three standard curves were prepared on each of two days. Stock solution was dried under a nitrogen stream, and the residue was re-dissolved in 150uL pyridine with 50ng/uL internal standard (N-acetyl-D-penicillamine). Derivatization was carried out by adding 50uL MTBSTFA and heating at 75°C for 30min. From the organic DAC ethyl ester stock, three standard curves were prepared. Stock solution was diluted with pyridine to the appropriate concentration following the addition of internal standard to a concentration of 40ng/uL. Derivatization was completed as above. Samples were analyzed by GC-MS using SIM. The average (product peak area / internal standard peak area) ratio was plotted vs. concentration of DAC ethyl ester.

2.5 System Suitability

Two standard solutions of DAC (parent drug compound) and DAC ethyl ester (a drug derivative) were prepared by drying 20uL stock solution (1ug/uL in H_2O) under a nitrogen stream. The residue was re-dissolved in 150uL pyridine with 50ng/uL internal standard (N-acetyl-D-penicillamine). Derivatization was carried out by adding 50uL MTBSTFA and heating at 75°C for 30min. To evaluate system suitability six consecutive injections of the same sample were made with the same equipment, on the same day.

2.6 Extraction Optimization

To assess the pharmacokinetic behavior of DAC derivatives through skin, an optimized procedure for extracting drug from homogenized skin samples was needed. Skin was surgically excised from SKH-1 hairless mice and cut into sections of approximately 200-300mg each. Skin patches were incubated for 24 hours in 340uL aqueous solution with [DAC ethyl ester] = 2.5ug/uL. After incubation, the treatment solution was removed and the skin was cleaned with ethanol wipes to remove excess drug from the surface. Skin patches were weighed, homogenized with mortar and pestle under liquid nitrogen, and reweighed to determine recovery.

Extraction from skin homogenate was tested by incubating with micro stir bars for 4 hours in 1mL of the following solvents: MilliQ H_2O (pH 4.7); neutral H_2O (pH 7); 9:1 ethyl acetate / THF; and isopropyl alcohol. Samples were then centrifuged for 5 minutes at 10,000rpm, and the supernatant was aliqoted and stored at -80°C. A two-phase extraction procedure was evaluated as well. Skin homogenates were first extracted as previously with both 1mL MilliQ H_2O and 1mL neutral H_2O. Each aliqoted supernatant was then added to an equal volume hexanes or diethyl ether so that all four aqueous/organic combinations were tested. The two-phase mix was vortexed rigorously and centrifuged for 5 minutes at 10,000rpm. The aqueous layer was then aliquoted and stored at -80°C. Extraction solutions were processed by drying 200uL under a nitrogen stream and re-dissolving the residue in 50uL pyridine. Samples were derivatized by adding 150uL MTBSTFA and heating for 30 minutes at 75°C, and assayed for the presence of DAC ethyl ester by GC-MS using the SIM method. Note that a higher dosage of MTBSTFA was necessary in skin samples to account for derivatization of excess amino acids and other organic molecules found in skin.

A quantitative analysis of drug uptake was performed on hairless mouse skin samples excised as previously outlined. Skin patches were prepared in triplicate and incubated for 20 hours in 1mL of a 5ug/uL solution of DAC ethyl ester in H_2O to ensure

maximal drug absorption into skin layers. The samples were homogenized and extracted with MilliQ H_2O, as previously outlined. Aliquoted extraction solutions were processed by drying 50-200uL under a nitrogen stream and re-dissolving the residue in 100uL pyridine with 40ng/ul internal standard. Samples were derivatized by adding 100uL MTBSTFA and heating for 30 minutes at 75°C, and analyzed by GC-MS using the SIM method. The average (product peak area / internal standard peak area) ratio was used to determine the concentration of DAC ethyl ester in the extract from the organic DAC ethyl ester standard curve (*Section 2.4*).

