**RESISTANCE TO VESICANT INJURY BY EFFLUX TRANSPORTERS**

By

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

And

The Graduate School of Biomedical Sciences

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Toxicology

Written under the direction of

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New Brunswick, New Jersey

October 2015

ABSTRACT OF THE DISSERTATION

Resistance to Vesicant Injury by Efflux Transporters

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Sulfur mustard and nitrogen mustard (mechlorethamine, HN2) are potent vesicants used in chemical warfare and cancer chemotherapy that primarily target skin, eye, and lung. These electrophilic, bifunctional alkylating agents cause oxidative stress and persistent tissue damage. Toxicity of related mustards, chlorambucil and melphalan, is limited by clearance from cells by multidrug resistance-associated protein 1 (MRP1/Mrp1), a transmembrane ATPase that stimulates efflux of glutathione-conjugated electrophiles. HN2 causes injury by covalently modifying biomolecules including glutathione. Monofunctional glutathione adducts contribute to cytotoxicity or are exported by MRP1/Mrp1. In A549 lung epithelial cells, which express MRP1 and MRP2, HN2 inhibits growth ($IC_{5o}$ = 0.18 μM), and inhibition of MRPs by MK-571 increases sensitivity to HN2 ($IC_{50}$ = 0.045 μM). Similar effects are seen for other bifunctional mustards chlorambucil and melphalan. Using human embryonic kidney (HEK) 293 cells overexpressing MRP1 and MRP2, we determined that MRP1 provides resistance to HN2 while MRP2 does not protect the cells. HN2 inhibited MRP functional activity in both A549 cells and HEK cells overexpressing MRP1, and increased sensitivity to growth inhibition induced by MRP1/Mrp1 substrates etoposide, methotrexate, and vincristine in A549 cells. PAM212 cells and primary mouse keratinocytes express Mrp1 mRNA and protein. Activation of the transcription factor Nrf2 by sulforaphane increased Mrp1

mRNA, protein expression and activity in PAM212 cells and protected cells against HN2-induced growth inhibition ($IC_{50}$ = 1 and 13 µM without and with sulforaphane, respectively). This protection was reversed by MK-571 ($IC_{50}$ = 0.63 µM). Sulforaphane increased Mrp1 mRNA and protein expression and activity and decreased HN2 growth inhibition in primary keratinocytes ($IC_{50}$ = 1.4 and 4.8 µM without and with sulforaphane, respectively). This attenuation was reversed by MK-571 ($IC_{50}$ = 0.27 µM). MK-571 increases HN2-induced cytotoxicity in primary mouse keratinocytes (growth inhibition $IC_{50}$ = 1.4 and 0.48 µM, without and with MK-571, respectively). Sulforaphane did not protect keratinocytes from $Nrf2^{-/-}$ mice ($IC_{50}$ = 0.31 and 0.14 µM without and with sulforaphane, respectively). These data show MRP1/Mrp1-mediated efflux is important for regulating HN2 injury. Inhibiting MRP1/Mrp1 may increase mustard efficacy in cancer chemotherapy, while enhancing transport may represent a promising route to mitigate vesicant-induced cytotoxicity.

DEDICATON

To my parents Gary and Iris, who never failed to encourage me to be intellectually curious but also to be well-rounded and pursue interests outside of science. As well as of course for their financial support all these years.

To my sister Sharon and brother-in-law Ravid, for always being there for me and also encouraging my interest in Israel. And also to their cat Moshie for being completely crazy.

ACKNOWLEDGEMENTS

I would like to acknowledge the many contributions of my mentor and advisor, Dr. Jeffrey Laskin both to the success of this project and to my personal success as a researcher. His guidance has been indispensible in my development. I would also like to thank Dr. Lauren Aleksunes for always being there for advice and being willing to allow me to use various aspects of her laboratory even though I was not technically her student. I would also like to acknowledge Dr. Michael Gallo, not only for his scientific mentorship but really for his personal mentorship throughout my entire life. It is always enlightening to have conversations where the topics range from multidrug resistance transporters to ice hockey to marathon running to the aryl hydrocarbon receptor (and of course, to Ray Lucas). I would like to acknowledge the important contributions to the other members of my thesis committee, Dr. Marion Gordon and Dr. Grace Guo.

Thanks also to all members of our laboratory, past and present. Special thank you to Dr. Karma Fussell for teaching me all of the basics of laboratory work, including teaching me how to use a pipette my first day. Thank you to Dr. Wendy Yun Wang, Dr. Shaojun Yang, Dr. Ruijin Zheng for always being there to give me advice including translating some Chinese papers for me, and Dr. Yi-Hua Jan for always being willing to provide advice to help with experiments. Thank you to John Szilagyi for helping me with some experiments and always being able to listen to my ideas, as well has for his helpful and thorough review of this document. Especially thank you to Dr. Vladimir Mishin for always being willing to share his vast knowledge of HPLC systems that helped with a variety of experiments.

I would like to acknowledge Dr. Debra Laskin for contributing many ideas and always being willing to review my work and provide advice. I would also like to thank

Alessandro Venosa for helping me refine some presentations, as well as Dr. Carol Gardner for her valuable advice.

I would also like to acknowledge Dr. Michael Shakarjian for sharing his knowledge of the culture of mouse keratinocytes and all his help maintaining those cells, as well as his advice on various aspects of my project. Thank you to members of the Aleksunes laboratory, especially Kristin Bircsak for always being willing to answer my stupid questions when I first started working with transporters, and to Dr. Xia Wen, especially for helping my with qPCR experiments, as well as Jamie Moscovitz. Also, special thanks to Dr. Ned Heindel and Dr. Christophe Guillon of his laboratory for always being there and being able to answer all of my chemistry questions. Thank you to Dr. Joshua Gray for always being there with advice whenever I needed.

I would also like to thank the contribution of the EOHSI support staff, past and present: Bernardine Chmielowicz, Liz Rossi, Eva Link, Sandy Selby, Toni Myers, Kristin Borbely, Wilson Rodriguez, and Mitchell Gayer in helping me navigate the bureaucracy of the university. Dr. Kenneth Reuhl and Dr. Jason Richardson were also immensely helpful in achieving this aim, as well as with their classroom teaching throughout the years.

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ABBREVIATIONS

| | |
|---|---|
| A549 | human lung tumor cell line |
| ABC | ATP-binding cassette |
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| BCA | bicinchoninic acid |
| Bcrp | breast cancer resistance protein (non-human) |
| BeWo | human placental choriocarcinoma |
| bimane-GS | bimane (Pyrazolo[1,2-a]pyrazole-1,7-dione) conjugated to glutathione |
| cDNA | complimentary DNA |
| CEES | chloroethyl ethyl sulfide |
| CHOP | cancer chemotherapeutic regimen consisting of cyclophosphamide, doxorbucin (hydroxydaunorubicin), vincristine (oncovin), and presdnisone |
| $CO_2$ | carbon dioxide |
| $C_t$ | threshold cycle |
| DMEM | Dulbecco's modified Eagle's medium |
| ECL | electrochemiluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| ethacrynic acid | [2,3-dichloro-4-(2-methylenebutanoyl)phenoxy]acetic acid |
| FBS | fetal bovine serum |
| G418 | geneticin, *O*-2-Amino-2,7-didesoxy-D-glycero-α-D-gluco-heptopyranosyl-(1→4)-*O*-(3-desoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl- (1→6))-D-streptamin |
| Gapdh | glyceraldehyde 3-phosphate dehydrogenase (nonhuman) |

| | |
|---|---|
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase (human) |
| GSH | glutathione, reduced |
| GST | glutathione *S*-transferase |
| GSTA1 | glutathione *S*-transferase A1 |
| GS-X | glutathione *S*-conjugated to a xenobiotic |
| $H_2O_2$ | hydrogen peroxide |
| HBSS | Hank's balanced salt solution |
| HEK | human embryonic kidney-293 |
| HEK control | human embryonic kidney-2903 transfected with empty pcDNA (control for MRP1 transfection) or pCMV6-NEO (control for MRP2 and MDR1 transfections) |
| HEK MDR1 | human embryonic kidney-293 overexpressing MDR1 |
| HEK MRP1 | human embryonic kidney-293 overexpressing MRP1 |
| HEK MRP2 | human embryonic kidney-293 overexpressing MRP2 |
| HeLa | human cervical cancer cell line |
| HepG2 | human transformed hepatocyte cell line |
| HN2 | nitrogen mustard, mechlorethamine, methylbis(2-chloroethyl)-amine |
| Ho-1 | heme oxygenase-1 (non-human) |
| hr | hours |
| $IC_{50}$ | concentration at which cell growth or calcein efflux is half of control value |
| Keap1 | Kelch-like ECH-associated protein 1 |
| $K_M$ | Michaelis-Menten kinetic constant |
| MCF7 | human breast cancer cell line |
| Mdr1 | multidrug resistance protein 1 or P-glycoprotein (non-human) |

| | |
|---|---|
| MDR1 | multidrug resistance protein 1 or P-glycoprotein (human) |
| min | minutes |
| mg | milligrams |
| miRNA | micro RNA |
| MLE15 | mouse lung epithelial transformed cell line |
| MK-571 | 5-(3-(2-(7-Chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid |
| mL | milliliters |
| mM | millimolar |
| Mrp1 | multidrug resistance-associated protein 1 (nonhuman) |
| MRP1 | multidrug resistance-associated protein 1 (human) |
| Mrp2 | multidrug resistance-associated protein 2 (nonhuman) |
| MRP2 | multidrug resistance-associated protein 2 (human) |
| Mrp3 | multidrug resistance-associated protein 3 (non-human) |
| MRP3 | multidrug resistance-associated protein 3 (human) |
| Mrp4 | multidrug resistance associated protein 4 (non-human) |
| MTX | methotrexate |
| NAD(P)H | nicotinamide adenine dinucleotide (phosphate) |
| Nqo1 | NAD(P)H dehydrogenase [quinone 1] (non-human) |
| Nrf2 | nuclear factor (erythroid derived 2)-like 2 or NFE2L2 |
| $Nrf2^{-/-}$ | Nrf2 knockout |
| nM | nanomolar |
| $O_2^-$ | superoxide anion |
| oltipraz | 4-methyl-5-(2-pyrazinyl)-3-dithiolethione |
| $P_i$ | inorganic phosphate |
| PAM212 | mouse epidermal keratinocyte cell line |

| | |
|---|---|
| PCR | polymerase chain reaction |
| pmoles | picomoles |
| PSC833 | Valspodar, 6-[(2*S*,4*R*,6*E*)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A |
| qPCR | quantitative real-time reverse transcriptase polymerase chain reaction |
| RNAi | RNA interference |
| RNA pol II | RNA polymerase 2 |
| Rpl13a | 60S ribosomal protein L13a |
| SEM | standard error of the mean |
| siRNA | small interfering RNA |
| SFN | $_{D,L}$-sulforaphane |
| SLC | solute carrier |
| SNP | single nucleotide polymorphism |
| TMD | transmembrane domain |
| tris | tris(hydroxymethyl)aminomethane, THAM |
| tTBS | tris-buffered saline supplemented with 0.1% Tween 20 |
| VCR | vincristine (Oncovin) |
| $V_{max}$ | maximal rate of transport |
| $V_{max}/K_M$ | apparent efficiency of transport |
| VP-16 | etoposide |
| WT | wild-type |
| µM | micromolar |