Results

Derivatization Conditions

Derivatized DAC ethyl ester eluted between 9.4 and 9.5 minutes with characteristic peaks at mass / charge ratios (m/z) of 115, 189, and 216; the internal standard (N-acetyl-D-penicillamine) at 12.2 minutes with peaks at m/z 189, 214, and 362; and DAC at 14.1 minutes with peaks at m/z 115, 189, and 302 (Figure 3). The analytical peaks of both DAC and DAC ethyl ester were well resolved from the internal standard, allowing quantitative analysis by integration of peak area. Optimal instrument response to DAC occurred when derivatization was carried out in pyridine without triethylamine, followed by heating at 75°C for 30 minutes (Table 1).

System Suitability and Linearity

To determine system suitability, six consecutive injections of DAC and DAC ethyl ester standard solutions were made on each of two separate days. Quantitation was performed by evaluating the peak area ratio of the analyte to the internal standard. Precision was measured by calculating the coefficient of variation (C.V.) for each day and between days.

$$C.V. = \frac{Std.Dev.}{Avg.}$$

Successive injections of DAC yielded increasing peak area ratios, with a C.V. of 20.4% and 6.2% on the first and second day, respectively. Successive injections of DAC ethyl ester also yielded increasing peak area ratios, with a C.V. of 28.2% and 25.7% on the first and second day, respectively (Table 2).

Statistical analysis of linearity is outlined in Table 3. A calibration curve prepared from aqueous DAC ethyl ester standards dried under nitrogen before derivatization was constructed in triplicate on two separate days. Linearity of each standard set was evaluated by averaging the three data points at each concentration and fitting to a line. The R^2 values were 0.9458 and 0.8003 on the first and second day, respectively. Comparison of the slopes gave a C.V. of 20.2%. A calibration curve prepared in triplicate from organic DAC ethyl ester standards derivatized directly without drying under nitrogen was also evaluated. The corresponding R^2 value was 0.9606.

Extraction Conditions

Of the single-phase extraction conditions evaluated, MilliQ H_2O was the only solvent to recover DAC ethyl ester. No peak at 9.4 minutes was observed in the

chromatograms of neutral H_2O (pH 7), 9:1 ethyl acetate / THF, or isopropyl alcohol extraction mixes (data not shown). Also, no peak at 9.4 minutes was observed in any of the aqueous layers following a two-phase extraction (data not shown). Therefore, a single phase extraction with MilliQ H_2O was used in quantitative experiments.

Quantitative Analysis

Three skin samples were extracted and aliquoted as outlined in the methods section 2.6. From each extraction aliquot, volumes of 50uL, 100uL, and 200uL were analyzed. The concentration of DAC ethyl ester in each derivatization mix was determined from the standard curve with the equation: *integration ratio = 0.008[DAC ethyl ester (ng/ul)]*. The expected concentration of drug in the original extraction mix was back-calculated using the volumes of the extraction mix (200uL) and the aliquot volume dried down:

$$[DAC.et.est.]_{extraction\_mix} = [DAC.et.est.]_{derivatization\_mix} \cdot \frac{Vol_{deriv-mix}(uL)}{Vol_{dry-down}(uL)}$$

Calculated concentrations were normalized between samples by dividing by the mass of homogenized skin recovered prior to extraction. The C.V. between the three dry-down volumes was 48.1% and 47.2% for the first and third skin samples, respectively. The 100uL and 200uL aliquots of the second skin sample yielded no peaks corresponding to DAC ethyl ester (Table 4).