## SUMMARY AND RATIONALE

Nitrogen mustard (HN2) is a bifunctional, nonspecific alkylating agent with the potential to be used as a chemical warfare agent with very few countermeasures. Recent evidence suggests that structurally similar compounds chlorambucil and melphalan may be exported from cells via ATP-dependent efflux pumps MRP1/Mrp1 and MRP2/Mrp2. More generally, these proteins are known to facilitate the export of electrophiles conjugated by glutathione. At present, the export of HN2 from cells has not been well-characterized. We hypothesize that like many other electrophiles, HN2 can be conjugated with glutathione and transported out of cells by MRP1/Mrp1 and MRP2/Mrp2. If glutathione-conjugated HN2 can act as a ligand for MRP/Mrp transporters, this characterization would help achieve a greater understanding of the molecular mechanism of action of HN2. Knowledge of the process by which HN2 is exported from cells may also lead to breakthroughs in developing new countermeasures against HN2 toxicity because these transporters can be induced through the Nrf2 pathway. Since HN2 is also relevant as a cancer chemotherapeutic agent, inhibition of MRP/Mrp transporters may serve to increase tumor cells' sensitivity to HN2 as an antitumor agent. Inhibition of these transporter proteins may also make HN2 more specific to tumor cells because MRP/Mrp transporters are more highly expressed in tumor cells than they are in healthy cells.

***The central hypothesis of this proposal is that nitrogen mustard toxicity is limited by its efflux from target cells because glutathione-conjugated mustards can act as ligands for MRP1/Mrp1 and that their toxicity can be influenced by treatments that alter the activity of these efflux transporters*.** To test this hypothesis, we plan to:

**Specific Aim 1. Characterize the effects of HN2, chlorambucil, melphalan, and chloroethyl ethyl sulfide (CEES) on human lung tumor cell growth and determine how vesicant-induced cell growth inhibition can be impacted by pharmacological inhibition of MRP1 and MRP2.** We will examine how vesicants affect growth of A549 human lung tumor cells in culture. We will determine whether MK-571, an inhibitor of MRP1 and MRP2, alters growth inhibitory properties of the mustards.

**Specific Aim 2. Determine the relative roles of MRP1 and MRP2 in protecting cells from vesicant-induced cell growth inhibition and characterize the mechanism by which MRP1 activity impacts vesicant-induced toxicity.** Vesicant-induced cell growth inhibition in human embryonic kidney-293 (HEK) cells overexpressing the MRP1 transporter will be compared with control cells with respect to growth inhibition. We will determine if these compounds act as inhibitors of MRP transporters and investigate whether mustards are ligands for MRP transporters by comparison of the uptake of vesicants and their glutathione conjugates inside-out membrane vesicles prepared from HEK cells overexpressing MRP1 and compare that to the uptake in control vesicles.

**Specific Aim 3. Determine whether induction of Mrp1 protein expression alters vesicant induced cell growth inhibition in mouse keratinocytes.** We will determine whether induction of Mrp1 through the Nrf2 pathway can decrease sensitivity to HN2 using cells where constitutive Mrp1 activity is low. We will use sulforaphane, a compound known to induce Nrf2. We will pretreat cells with sulforaphane and then add HN2 to their medium and determine the impact of these pre-treatments on cell growth inhibition by these vesicants. We will then confirm an upregulation in Mrp1 activity and protein expression following treatment with sulforaphane. If, as we hypothesize, we

observe mitigation of toxicity, we will determine whether or not this protection can be reversed by pharmacological inhibition of Mrp1.

Taken together, these data will show that MRP1 and MRP2 efflux transporters mediate the export of vesicants from cells. This may lead to breakthroughs in new therapies both to alleviate the effects of mustards when used as chemical warfare agents and also to strengthen their sensitivity and specificity as cancer chemotherapeutic agents.

## INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, mustard gas) and the related vesicant nitrogen mustard (mechlorethamine, methylbis(2-chloroethyl)-amine, HN2) are nonspecific, bifunctional alkylating agents that were originally developed as chemical warfare agents. Because these compounds work by multiple mechanisms with a lack of specific molecular targets, there are very few effective countermeasures against the use of these agents in chemical warfare [1]. HN2 and related nitrogen mustards chlorambucil (4-[bis(2-chlorethyl)amino]benzenebutanoic acid) and melphalan (4-[bis(chloroethyl)amino]phenylalanine or phenylalanine mustard) are used in cancer chemotherapy, but chlorambucil and melphalan are known to be limited in their cytotoxicity by their clearance from cells by multidrug resistance transporters [2-4]. The role by which HN2 is exported from cells, and whether or not its export can be pharmacologically modulated, remains poorly understood.

### A. Sulfur mustard and the nitrogen mustards

#### 1. History of mustards

The term mustard was coined to describe sulfur mustard and later structurally similar compounds that share sulfur mustard's dichlorodiethyl motif after description of the compound's "distinctive mustard and garlic-like odor" [1]. Sulfur mustard, or mustard gas, was first synthesized by Despretz in 1822 and later by Guthrie and Niemann (both in 1860). In 1886, Meyer developed a process for synthesizing pure sulfur mustard that was used by the Germans to produce sulfur mustard and deploy the compound as a weapon during World War I, and its effects on skin characterized which included blistering were immediately described [1, 5, 6]. The French named the compound

Yperite because the first known use of sulfur mustard during World War I was an attack near a Belgian town called Ypres [6]. In addition to impacting the skin, sulfur mustard also causes extensive damage, mainly to the lungs and the eyes, specifically the cornea [7, 8].

Sulfur mustard remains a threat, largely because it can be easily and cheaply synthesized and weaponized, and this compound was used in chemical warfare as recently as the 1980s during the Iran-Iraq War [9]. In fact, sulfur mustard is among the chemical warfare agents known to be housed in Syria today [10]. Prior to World War II, HN2, a similar new compound, was synthesized and produced, replacing the sulfur atom with a nitrogen atom bound to a methyl group. HN2 itself has never been known to be used as a chemical warfare agent, although the threat exists.

The concept that this compound is cytotoxic in blood was conceived following observation that patients exposed to mustards show a decrease in white blood cell count. Mustards may therefore be useful as a means to kill tumor cells in cancer chemotherapy, leading to testing and use of HN2 to treat blood cancers [11, 12]. Since then, HN2 has also been used to treat many solid tumors. Sulfur mustard itself has not been used as an antitumor agent except in rare, experimental cases [13]. The lack of use of sulfur mustard as an anticancer agent largely results from the fact that it has been used in chemical warfare and therefore, access to sulfur mustard, even for laboratory study, is strictly regulated. After HN2 was synthesized, mustard agents were more thoroughly tested for potential use in cancer chemotherapy, and their basic mechanism of action as alkylating agents, or compounds that covalently modify biomolecules by adding a carbon chain, was described [14-18]. At that time, covalent modification of nucleophilic amino acid residues such as cysteine of proteins was characterized, and it

was not long after the discovery of DNA that adducts to nucleic acids were also thoroughly described [19].

More hydrophobic nitrogen mustards with greater potential to passively diffuse into the tumor cell across the plasma membrane have also been synthesized including chlorambucil and melphalan [20, 21]. These compounds, as well as other nitrogen mustards such as cyclophosphamide and bendamustine are somewhat effective and have been commonly used in cancer chemotherapy for over half a century [22]. In order for a compound to be considered a mustard agent, it must have the dichloroethyl motif seen in both HN2 and sulfur mustard (Scheme 1). In order to be considered nitrogen mustard, the atom linking the two chloroethyl groups must be nitrogen. Because nitrogen forms three chemical bonds, the structural groups attached to this third bond is where the many nitrogen mustards differ.

## 2. Target Tissues and Clinical Implications

The primary target tissues of concern for clinical symptoms of exposure to mustard agents are the skin, eyes, and lungs. These three tissues are the most commonly effected simply because these are the tissues that will be directly exposed to mustards when they are used in chemical warfare. Other tissues require distribution through the systemic circulation before they can be reached by HN2 and other mustard agents.

a. The lung

There is generally a brief latency period following exposure before any clinical symptoms are observed. In the lung, this latency period typically lasts approximately four to six hours, before observation of acute symptoms such as chest tightness, hacking cough, and rhinorrhea. Persistent clinical symptoms of mustard exposure in the respiratory tissues include chronic bronchitis, airway hyperreactivity, lung fibrosis, and bronchopneumonia [23].

b. The skin

In the skin, there is an initial asymptomatic period that typically lasts between two hours and one day. The earliest clinical symptoms following mustard exposure in the skin include erythema or redness, which are often associated with itching. Then, vesicles, which are sacs filled with a pale yellow fluid, form. These vesicles will eventually develop into blisters. Over a period of ten to fifty days, these wounds may resolve but generally leaves changes to the pigmentation of the skin and scarring that can persist months or even years [1, 24]. If the area of scarring is in close proximity to a joint, this can lead to severe restrictions in mobility for the person or animal exposed to mustard [25].

c. The eye

The latency period has its shortest duration in the eye, only two to four hours, where redness and irritation will eventually lead to photophobia or discomfort and pain associated with light, blepharospasm or an abnormal twitch in the eyelid, and temporary

blindness [1, 26]. Exposure to mustards in the eye is also associated with separation between the epithelial and stromal cell layer [26].

## 3. Mustards as nonspecific, bifunctional alkylating agents

On the molecular level, sulfur mustard and related vesicants are nonspecific, bifunctional alkylating agents [27]. The purported mechanism of action for mustard-induced alkylation (Scheme 2) is as follows: first, a chlorine atom will rapidly leave via a first-order, SN1 intramolecular cyclization reaction, and the compound will readily react with water to form a highly electrophilic three-membered ring – a thiiranium or ethylsulfonium in the case of sulfur mustard and an aziridinium in the case of nitrogen mustards. These rings are highly unstable and will quickly react with nucleophilic groups on a wide variety of molecular targets, such as a nitrogen atom on a nucleic acid (typically N7 of 2-hydroxyguanosine) or the thiol group on a cysteine residue where the mustard will replace hydrogen. Either following or concurrent to this reaction, the other chlorine atom will leave the molecule, and a second cyclization will occur, forming a thiiranium or an aziridinium ring. As this ring is also extremely unstable, the mustard will similarly react with nucleophilic groups. This formation of a covalent linkage has the potential to create a cross-link between either two nucleic acid bases on the same molecule, two nucleic acid bases on different molecules, a nucleic acid and amino acid residue, two amino acid residues on the same protein molecule, or two amino acid residues on different protein molecules [28]. It is important to note that these covalent modifications also are not limited to nucleic acids and proteins, as the alkyl chains of mustard agents can be added not only to protein and DNA but also to nucleophilic acceptors found on various lipids and carbohydrates [1]. Thus, alkylation of intracellular

biomolecules can lead to a wide variety of damaging effects to the cell, including DNA damage, oxidative stress, and recruitment of inflammatory cells such as macrophages and granulocytes, all of which have the potential to lead to cytotoxicity. Because of the wide variety of molecular targets for mustards, the ability to develop effective countermeasures to mitigate mustard toxicity remains elusive [1, 29].