Discussion

Method Development

Here, a novel method of monitoring transcutaneous absorption of a novel drug through full-thickness skin is presented. The GC-MS assay is capable of detecting the ethyl ester drug derivative, and should work similarly for esters of various chain length. However, the procedure outlined does not give highly quantitative results. High variability between repeated injections and between replicated standard sets (demonstrated by C.V. between 6.2% and 28.2%) poses an analytical problem that must be addressed before a quantitative pharmacokinetic study can commence. The high C.V. of the calculated concentrations in skin extraction mixes (42.8% to 48.1%) could be a result of system variability as well as further variability in the extraction procedure. Additionally, measurement of esterified DAC compounds in skin samples is complicated by the action of esterase enzymes in the skin. A significant peak at 14.19 minutes in skin extraction samples (Figure 4) indicates that the parent compound (DAC) was present in the extraction mix. This esterase activity could be quite variable from sample to sample, and quantitation of DAC will require a separate analytical calibration curve.

Further method development will include a more exhaustive study of alternate extraction solvents. MilliQ H_2O was the most effective solvent evaluated thus far, likely because DAC ethyl ester is amphipathic due to the ethyl chain but still fairly polar due to the amino, thiol, and carbonyl groups. Additionally, a polar solvent has the benefit of minimizing the amount of fats, lipids and other hydrophobic molecules partitioned into the extraction mix. The two-phase extraction was devised to remove excess lipids and other non-polar molecules in a second extraction step; however, if samples are analyzed

with an SIM method this step is not crucial as almost all extraneous peaks are minimized (Figure 4). Also, while MilliQ H_2O has proven to be an effective extraction solvent, it complicates derivatization procedures. The silylating reagent, MTBSTFA, works only in an anhydrous solvent, and any water in the derivatization mixture can degrade the reagent and reaction products (Pierce, 2003-2004). Drying the aqueous extraction mixture under nitrogen is therefore crucial, yet it is hard to monitor and ensure the complete removal of water.

Future Direction

Once an analytical assay is developed, a pharmacokinetic study using a skin penetration system (Dvorakova et. al., 1999) will allow the selection of a set of optimal drug derivatives. Extraction and quantitation of drug content in skin samples at various time points will demonstrate the ability of each derivative to partition into the skin, and will allow calculation of the resonance time of each derivative. Additionally, flux of drug through the skin can be measured by analyzing the receptor fluid at each time point. Either water or PBS is used in the receptor compartment, and detection of drug from an aqueous solution has been demonstrated in this report.

After selecting the drug derivatives with optimal pharmacokinetic properties, an *in vivo* study will be conducted to evaluate the efficacy of the treatment and any systemic side effects. Non-melanoma skin cancer can be induced in SKH-1 hairless mice by acute exposure to UVB radiation (Bair III et. al., 2002). Mice will be irradiated until tumors form and treated with either drug or vehicle (placebo) to determine the therapeutic effect. Experimental groups will also be treated prior to exposure with either drug or vehicle to assess any preventative effect. Tumors will be measured by volume, incidence, and multiplicity before and after treatment and exposure periods; and regression will be analyzed quantitatively. Topical treatment of mouse model UVB-induced tumors with the selected DAC derivatives should result in: tumor growth delay and regression when existing tumors are treated; and slowing of tumor formation when the treatment is applied prior to UV exposure.

Perspectives

There is a pressing need for a topical therapeutic that targets tumor cells more directly with maximal efficacy and fewer side effects. Several topical treatments offer an alternative to surgical removal, but are accompanied by other shortcomings and undesirable side effects. The most common topical treatment, chemotherapeutic 5-fluorouracil, blocks DNA synthesis by inhibiting the enzyme thymidylate synthase; but this non-specific inhibition of quickly-dividing cells produces an intense local inflammatory reaction that often prevents compliance with prolonged treatment. Alternatively, Imiquimod exhibits anti-tumor activity by stimulating the local release of cytokines that stimulate immune response; but the treatment often causes pain, erythema, ulceration, edema, and desquamation (Barrera and Herrera, 2007).