As an alternative to forming nucleic acid adducts and thioester bonds with cysteine residues on proteins, these alkylating agents can also form similar thioester bonds with the tripeptide electrophile scavenger glutathione (Scheme 3) [30]. This conjugation reaction protects the cell from mustard injury because glutathione is acting as a scavenger for the alkylating agent and preventing it from reacting with other biomolecules and damaging cellular components.

## 4. Chlorambucil and Melphalan

Other nitrogen mustard-derived compounds such as chlorambucil and melphalan can form adducts with DNA, nucleophilic amino acid residues, as well as lipids and carbohydrates by the same mechanism as HN2 and sulfur mustard can form these complexes. Chlorambucil and melphalan are less potent than sulfur mustard and HN2 because the larger group attached to the third bond on the nitrogen atom decreases their accessibility to many molecular targets, as well as their solubility [31]. In contrast to sulfur mustard and the nitrogen mustards, related “half mustard” chlorethylethyl sulfide (CEES), an analog used for laboratory study where use of sulfur mustard is restricted, is only a monofunctional alkylating agent and cannot react after interaction with its first nucleophilic target [32].

Two derivatives of HN2, chlorambucil and melphalan, were synthesized in the 1950s and continue to be used in cancer chemotherapy, especially to treat blood cancers. Chlorambucil was the standard of care for chronic lymphocytic leukemia for over four decades [33]. These agents were synthesized because their bulkier "R-groups", which increase their lipophilicity and thereby increase their ability to diffuse into cells [34]. Only 60-80 percent of patients respond successfully to chlorambucil therapy, and a major factor limiting the efficacy of chlorambucil and other mustard agents in treating tumors is the modification of these compounds by glutathione and clearance of these conjugates from cells by primary active transport [35].

Several other nitrogen mustard derivatives besides chlorambucil and melphalan have also been synthesized, and many have been tested and used in cancer treatment. Some of these derivatives include cyclophosphamide [36], bendamustine [22], ifosfamide [37], a thymine-derived nitrogen mustard [38], and benzaldehyde nitrogen mustard pyridine carboxyl acid hydrazones [39].

## B. Multidrug resistance-associated protein 1

### 1. Introduction

Pharmacokinetics is defined as the study of how the body affects a drug. In pharmacokinetics, the four classical steps that alter the xenobiotic are absorption, distribution, metabolism, and excretion. Absorption refers to the mechanism by which an exogenous compound gets into the bloodstream. For drugs administered orally, this involves moving through the digestive tract before reaching the blood with a "first pass" through the liver. For drugs injected intravenously, the drug originates in the blood, thereby skipping the absorption step and proceeding directly to the distribution step [40].

Distribution is defined as the means in which the compound of interest moves from the blood to the target tissue [41]. The next step, metabolism, refers to the process by which compounds are broken down by the body. Xenobiotic metabolism occurs in three distinct, classically defined phases. Phase I of drug metabolism refers to reactions such as hydroxylation and dehalogenation mainly by mixed function oxidase enzymes, particularly cytochromes P450. Phase II describes the covalent addition of a larger functional group or conjugation. Examples of such conjugation reactions are glucoronidation, acetylation, and the formation of a thioester linkage with the electrophile scavenger glutathione [42, 43]. The final step of pharmacokinetics, excretion refers to the substance of interest exiting the body. Excretion occurs primarily through processes such as expiration from the lungs, perspiration from the skin, and urination from the kidney [44].

Transporters are specialized proteins that span the plasma membrane of the cell, as well as some intracellular membranes such as the nuclear and mitochondrial membranes. These proteins facilitate the translocation of chemicals across membranes. Very broadly, transport proteins function in a manner similar to enzymes, where their “substrate” is a compound on one side of the plasma membrane and their product is the same compound on the other side of the membrane [45]. When the substrate is a xenobiotic, and the direction of transport is efflux from the cell, this process is often considered phase III of drug metabolism [43].

Both xenobiotics and endogenous compounds can be transported into and out of cells by either passive or active mechanisms. In general, passive diffusion requires the compound to be hydrophobic, relatively small, and along a concentration or electrochemical gradient [45]. When transport against a concentration gradient is required, this process needs energy, generated in primary active transport through the

hydrolysis of adenosine triphosphate (ATP). Secondary active transport requires both hydrolysis of ATP and a co-substrate from transporter, typically an ion [45]. This process of coupling substrates is referred to as cotransport and is classified as symport when the substrates move in the same direction and antiport when the move in opposite directions.

There are two main superfamilies of transport proteins that mediate the disposition of xenobiotics and endogenous chemicals such as cholesterol and bile acids. Proteins that catalyze the uptake of these compounds belong to the solute carrier (SLC), though they can also facilitate some bidirectional transport, generally using secondary and tertiary active transport. The main biological export pumps are members of the ATP-binding cassette (ABC) superfamily, which function by primary active transport [45].

## 2. ABC Transporters

The first ABC transporter described was multidrug resistance protein 1 (MDR1 in human, Mdr1a/b in rodents, or P-glycoprotein) in 1976 [46]. This 170 kilodalton transmembrane protein, encoded by the gene name ABCB1, spans the plasma membrane six times in two distinct regions [47]. When this protein was first characterized, it was discovered that cells overexpressing Mdr1a/b were resistant to colchicine, doxorubicin, and actinomycin D [48]. Its original name, P-glycoprotein describes the protein structurally, while the more modern name, multidrug resistance protein 1, refers to the correlation between protein expression and desensitization of tumor cells to a wide variety of antitumor agents. Many details relating to exactly how ligands are transported by this protein remain unknown. Because of the difficulty obtaining stable crystals to analyze the three-dimensional structure of MDR1, it took

thirty-three years from its initial discovery until the crystal structure of this protein was finally characterized [49]. However, much information has been learned about substrate specificity and the functions of this transporter through functional characterization of the effects of overexpression, induction, pharmacological inhibition, and genetic knockdown on various endpoints ranging from cytotoxicity to efflux activity to accumulation of potential ligands [45].

After the discovery of MDR1/Mdr1, there was a realization that efflux pumps exist that desensitize cells to cancer chemotherapy; however, there were many cancers that did not overexpress this ATPase [45]. Because of this resistance of many tumors to chemotherapy, the discovery of additional xenobiotic efflux pumps was pursued, leading to the discovery and characterization of multidrug resistance-associated protein 1 (Mrp1/MRP1) in 1992 [50]. Not long after its initial discovery, MRP1 was linked to resistance to anticancer drugs [51-53]. With the exception of some very low resolution structural images (22 angstroms), the crystal structure remains unknown for all ABC transporters besides MDR1, and therefore most of the mechanistic information learned about these transporters comes from various types of functional studies [45, 54]. It is notable that no correlation has been observed between expression of MRP1 and that of MDR1 in human tumors [55].

In total, there are seven subfamilies of ABC transporters that are grouped into classes A to G based on sequence homology [56]. The multidrug resistance-associated protein class (MRPs) is encoded by the gene name ABCC and consists of nine proteins. Structurally, MRP transporters all contain regions named Walker A, Walker B, and Signature C that are required for ATP binding (Scheme 4) [45]. Mrp1/MRP1, along with Mrp2/MRP2, Mrp3/MRP3, Mrp6/MRP6, and Mrp7/MRP7 are 190 kilodalton proteins that

contain 17 transmembrane regions in three distinct domains [57]. For these proteins, the amino terminus is extracellular, and the carboxyl terminus is intracellular [45].

In general, MRP1/Mrp1 localizes to the basolateral membrane in polarized cells, in contrast to MDR1/Mdr1 and MRP2/Mrp2; however, in some cells such as placental syncytiotrophoblasts and brain microvessel endothelial cells, MRP1 is localized on the cell's apical membrane [58, 59]. Tumor cells are not polarized in this way, so this characterization of an apical and basolateral membrane becomes irrelevant when considering Mrp1/MRP1 in solid tumors. MRP1/Mrp1 and analogous Mrp1-like proteins, have been identified in all eukaryotes including plants [60, 61], Drosophila [62, 63], skate [64], sea urchin [65], red mullet [66], fluke [67], the marine sponge agosterol A [68], zebrafish [69], and yeast [70-74]. However, they have not yet been found in bacteria or archaea [70, 75, 76].

The first and most described endogenous ligand for MRP1/Mrp1 is leukotriene $C_4$, an eicosanoid inflammatory mediator conjugated by the electrophile scavenger glutathione [77]. In fact, the human mast cells that secrete leukotriene $C_4$ express high levels of MRP1, indicating a vital physiological function for the transporter [78]. Another endogenous compound later determined to be transported by MRP1/Mrp1 is cobalamin, or vitamin $B_{12}$ [79]. Many of the xenobiotics transported by MRP1/Mrp1 and MRP2/Mrp2, and to a somewhat lesser extent MRP3/Mrp3, are electrophiles modified by glutathione, and these proteins are often associated with resistance to cancer chemotherapeutic agents [3, 45, 80-83]. A recent study has shown that a glutathione conjugate of cisplatin, an antitumor cross-linking agent that acts by a generallyy similar mechanism of action to mustards, can likely act as a ligand for MRP2/Mrp2 [84]. Mrp1/MRP1 is known to transport glutathione conjugates of nitrogen mustard-derived compounds chlorambucil and melphalan [2-4]. Other ligands for MRP1/Mrp1 include

oxidized glutathione [85], the topoisomerase II inhibitors doxorubicin, idarubicin [86], and etopiside, the lipid peroxidation product glutathione-conjguated 4-hydroxynonenal [87, 88], glutathione-conjugated aflatoxin B1, estradiol-17β-glucuronide [89], and glutathione-conjugated methylmercury [45, 90, 91]. There are also some heavy metals, likely conjugated by glutathione, that have been shown to act as substrates for human MRP1, including antimony salts [92, 93], the mercuric ion [94], arsenate, and arsenite [95, 96]. Even for compounds transported by MRP1/Mrp1 that are not conjugated to glutathione, co-transport of glutathione, or in some cases, S-methyl glutathione [97], is still required for MRP1/Mrp1 to function [98].

Despite the fact that glutathione is a required co-factor, increasing cellular glutathione concentrations has been found to have no impact on the ATPase activity of MRP1 in human small cell lung cancer $GLC_4$ cells overexpressing MRP1 [99]. The fact that there is no stimulation of MRP1 activity in this system is not very surprising since circulating concentrations of glutathione in lung cells are already high, but the finding that competitive inhibition does not occur supports the postulation that glutathione interacts with the MRP1/Mrp1 protein at a different site than the co-transported substrate. Ligands for MRP2/Mrp2 overlap quite often with those for MRP1/Mrp1, though MRP2/Mrp2 also transports many glucuronide conjugates in addition to glutathione [45]. The effect of increasing glutathione concentrations is likely cell-type specific due to differences in constitutive glutathione expression. For example, treatment of HEK cells transfected to overexpress MRP1 with glutathione precursor *N*-acetylcysteine has been determined to increase MRP1-mediated efflux of doxorubicin [100].

Glucuronide transport, with the exception of estradiol-17β-glucuronide mentioned above, is rare for MRP1/Mrp1 [45]. MRP3/Mrp3 has also been reported to facilitate the

transport of one glutathione-derived compound, glutathione-conjugated prostaglandin J2 [101]; however, the vast majority of its substrates are thought to be glucuronides-derived metabolites and endogenous compounds such as bile acids and glycine-conjugated cholic acid [102, 103].

3. Tissue Distribution

In the mouse, Mrp1 has been observed at high levels in the testes and ovaries, and at lower levels, in areas of the brain such as the choroid plexus [104, 105], astrocytes [106], cortical neurons [107], and the endothelial cells of the blood-brain barrier [108], as well as in the placenta, stomach, and skin [109-111]. MRP1 is more widely expressed in many human tissues than those of mice. It has been shown to be present in humans in the testes, prostate, lungs, heart, bladder, spleen, adrenal glands, placenta, kidneys, monocytes, skeletal muscle, and skin [45, 50, 110, 112, 113]. It is notable that human MRP1 expression is thought to be elevated in human tumors than healthy tissues. One such example is the liver, where MRP1 is barely expressed; whereas, in HepG2 hepatocellular carcinoma cells, the protein expression of MRP1 is considerably higher [114]. In the human liver, MRP1 is expressed at low levels on the basolateral membrane, while related GS-X pump MRP2 is localized on the canalicular membrane [115]. The overexpression of MRP1/Mrp1 in tumor cells relative to healthy cells does, however, vary by the type of tumor. Examples of human tumors thought to express elevated levels of MRP1 are chronic lymphocytic leukemia, where 60 percent of tumors expressed twenty-five-fold elevated MRP1 mRNA compared with normal cells, and prolymphocytic leukemia, where 40 percent of tumors expressed twenty-five-fold elevated MRP1 mRNA compared with normal cells [116]. Other tumor types where this

differential, as well as elevated MRP1 protein level and functional activity was demonstrated, include non-small cell lung carcinoma and esophageal squamous cell carcinoma [116, 117].

In the lung, an organ particularly relevant when considering the mustards in terms of their potential use as chemical warfare agents, MRP1/Mrp1 is highly expressed in humans and rats but mouse expression is extremely low [45, 118]. Several groups showed that patients with small cell lung cancer with high MRP1 expression have a weaker response to cancer chemotherapy consisting of combination therapy of the topoisomerase II inhibitor etoposide and the crosslinking agent cisplatin [119-123]. The relative response rates for patients highly expressing MRP1 ranged from -0.78 to -0.40. Another group showed a correlation between MRP1 expression and resistance to vinorelbine and carboplatin to treat lung cancers [124]. MRP1 has also been shown to be expressed in primary human lung epithelial cells and A549 cells, a human lung tumor cell line [125].

Little characterization of transporters has been done in the skin, another important area relating to mustard injury. Evidence suggests that Mrp1/MRP1 is the predominant multidrug resistance transporter in both human and mouse skin, while Mrp2/MRP2 and Mdr1/MDR1 are expressed at much lower levels [110, 126]. It is also believed that MRP1 is the only transporter with appreciable expression on the epidermal layer of human skin, and that the other transporters observed are mainly distributed deeper within the skin [110].

4. Structural Features of Mrp1/MRP1

Although discovery of the crystal structure for MRP1/Mrp1 remains elusive, many inferences have been made regarding the three-dimensional structure of MRP1/Mrp1, mainly as the result of functional analyses using systemically mutated Mrp1/MRP1 protein. Very broadly, the human protein is thought to be made up of 46 percent alpha helices, 26 percent beta sheets, 12 percent beta turns, and 17 percent random coils [127]. The entire protein contains an "MDR-like core" consisting of amino acids 281 to 1531 and an "extra" membrane spanning domain not present in many other multidrug resistance proteins consisting of amino acids 1-203 (transmembrane domain 0 or $TMD_0$) starting at the extracellular amino terminus [128]. This amino terminal transmembrane domain and the MDR-like core are combined by a "cytoplasmic linker" consisting of amino acids 204-281 [128]. It has been demonstrated that the cytoplasmic linker is essential, but $TMD_0$ is not for MRP1-mediated transport of leukotriene $C_4$ or *N*-ethylmaleimide *S*-glutathione in Sf9 cells transfected to overexpress the whole or truncated human protein. The human MRP1 protein reconstitutes effectively when the fragments consisting of amino acids 1-281 and 281-1531 are inserted in the membrane independently [128]. This indicates that proximity of these domains is required for function, but it is not necessary for them to be covalently linked [129]. In contrast, another group found that removing either $TMD_0$ or simply the first amino-terminal transembrane helix completely diminished MRP1 function in Sf21 cells; however, their truncated protein consisted of only amino acids 229-1531 [130]. These data suggest that there may be an essential feature for transport activity in amino acids 204-229; however, it is not impossible that there also could be slight differences between Sf9 and Sf21 cells used in the two independent experiments that can account for these differential results.

The human MRP1 protein contains twenty-five total cysteine residues [131], and two of these, cysteine-7 and cysteine-32 are conserved in the amino-terminus among human MRP1, MRP2, MRP3, MRP6, and MRP6, as well as mouse Mrp1 [132]. In total, there are seven cysteine residues in the extra cellular amino terminal region of the protein [133]. Both cysteine-7 and cysteine-32 contribute to human MRP1 functional activity to different degrees. Replacement of cysteine-7 with alanine reduces MRP1-mediated transport of leukotriene $C_4$ by 80%, while making an analogous replacement of cysteine-32 reduces transport only by 20-40% [132]. Another confounding result is that one group determined that a human MRP1 mutant containing no cysteine residues in this region can still transport leukotriene $C_4$ in yeast cells [131]. It was later found that this cysteine-less mutant will not leave the endoplasmic reticulum and insert itself appropriately in the cell's plasma membrane following protein folding in human embryonic kidney 293 cells, and this cysteine-less protein will have 20% activity in Sf21 cells compared to cells transfected with wild type human MRP1 [133]. Cells containing a modification of cysteine-7 are also no longer resistant to a wide range of MRP1 ligands, including colchicine, doxorubicin, vincristine, and etoposide [103, 132]. Because these modifications are both in the extracellular portion near the amino terminus of the protein, it is likely that this modification in activity is the result of a conformational change, rather than these cysteine residues acting as a binding site for the substrate [132].

In the next transmembrane domain ($TMD_1$), there are four cysteine residues, and replacing all four with alanine decreases the transport of leukotriene $C_4$ by 40% [133]. Photoaffinity labeling has shown a substrate-binding site is likely between the tenth and eleventh sequence in which the protein crosses the plasma membrane in $TMD_1$ (serine-542 to arginine-593) and between the sixteenth and seventeenth sequence in $TMD_2$ (cysteine-1205 to glutamate-1253) [134]. Replacement of one amino acid in $TMD_2$,

tryptophan-1246, with cysteine, has been found to result in a complete loss of drug resistance [135]. It is possible to successfully replace individual cysteine residues and construct an MRP1 mutant where there were only five cysteine residues in nucleotide binding domain 1 and transmembrane domain 2. It has further been determined that cysteine-1439, which is located at the carboxyl terminus and is completely conserved in all MRP transporters, is essential for leukotriene $C_4$ transport [133]. Another cysteine residue in the carboxyl terminus, cysteine-1479 is conserved in all MRPs except MRP7/Mrp7; however, replacement with serine or alanine does not have a significant effect on leukotriene $C_4$ transport activity in other MRPs but not in MRP1 [136].

Other information about the three dimensional structural features of MRP1/Mrp1 have been learned from examination of Sav1866, a bacterial ABC transporter found in *Staphylococcus aureus* [137]. Sav1866 exists in a homodimeric form and was crystalized while bound to ATP [138]. Study of this protein has isolated tyrosine-324 as another important amino acid residue in MRP1/Mrp1. Using site-directed mutagenesis and replacing this residue with alanine, phenylalanine, and tryptophan, it was determined that replacement with alanine or tryptophan does not affect transport of MRP1/Mrp1 substrates estradiol 17-β-glucuronide, estrone-3-sulfate, leukotriene $C_4$, or methotrexate in inverted membrane vesicles prepared from HEK cells overexpressing MRP1; however, replacement with phenylalanine caused a 1.5 to 1.8-fold increase in transport activity, indicating that the polar aromatic properties of this residue have an influence on the substrate specificity of MRP1/Mrp1 [139]. Also, other groups have used a mutation-based study to suggest that four intracellular, polar residues, lysine-513, lysine-516, glutamate-521, and glutamate-535, are critical for the proper folding of the human MRP1 protein [140].

Site-directed mutagenesis has also been used to study the two nucleotide binding domains in the human MRP1 protein. These are the areas where ATP is converted to ADP and inorganic phosphate. Using vanadate-induced trapping of 8-azido-α-[$^{32}$P]ADP, which can also bind in the same place as ATP, it has been shown that the majority of the nucleotide binds in nucleotide binding domain 2, with very little binding in nucleotide binding domain 1 [141]. However, uptake experiments in inverted membrane vesicles expressing only the carboxyl-terminal half of the protein, which does not include nucleotide binding domain 1, showed no uptake of leukotriene $C_4$, indicating a necessity of nucleotide binding domain 1 for functional activity [141]. In fact, the ATPase activity of each individual nucleotide binding domain was found to be fifty to one hundred-fold lower than the ATPase activity of the parent transporter [142]. Further studies showed that replacement of lysine-684 in nucleotide binding domain 1 decreased leukotriene $C_4$ transport by approximately 70 percent, while replacing the comparable amino acid in nucleotide binding domain 2, lystine-1333 caused a complete inactivation of the protein [141, 143].