Carbonyl scavenger drugs offer an advantage over these existing treatments because they target tumor metabolism specifically. However, unlike chemotherapeutics that induce cell death in quickly dividing cells, carbonyl scavengers do not directly kill cancer cells. Instead, they are designed to re-open an apoptotic pathway that is blocked due to the accumulation of methylglyoxal. Nevertheless, this action alone could block

cancer progression and reverse tumor growth. Epithelial cancer cells are in a highly active metabolic state, and survive and divide quickly despite numerous mutations. This metabolic hyperactivity could be very stressful to cells, but they are able to evade death because some apoptotic pathway has been bypassed. By trapping methylglyoxal, the intrinsic apoptotic pathway (through mitochondrial signaling) can be reactivated, and metabolically stressed tumor cells could then undergo apoptosis.

Topical drug delivery is an optimal system for treating a tumor locally. Systemic drug delivery through the blood via an oral or intravenous treatment is very inefficient in targeting Non-Melanoma Skin Cancer because Squamous and Basal cells are non-vascular (Jacobson et. al., 2005). Esterification of the DAC parent compound produces more lipophilic derivatives that will theoretically partition more optimally into the skin. The main barrier to topical drug delivery is the stratum corneum, the keratinized outermost layer made up of dead skin cells (Anderson et. al., 1988). Previous studies with the Non-Steroidal Anti-Inflamatory naproxen have demonstrated that carboxyl group esterification of a polar drug compound can increase dermal penetration (Rautio et. al., 2000). Such studies have relied on skin esterases to convert the esterified "prodrug" to the active therapeutic. However, the carboxylic hydroxyl group of DAC does not appear to be involved in the scavenging g activity (Wondrak et. al., 2006). DAC ester derivatives therefore have the potential to be highly efficient—with the proper esterification they will partition well into the skin, and both the esterified drug and the parent compound liberated by esterases should be pharmacologically active.