## 5. Anticancer agents and MRP1/Mrp1

The human MRP1 gene was first isolated from a doxorubicin-resistant small-cell lung carcinoma, and it was determined to be a member of the ATP-binding cassette family based on its primary sequence [50]. This transmembrane protein is overexpressed in multidrug resistant cervical cancer HeLa cells and non-small cell lung carcinoma cell lines [50]. Another group demonstrated that MRP1 was overexpressed in a number of human tumor cell lines that do not express MDR1 but possess the "multidrug resistant" phenotype [144]. It has also been shown that the human breast

cancer cell line MCF7 overexpresses MRP1 and is resistant to etoposide, doxorubicin and has a small amount of cross-resistance to vincristine and mitoxantrone [145]. HeLa cells transfected with MRP1 overexpression are resistant to doxorubicin, vincristine, and etoposide as well [52]. Two glioma cell lines, IN500 and T98G, have elevated MRP1 expression and are resistant to etoposide, vincristine, and doxorubicin and have a decreased accumulation of etoposide following treatment [146, 147]. Various inhibitors of MRP1 activity were shown to reverse resistance to etoposide and doxorubicin in human glioma cells [148]. Clinically, high-grade gliomas have been demonstrated to express more MRP1 than those of a lower grade [149]. Research has shown a correlation between MRP1 expression and a poor prognosis for patients with breast cancer [150, 151], ovarian cancer [152], and neuroblastoma [153-156]. Neuroblastoma cells were also shown to have an increased MRP1 expression compared to healthy cells [157]. A correlation was also shown between MRP1 expression and a poor response to chemotherapy in acute lymphocytic leukemia, though this study combined several different treatment regimens, some of which included MRP1/Mrp1 substrates and some of which did not [158]. A further study of acute lymphocytic lymphoma examined patients treated with the BFM-95 protocol that includes MRP1/Mrp1 ligands methotrexate, vincristine, daunorubicin, and cyclophosphamide, and a statistically significant correlation was found between MRP1 expression and poor response to therapy [159]. Clinical research also showed that MRP1, as well as MDR1, MRP2, and MRP3 are elevated in residual tumors following treatment with MRP1 substrate doxorubicin compared with the untreated primary tumors [160].

Another factor involved in determining whether a tumor will show a multidrug resistance phenotype is the degree of differentiation of the tumor. One group showed that there is a correlation between MRP1 expression and degree of differentiation in

esophageal, gastric, and colorectal carcinomas, indicating that when a tumor is still well-differentiated, it will show less sensitivity to antitumor agents that are substrates for MRP1, but once it becomes more poorly differentiated, it will no longer be resistant [161]. It is worth noting that current therapy for colorectal cancer is 5-fluorouracil, which is transported by neither MDR1/Mdr1 or MRP1/Mrp1, making some discussion of transporter function in this tumor type irrelevant [162]. However, therapy regimens constantly evolve, oxaliplatin, a substrate for MRP1/Mrp1, has been shown to increase survival for patients with colorectal cancer in some cases [162]. In some other tumors, such as oral squamous cell carconioma, opposite results have been found where loss of differentiation was associated with increased expression of MRP1 [163].

It was determined that MRP1/Mrp1 transports mainly anionic glutathione *S*-conjugates such as the endogenous leukotriene $C_4$ and glutathionyl dinitrophenol [164]. Even unconjugated substrates including vincristine require the co-transport of glutathione to be translocated by MRP1/Mrp1 [165]. Interestingly, the thiol of the cysteine residue in glutathione is not required for this co-transport, since MRP1/Mrp1 can still transport organic anions when the cysteine residue in glutathione is replaced with leucine [166]. That thiol, however, is typically necessary for the conjugation of glutathione to electrophilic MRP1/Mrp1 ligands.

Further investigations focused more on whether this transport protein provided resistance to antitumor agents that can be conjugated by glutathione. It was determined that doxorubicin itself cannot act as a direct substrate for MRP1, and the addition of a negative charge by forming a covalent bond with glutathione is critical for the transport of doxorubicin and similar compound duanorubicin [70]. The preference of MRP1/Mrp1 to catalyze the export of polar substrates likely stems from the fact that many of the amino acids in this protein's transmembrane domains are amphiphatic and have polar or

ionizable amino acids [165]. This is in contrast to MDR1/Mdr1, a protein that contains many more hydrophobic amino acid residues in accessible sites for substrate binding [167].

a. Alkylating and crosslinking agents and MRP1/Mrp1

In 1998, it was first shown that two mustard-derived alkylating agents, melphalan and chlorambucil, can be transported across the membrane of inside-out membrane vesicles prepared from HeLa cells transfected to overexpress MRP1 [2]. Transport of monoglutathionyl chlorambucil in Chinese hamster ovary cells that overexpress glutathione *S*-transferases, an enzyme that can catalyze the reaction between many electrophiles and glutathione was also demonstrated in this work [2]. This group inferred that although these cells did not overexpress Mrp1, they effectively had an increased substrate concentration by overexpressing the enzyme that conjugates chlorambucil to glutathione, thereby increasing amount of substrate that could interact with the transporter, pushing the efflux “reaction” to the right. It was further observed that human promyelocytic leukemia HL60 cells and human $GLC_4$ small cell lung carcinoma cells that overexpress MRP1 had greater resistance to chlorambucil-induced cytotoxicity compared with control cells [2]. Another group, using MCF7 cells transfected to overexpress MRP1, found an increased resistance to chlorambucil but not to similar compound melphalan. They did observe transport of monoglutathionyl chlorambucil and monoglutathionyl melphalan with a similar $K_M$; however the $V_{max}$ for the chlorambucil conjugate was approximately 3-fold higher [4]. Another group showed that monoglutathionyl chlorambucil can be transported by both MRP1 and MRP2, though the apparent efficiency of transport ($V_{max}/K_M$) is approximately 25-fold higher in vesicles prepared from MCF7 cells transfected to overexpress MRP1 than those transfected to overexpress MRP2 [168].

6, Pharmacological Inhibition of MRP1/Mrp1

Several small molecule, pharmacological inhibitors of the functional activity of MRP/Mrp transporters, have been characterized and a commonly used example of an MRP1/Mrp1 antagonist is MK-571. This compound was first discovered and described as an antagonist for the leukotriene $D_4$ receptor before MRP/Mrp transporters were first cloned and discovered [169]. Leukotriene $C_4$, which is endogenously conjugated by the tripeptide glutathione, was later discovered to be a ligand for MRP1/Mrp1 [4]. MK-571 decreases the signaling from the leukotriene $D_4$ receptor by inhibiting efflux of leukotriene $C_4$, a process mediated by MRP1/Mrp1 [170]. When leukotriene $C_4$ accumulates inside cells, the higher concentration causes it to compete with leukotriene $D_4$ more favorably for receptor binding sites, decreasing the degree to which leukotriene $D_4$ can interact with its receptor. Therefore, inhibition of MRP1/Mrp1-mediated efflux of leukotriene $C_4$ will antagonize the signaling of this G-protein coupled receptor [81]. At the same time as MK-571 was being developed and characterized, another group described the ATP-dependent efflux of glutathione-conjugated leukotrienes in the heart [171]. The efflux pump catalyzing this export was also later determined to be MRP1 [45]. In addition, MK-571 is effective inhibiting MRP1 functional activity in A549 human lung epithelial cells [172].

Another example of a small molecule inhibitor of MRP1/Mrp1 ibrutinib, an anticancer drug used to treat chronic lymphocytic leukemia, which is effective at concentrations as low as 1 μM and 5 μM in inhibiting MRP1 activity in both HEK and HL60 cells that overexpress MRP1, an approximately order of magnitude improvement in sensitivity compared with MK-571 [173]. In fact ibrutinib showed some efficacy in a mouse xenograft model, enhancing the ability of vincristine to shrink the tumors with no significant pathologies or phenotypic changes [173]. Other examples of MRP1/Mrp1

inhibitors include methylenedioxymethamphetamine, caffeic acid, as well as the glutathionyl *S*-conjugates of 2-hydroxy- and 4-hydroxy- metabolites of estrone, estradiol, and estriol [174]. Of these estrogen metabolites, only 2-hydroxy-1-glutathonyl-estradiol and 2-hydroxy-1-glutathionyl-estradiol were also inhibitors of MRP2 in a similar concentration range, indicating an MRP1 specificity for 2-hydroxy-4-glutathionyl-estradiol, and 4-hydroxy-2-glutathionyl-estradiol [175]. Another example of an inhibitor of MRP1/Mrp1 is 3β-acethyl tormentic acid, which blocks the efflux activity of Mrp1 in B16 cells, a mouse melanoma line that expresses Mrp1, and Ma104 monkey kidney epithelial cells [176].

## C. Nuclear factor (erythroid derived 2)-like 2

### 1. Introduction

It is known that MRP1/Mrp1 protein expression and functional activity can be regulated by the transcription factor nuclear factor (erythroid derived 2)-like 2 (Nrf2 or NFE2L2) [177-179]. Nrf2 is a protein constitutively expressed in the cytosol and bound to a “chaperone protein” known as Kelch-like ECH-associated protein 1 (Keap1). Interaction between Keap1 and reactive oxygen species or sulfur-containing compounds such as isothiocyanates that may form disulfide bonds with one of the twenty-seven cysteine residues in the Keap1 protein can cause dissociation between Keap1 and Nrf2 [180]. When unbound and activated, Nrf2 translocates to the nucleus (Scheme 5). After entering the nucleus, Nrf2 will bind to specific sequences in the promoter region of target genes. These sequences are known as antioxidant response elements or electrophile response elements [181]. Agonism of this transcription factor will induce a wide variety of genes. Genes that contain antioxidant or electrophile response elements include but

are not limited to glutathione synthesis enzymes such as glutamate-cysteine ligase, glutathione synthetase; phase II metabolizing enzymes such as glutathione *S*-transferases and UDP-glucuronosyltransferases; antioxidant enzymes such as NADPH quinone oxidoreductase 1, thioredoxin reductase, and heme oxygenase-1; and transporters such as Mrp1/MRP1 [182].

2. Characteristic Nrf2 target genes

The most commonly investigated target genes for Nrf2 are NAD(P)H dehydrogenase [quinone 1] (NAD(P)H quinone oxidoreductase 1, DT diaphorase, or NQO1/Nqo1) and heme oxygenase-1 (HO-1/Ho-1). Increases in NQO1/Nqo1 and HO-1/Ho-1 mRNA and protein is one way to determine that the Nrf2 transcription pathway has been activated [183-187]. Briefly, NQO1/Nqo1 is an oxidoreductase enzyme that catalyzes the two-electron reduction of oxygen. At the expense of NADH or NADPH, this protein will catalyze the conversion of a quinone to a hydroquinone and two oxygen molecules to two superoxide anions (Scheme 6). These superoxide anions are further reduced to generate hydrogen peroxide [188, 189]. NQO1/Nqo1 is generally considered an antioxidant enzyme because even though it generates hydrogen peroxide, it will prevent the quinone from reacting with an enzyme that can catalyze one-electron reductions, which would create superoxide anions with a much longer biological half-life.

The other main characteristic target gene for Nrf2 is Ho-1/HO-1. This enzyme, in conjunction with cytochrome P450 oxidoreductase, catalyzes the NADPH-dependent breakdown of heme to generate biliverdin. Biliverdin is then further metabolized by biliverdin reductase to form bilirubin, a compound that has the ability to scavenge reactive oxygen species [190].

3. Induction of transport proteins through the Nrf2 pathway

In addition to antioxidants, phase II metabolizing enzymes, and glutathione synthesis enzymes, Nrf2 activation has also been associated with upregulation of multidrug resistance transporters such as MDR1/Mdr1, BCRP/Bcrp, and MRP1/Mrp1 [6, 191]. More generally, it has been shown that conditions of oxidative stress result in higher levels of MRP1/Mrp1 expression [192]. The idea that MRP transporters are regulated by the Nrf2 pathway was conceived following observation that HepG2 hepatocellular carcinoma cells overexpressing Keap1 had decreased efflux of ligands for MRPs [193]. Overexpression of Keap1 correlates with a downregulation of Nrf2 because there is more of the "chaperone" protein to bind Nrf2 and prevent this transcription factor from translocating Nrf2 into the nucleus. Another group showed increased Mrp1 mRNA level in mouse fibroblasts following treatment with the electrophilic diethyl maleate. They demonstrated that induction was related to the Nrf2 pathway when they were unable to activate Mrp1 mRNA in Nrf2 knockout fibroblasts [179]. It has also been shown that 15-deoxy-delta 12, 14-prostaglandin J2 can induce the upregulation of MRP1 mRNA in MCF7 human breast cancer cells through an Nrf2-mediated pathway [194]. Another group showed that the flavone wogonin decreases MRP1 mRNA and protein expression, as well as functional activity by antagonizing the Nrf2 pathway in K562 human leukemia cells [195]. Another group examined the sequence of the MRP1 gene and found two antioxidant/electrophile response elements in the promoter region of for MRP1 [196]. These two sequences are TGAGCGGGC starting from base pairs -66 to -57 and GTGACTCAGC from positions -499 to -490 [196].

An example of a small molecule known to activate Nrf2 is $_{D,L}$-sulforaphane, an isothiocyanate found in cruciferous vegetables such as Brussels sprouts, cauliflower, and broccoli, which forms disulfide bonds with cysteine residues on Keap1 and causes a conformational change that leads to the dissociation between Keap1 and Nrf2 [197]. One group showed that sulforaphane increases MRP1 mRNA and protein expression in A549 and HepG2 cells and increases the efflux of 5-carboxy-2'-7'-dichlorofluoroscein, a model substrate for MRP1 [198]. To date, there exists no published data demonstrating induction of MRP1/Mrp1 through the Nrf2 pathway in the skin; however, the fact that this induction could occur is plausible. Another important factor is that in addition to modifying cysteine residues on Keap1, sulforaphane can also react with glutathione and be transported from cells by MRP1/Mrp1 [199, 200]. Therefore, because sulforaphane will not continue to remain inside the cell and will be exported more rapidly as MRP1/Mrp1 and other GS-X pumps are transcribed, necessitating relatively short treatment times in order to observe upregulation of downstream targets.

D. Mustards and MRP1/Mrp1

One major gap in our understanding of the molecular mechanism of action of mustards is a characterization of how these compounds can be conjugated and cleared from cells. The efflux of these compounds from cells limits toxicity by decreasing their concentrations inside the cells, thereby allowing fewer molecular targets to be reached. Recent research has shown that MRP1 may be able to catalyze the efflux of glutathione conjugates chlorambucil and melphalan [2, 201, 202]. Another group has shown that overexpression of MRP1 may provide resistance to chlorambucil cytotoxicity in MCF7 breast cancer cells [203]. Because of their structural and mechanistic similarity, we

hypothesize that a glutathione conjugate of HN2 may interact with Mrp1/MRP1. It is important to note that there is significant substrate overlap between Mrp1/MRP1 and MRP2/Mrp2, another efflux pump that commonly transports glutathione-conjugated electrophiles, such as cisplatin [84]. One study showed that monoglutathionyl chlorambucil is transported by MRP2 in inside-out membrane vesicles prepared from MCF7 cells, though with a much higher $K_M$ than was observed for the transport of this conjugate by MRP1. In these studies, we will investigate the role of MRP2/Mrp2 in providing resistance to mustard agents; however, we hypothesize that MRP1/Mrp1 is the more important transporter regulating mustard toxicity. We predict that based on this interaction, pharmacological inhibition of MRP1/Mrp1 will lead to an increase in the sensitivity of cells to mustard-induced growth inhibition because the cells will have a decreased ability to export these alkylating agents. We also expect that increased transcription of MRP1/Mrp1 through the Nrf2 pathway will result in an attenuation of HN2-induced cell growth inhibition, at least partly because of an increased efflux of the glutathione-conjugated mustard. It is worth noting that the assay that is typically referred to as “growth inhibition” is really a measure of cytotoxicity and does not distinguish between dead cells and cells that are still alive but can no longer undergo mitosis.

It is worth noting that there are likely other transcription factors that can also mediate MRP1/Mrp1 protein expression; however, the interaction between other transcription factors and MRP1/Mrp1has not yet been well-characterized. It has been reported that treatment with tetrachlorodibenzodioxin, an agonist of the aryl hydrocarbon receptor, increases Mrp1 mRNA in rat large intestine but not in kidney or liver. Another activator of the aryl hydrocarbon receptor, β-naphthoflavone induces Mrp1 mRNA expression in rat kidney but not large intestine or liver, and pregnenolone 16α-carbonitrile an agonist of the pregnane X receptor increases Mrp1 mRNA expression in

rat liver [204]. In the mouse liver, where constitutive Mrp1 mRNA is almost unobservable, a down regulation in Mrp1 mRNA was found following treatment with activators of the peroxisome proliferator-activated receptor α, and that activators of the pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor have no impact on Mrp1 mRNA in mouse liver [205]. Another example of receptor-mediated MRP1 transcription is the epidermal growth factor receptor, which when agonized by epidermal growth factor in MCF7 breast cancer cells, has been shown to cause an upregulation in MRP1 mRNA as well as promoter activity [206]. There exists conflicting data relating to another transcription factor, hypoxia-inducible factor-1-α, which may be associated with an upregulation of MRP1 in HepG2 human hepatocellular carcinoma cells [207], but no effect was seen in MRP1 in MCF7 cells exposed to hypoxia [208]. Other groups found that hypoxia significantly increased MRP1/Mrp1 expression in SGC7901 human gastric cancer cells [209], as well as T98G human glioma cells [210]. To add further confusion, there was even a group that demonstrated a 25 percent reduction in Mrp1 activity in R3327-AT1 rat prostate cancer cells exposed to hypoxia [211]. Agonism of another transcription factor, the peroxisome proliferator-activated receptor α, has been shown to downregulate Mrp1 mRNA in mouse small intestine [212].

## E. Significance and Innovation

1. Significance

Mustards are nonspecific alkylating agents that cause oxidative injury and persistent damage to tissues such as lung, skin, and eye. Sulfur mustard was first used as a chemical warfare agent in World War I and has been used for that purpose as recently as the Iran-Iraq War in the 1980s. HN2 was developed but never used as a chemical warfare agent during World War II. Few effective countermeasures exist to attenuate the toxicity of these compounds. HN2 and related mustards chlorambucil and melphalan, as well as other mustard derivatives, are also used as cancer chemotherapeutic agents [1, 23, 26].

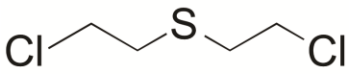
MRP1/Mrp1 is an ATP-dependent, membrane-bound efflux pump that commonly facilitates the export of a wide variety of organic anions from cells, including glutathione *S*-conjugated electrophiles including chlorambucil and melphalan [2]. It therefore is likely that glutathione conjugates of HN2 and sulfur mustard may act as substrates for these transport proteins. When considering these alkylating agents as potential chemical warfare agents, elucidating the molecular mechanism of action for their conjugation and efflux serves to increase understanding of the full molecular mechanism of action of the compounds. This understanding can lead to novel therapies. There are also several known naturally occurring mutations in the human MRP1 protein; therefore, this research could be critical in determining susceptible populations to mustard toxicity [213-217]. If, on the other hand, we consider these compounds in terms of their application in cancer chemotherapy, pharmacological inhibition or transcriptional downregulation of these ATPases could serve as a means of increasing the sensitivity of these antitumor agents. It should also be noted that there are many other anticancer drugs, such as the topoisomerase II inhibitor etoposide [148], the anti-mitotic microtubule-disrupting agent vincristine [103, 218], and the dihydrofolate reductase

inhibitor methotrexate [219-221] that are also transported from cells by MRP1/Mrp1, so the impact of combining multiples of these drugs should be considered. This strategy could also very likely increase the specificity of these alkylating agents because MRP1/Mrp1 is generally much more highly expressed in tumor cells than in healthy cells [45].

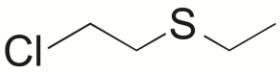
## 2. Innovation

The proposed research will adjudge the **novel hypothesis that glutathione *S*-conjugated nitrogen mustard and related alkylating agents can serve as ligands for MRP1/Mrp1**, and the planned studies will also determine the molecular mechanism of action for this interaction. These studies will also propose a novel mechanism to explain why combination therapy is effective in cancer treatment and have more than an additive impact on the efficacy in cancer treatment.