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Figures

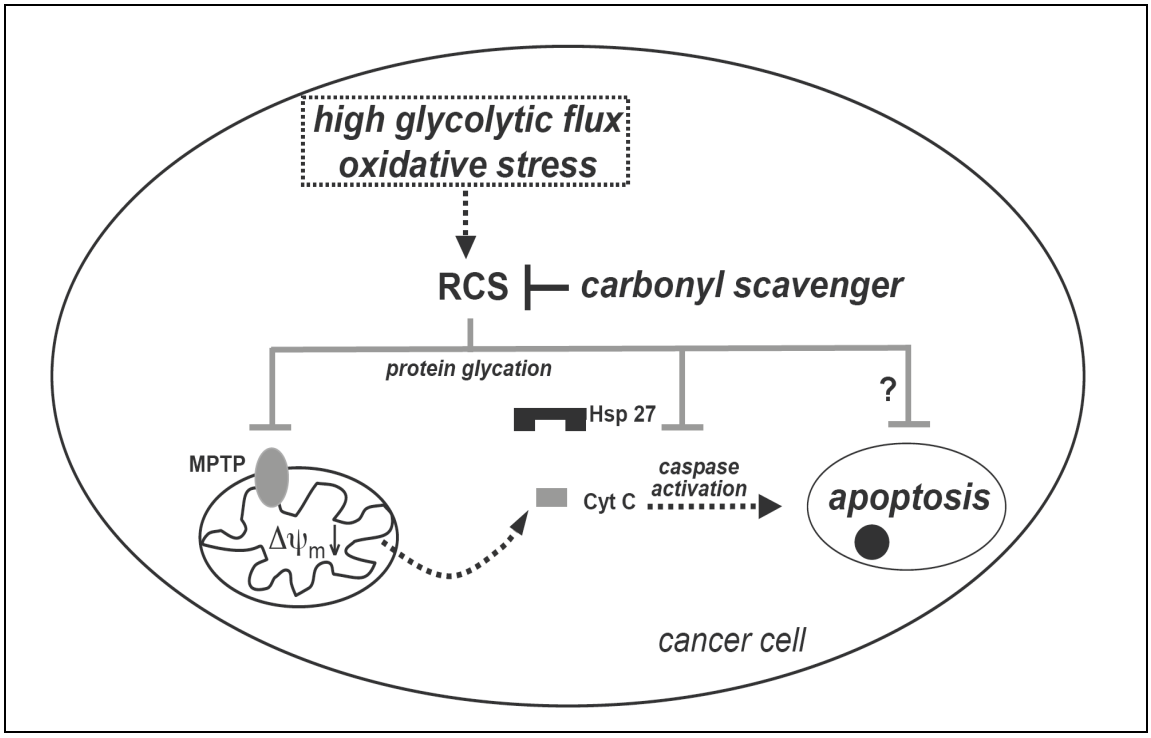

Figure 1.

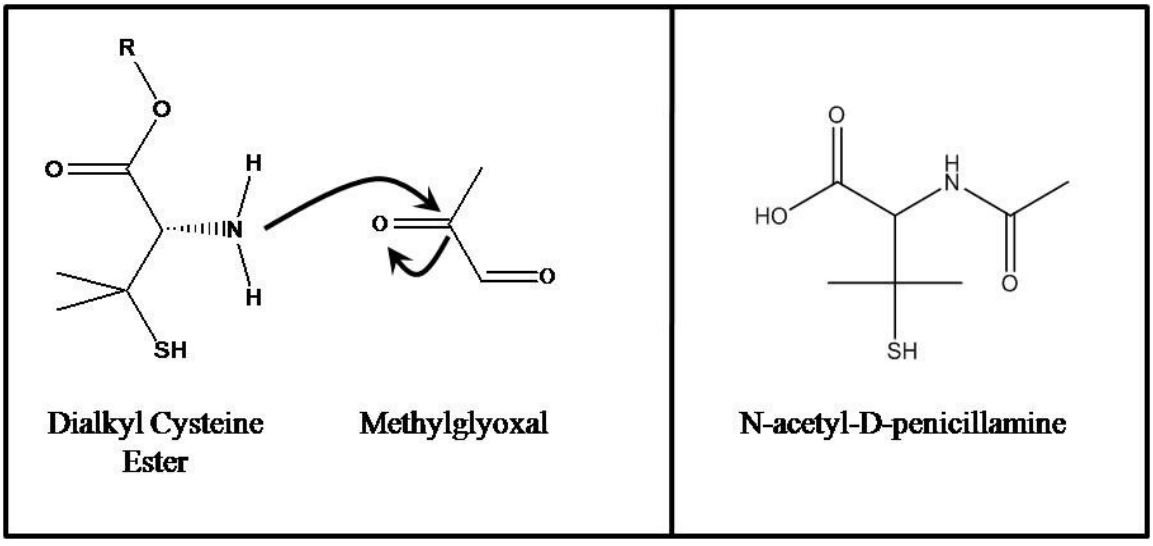

Figure 2.