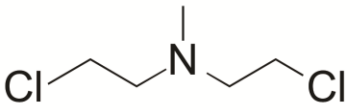
**Scheme 1. Structures of sulfur mustards and nitrogen mustards.**



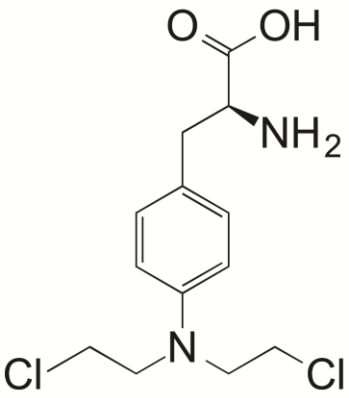

Sulfur Mustard



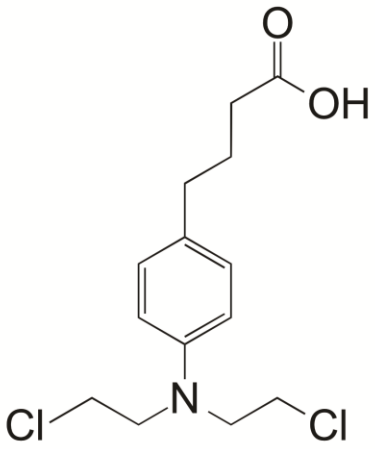

Chloroethyl ethyl sulfide




Nitrogen Mustard (HN2)

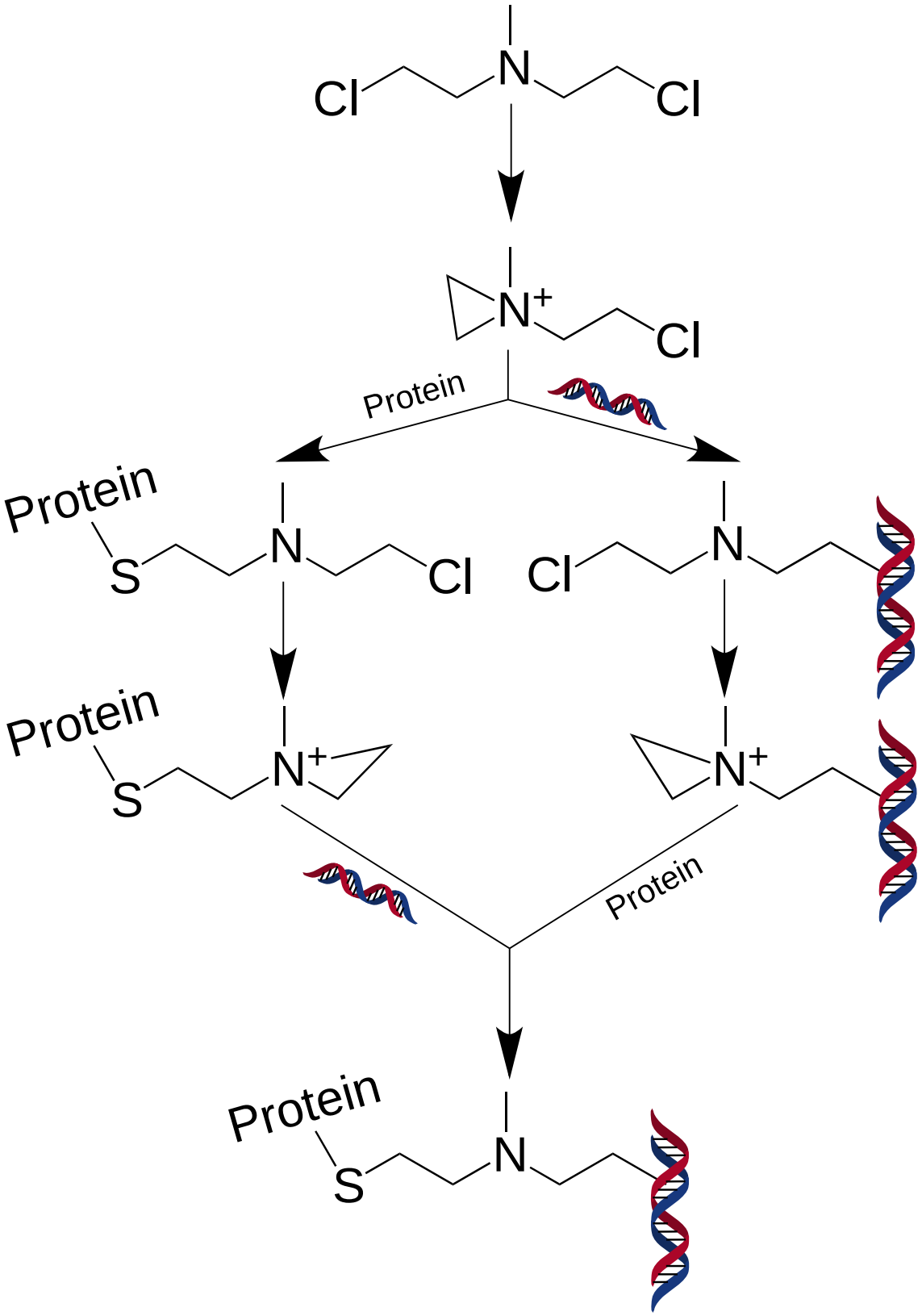



Melphalan

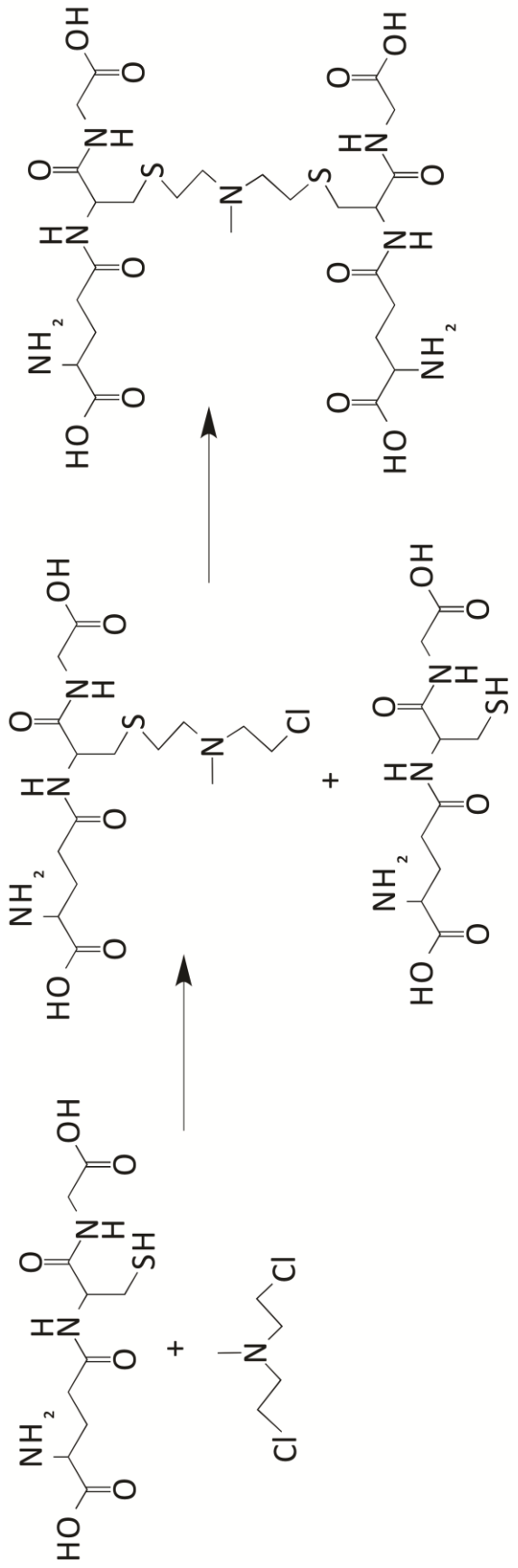



Chlorambucil

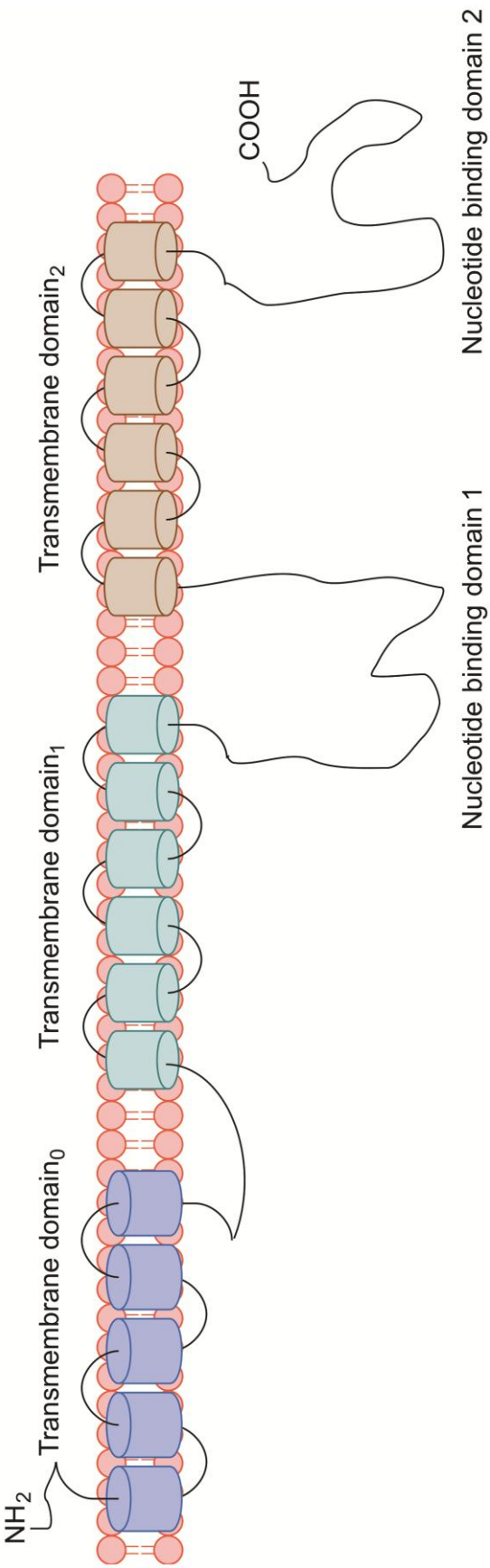
**Scheme 2. Mechanism by which HN2 alkylates DNA and proteins.** HN2 reacts rapidly with water in an SN1 intramolecular cyclization reaction to form an aziridinium ion. As a strong electrophile, this ion reacts with nucleophilic sites in cells including cysteine residues in proteins and DNA bases. HN2 is a bifunctional alkylating agent and can form cross-links in DNA and between DNA and proteins.