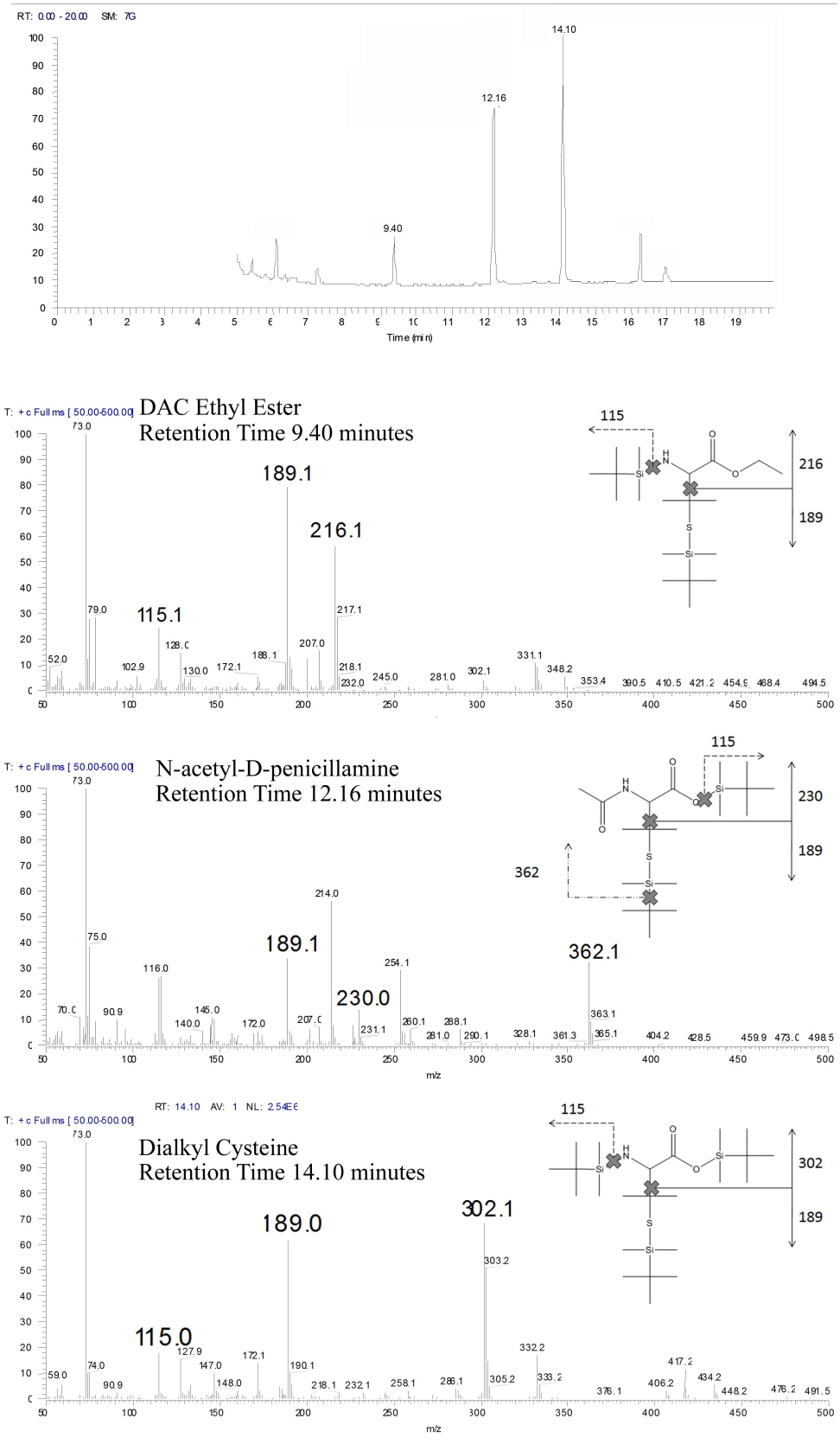
RT: 0.00 - 20.00 SM: 7G
14.10
12.16
9.40
Time (min)
T: + c Full ms [50.00-500.00]
DAC Ethyl Ester
Retention Time 9.40 minutes
189.1
216.1
115.1
115
216
189
T: + c Full ms [50.00-500.00]
N-acetyl-D-penicillamine
Retention Time 12.16 minutes
189.1
362.1
230.0
214.0
115
230
189
362
m/z
RT: 14.10 AV: 1 NL:
T: + c Full ms [50.00-500.00]
Dialkyl Cysteine
Retention Time 14.10 minutes
189.0
302.1
115.0
115
302
189
m/z

Figure 3.

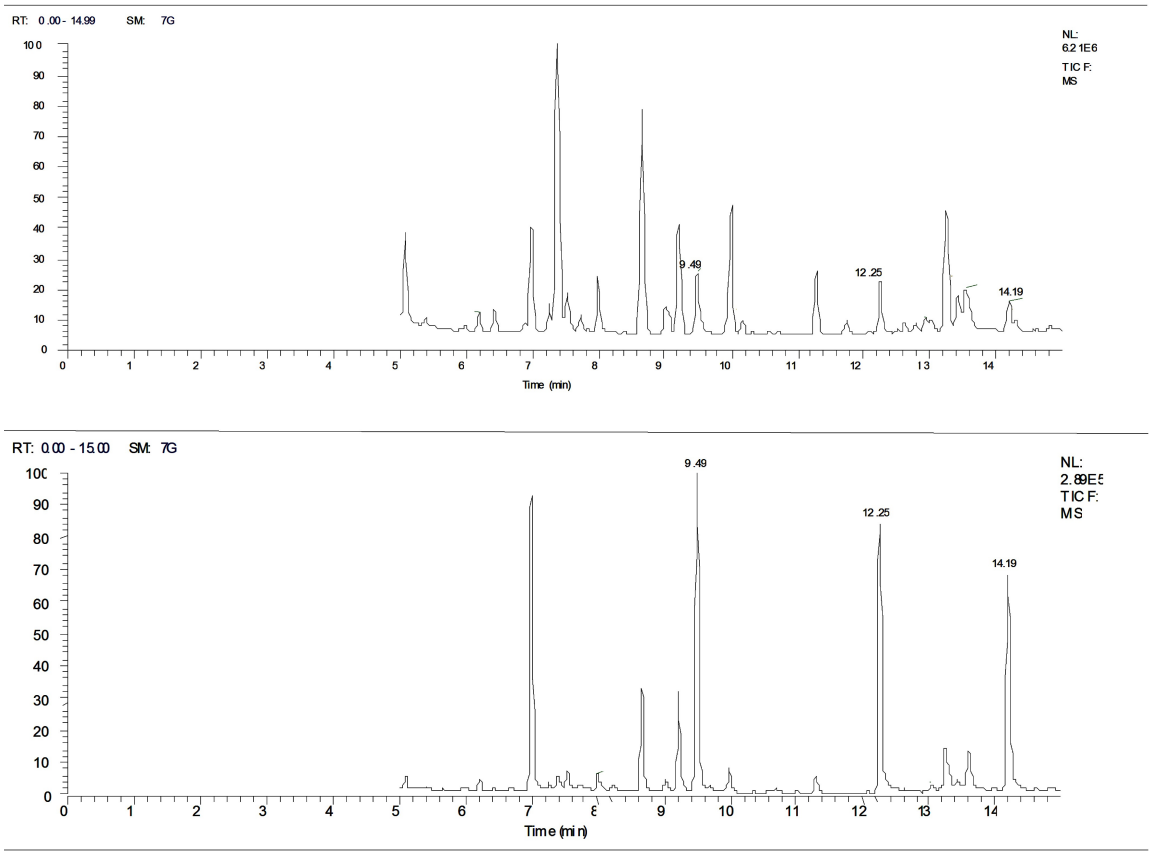
RT: 0.00 - 14.99 SM: 7G
NL:
6.21E6
TIC F:
MS
9.49
12.25
14.19
Time (min)
RT: 0.00 - 15.00 SM: 7G
NL:
TIC F:
MS
9.49
12.25
14.19
Time (min)

Figure 4.

Table 1 Derivatization Optimization. Optimal instrument response to DAC occurred when derivatization was carried out in pyridine without triethylamine, followed by heating at 75°C for 30 minutes..

Derivatization Conditions				
Conditions	**A**	**B**	**C**	**D**
Rxn Temperature	75	75	80	60
Rxn Time (min)	30	30	60	30
Acetonitrile (uL)	0	0	50	50
Pyridine (uL)	100	100	0	0
MTBSTFA (uL)	60	60	50	50
triethylamine (uL)	8	0	0	0
GC-MS Analysis				
Product Peak				
Retention Time (min)	9.32	9.35	9.36	9.35
Peak Integration Area	6.50 E6	1.21 E8	6.82 E7	1.79 E7
Peak Intensity	8.79 E5	6.77 E6	4.27 E6	1.08 E6

Table 2. System Suitability.

DAC *(parent compound)*				
Day	**N**	**Analyte Peak / Standard Peak (area %)**	**S.D.**	**C.V. (%)**
1	6	2.30	0.47	20.4%
2	6	4.49	0.28	6.2%
Inter-Day				
Mean	3.40			
S.D.	1.20			
C.V. (%)	35.3%			
DAC ethyl ester				
Day	**N**	**Analyte Peak / Standard Peak (area %)**	**S.D.**	**C.V. (%)**
1	6	0.20	0.06	28.2%
2	6	0.48	0.12	25.7%
Inter-Day				
Mean	0.34			
S.D.	0.17			
C.V. (%)	51.5%			

Table 3. Standard Curve Linearity. A calibration curve prepared from: (a) aqueous DAC ethyl ester standards dried under nitrogen before derivatization, constructed in triplicate on two separate days; and (b) from organic DAC ethyl ester standards.

Part (a) Dried down standards.

Day	N	Slope	R^2
1	3	0.006	0.9458
2	3	0.0045	0.8003
	S.D.	0.0011	
	C.V.	20.2%	

Part (b) Pyridine dissolved standards.

	N	Slope	R^2
	3	0.008	0.9606

Table 4. Quantitative Extraction Recovery.

Sample	**Volume Dried (ul)**	**Normalized [DAC Et Est] (ng/ul per mg skin)**
1	50	2.75
	100	1.15
	200	3.45
S.D.		1.18
C.V.		48.1%
2	50	4.41
3	50	4.59
	100	2.37
	200	1.98
S.D.		1.41
C.V.		47.2%
Overall		
Average		2.96
S.D.		1.27
C.V.		42.8%

Legends

Figure 1. Mitochondrial apoptotic signaling and the role of reactive carbonyl species and carbonyl scavengers. Reactive Carbonyl Species (RCS) block apoptotic signals by preventing the Mitochondrial Permeability Transition Pore (MPTP) from opening, and by causing Hsp27 to oligomerize. Carbonyl scavenging compounds can trap methylglyoxal, allowing these apoptotic pathways to become functional.

Figure 2. Chemical structures and proposed scavenging activity of esterified dialkyl cysteine against methylglyoxal. The nucleophilic amino of the pharmacophore attacks a carbonyl carbon of the RCS, leading to thiazolidine ring formation and deactivation of the RCS anti-apoptotic functionality (Wondrak et. al., 2002). The internal standard (N-acetyl-D-penicillamine) has a very similar structure to the parent DAC compound.

Figure 3. Chromatogram and full scan mass spectrum of DAC ethyl ester, DAC, and internal standard. Samples were injected into a Trace GC coupled to a DSQ Mass Spectrometer (Thermo Electron Co.) and run through a DB1 Capillary Column (J&W Scientific) with Helium as the mobile phase. Elution of the three compounds was well separated. Derivatized DAC ethyl ester eluted at 9.40 minutes with characteristic peaks at mass / charge ratios of 115, 189, and 216; the internal standard (N-acetyl-D-penicillamine) at 12.16 minutes with peaks at 189, 214, and 362; and DAC at 14.10 minutes with peaks at 115, 189, and 302. The fragmentation patterns corresponding to these peaks are displayed.

Figure 4. Full scan and SIM spectra of skin extraction. Extraction solutions were dried under a nitrogen stream and re-dissolved in 100uL pyridine with 40ng/ul internal standard. Samples were derivatized by adding 100uL MTBSTFA and heating for 30 minutes at 75^{o}C, and analyzed by GC-MS using full scan and SIM methods. Peaks corresponding to the drug compound (DAC ethyl ester at a retention time of 9.49 minutes) and the internal standard (N-acetyl-D-penicillamine at a retention time of 12.25 minutes) were well-resolved using the SIM method.