Cl
N
Cl
N+
Cl
Protein
Protein
S
N
Cl
Cl
N
Protein
S
N+
N+
Protein
Protein
S
N

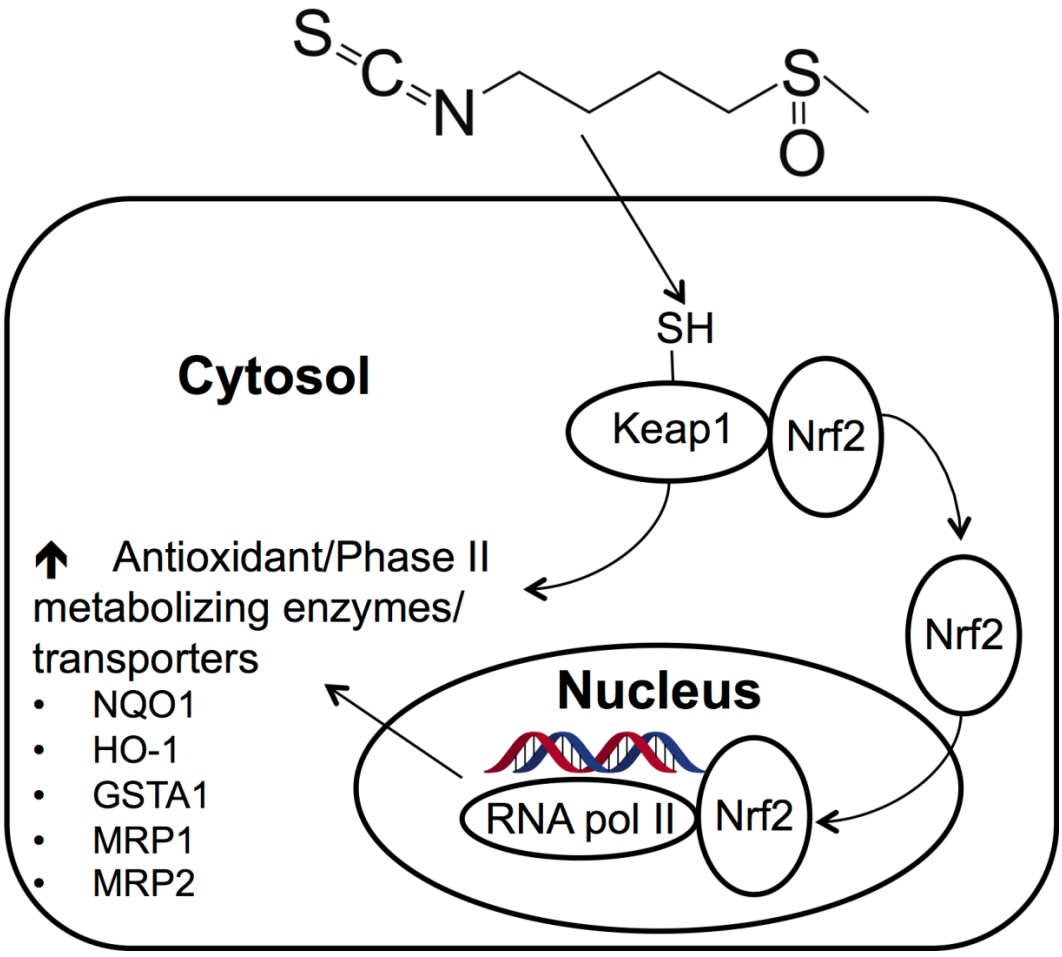
**Scheme 3. Conjugation of HN2 by glutathione.** Glutathione, a tripeptide consisting of glutatmate, cysteine, and glycine, forms thioester linkages with HN2. Because HN2 is a bifunctional alkylating agent, it has the potential to form monoglutathionyl or bisglutathonyl conjugates. This reaction can either be a spontaneous nucleophile-electrophile reaction or can be catalyzed by a member of the glutathione *S*-transferase class of enzymes.



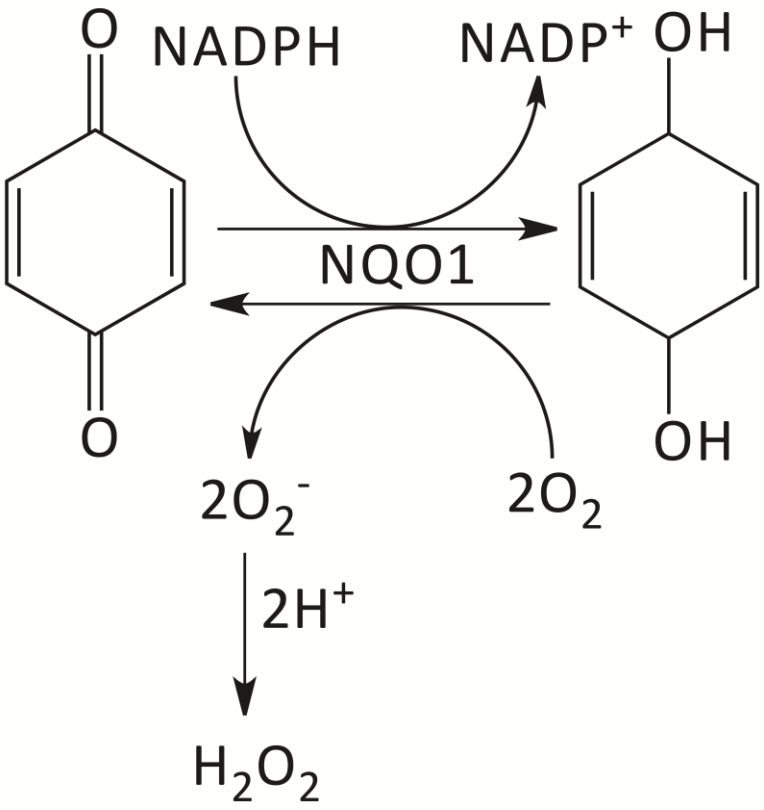
**Scheme 4. Structure of Mrp1/MRP1.** Mrp1/MRP1 contains an extracellular amino terminus, a transmembrane $domain_0$ that crosses the cell membrane five times, a transmembrane $domain_1$ that crosses the cell membrane six times, an intracellular nucleotide binding domain for ATP, a transmembrane $domain_2$ that crosses the cell membrane six times, another nucleotide binding domain, and an intracellular carboxyl terminus.


NH2
Transmembrane domain0
Transmembrane domain1
Transmembrane domain2
COOH
Nucleotide binding domain 1
Nucleotide binding domain 2

**Scheme 5. Sulforaphane induces antioxidants, phase II metabolizing enzymes, and transporters through the Nrf2 pathway.** Sulforaphane enters cells and bonds with cysteine residues on Keap1, causing dissociation between Nrf2 and Keap1. This dissociation induces translocation of Nrf2 from the cytosol to the nucleus, where it forms a complex with DNA and RNA polymerase II, leading to the transcription of antioxidants, phase II metabolizing enzymes, and transporters.


S=C=N
S
O
SH
Cytosol
Keap1
Nrf2
Antioxidant/Phase II metabolizing enzymes/ transporters
• NQO1
• HO-1
• GSTA1
• MRP1
• MRP2
Nrf2
Nucleus
RNA pol II
Nrf2

**Scheme 6. General reaction catalyzed by NAD(P)H dehydrogenase [quinone] 1.** In the presence of NADPH, a quinone will react to form a hydroquinone at the expense of two electrons from molecular oxygen. These two oxygen molecules, converted to the superoxide anion, will quickly react with each other and protons from water to form hydrogen peroxide.


O
NADPH
NADP+
OH
NQO1
O
OH
2O2-
2O2
2H+
H2O2

# METHODOLOGY

## A. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin/streptomycin, and G418 were purchased from Life Technologies (Rockville, MD). Rat primary antibody for MRP1/Mrp1, mouse primary antibody for Mrp2/MRP2, and rabbit primary antibody for Ho-1 were purchased from Enzo Life Sciences (Farmingdale, NY). Rabbit primary antibody for Nrf2 was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Goat primary antibody for NAD(P)H dehydrogenase [quinone] 1 (Nqo1) was from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody, pre-cast polyacrylamide gels, and the BCA (bicinchoninic acid) protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence reagent was purchased from Millipore (Billerica, MA). Nitrogen mustard (HN2), MK-517 (3-[[[3-[(1E)-2-(7-Chloro-2-quinolinyl)ethenyl]phenyl][[*3*-(dimethylamino)-*3*-oxopropryl]thio]propanoic acid), protease inhibitor cocktail (P2714) consisting of 4-(2-aminoethyl)benezenesulfonyl fluoride, pepstatin A, E-64 [trans-expoysuccinyl-l-leucylamido-(4-guanidino)butane], bestatin, leupeptin, and aprotinin, reduced glutathione, goat anti-rat secondary antibody, rabbit anti-goat secondary antibody, sulforaphane and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

## B. Cells

A549 human lung tumor cells were obtained from the American Tissue Culture Collection (Manassas VA) and maintained in DMEM growth medium supplemented with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 U/mL) as previously described [32]. Cells were cultured at 37°C in 5% $CO_2$ in a humidified incubator.

Human embryonic kidney 293 (HEK) cells stably transfected to overexpress MRP1 (HEK MRP1) and those transfected with an empty pcDNA 3 vector (HEK control) were kindly provided by Dr. Robert Robey (National Institute for Health, Bethesda, MD) and maintained in DMEM growth medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), and G418 (2 mg/mL) as previously described [222, 223]. HEK cells stably transfected to overexpress MRP2 (HEK MRP2) and those transfected with an empty pCMV6-NEO vector (HEK control) were prepared and maintained in DMEM growth medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), and G418 (2 mg/mL) as previously described [224]. HEK cells were confirmed to overexpress the transporters of interest by Western blotting (Figure 3, Panels A and B) and functional activity by examining efflux of the model MRP substrate calcein (Figure 3, Panels C and D).

PAM212 mouse keratinocytes were maintained in growth medium consisting of DMEM supplemented with 10% fetal bovine serum and penicillin (100 U/mL) and streptomycin (100 µg/mL) and cultured at 37°C in 5% $CO_2$ in a humidified incubator. Primary mouse epidermal keratinocytes were isolated from the skin of newborn C57BL/6J wild type mice (The Jackson Laboratory, Bar Harbor, ME) or C57BL/6J Nrf2$^{-/-}$ mice [225, 226] bred at Rutgers University animal care facility and were maintained as previously described [42, 187]. For experiments with primary keratinocytes, cells were grown on culture plates coated with collagen IV [187, 227]. The genotype Nrf2$^{-/-}$ keratinocytes was confirmed using PCR.

C. Western blotting

Western blotting was performed as previously described with some modifications [187, 228]. Briefly, cells were lysed in Triton-X-100 lysis buffer (20 mM tris HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, pH 7.4, and protease inhibitor cocktail), transferred

into 1.5 ml Eppendorf microcentrifuge tubes, and centrifuged (750 x g, 10 min, at 4°C) to remove cellular debris. Total protein in the supernatant fraction was determined by the BCA protein assay kit using bovine serum albumin as the standard. Nuclear extracts were prepared using the NE-PER Nuclear Protein Extract Kit (Thermo Scientific, Rockford, IL). Lysates (10-30 µg protein/well) were electrophoresed on 10.5-14% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes and blocked in Tris buffer supplemented with 5% milk at room temperature. After 1 hr, the blots were incubated overnight at 4°C with primary antibodies, washed with tTBS (Tris-buffered saline supplemented with 0.1% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies. After 1 hr at room temperature, proteins were visualized by ECL chemiluminescence (Millipore, Billerica, MA).

D. Assays for Mrp/MRP Functional Activity

The fluorescent ligand calcein was used to monitor Mrp/MRP functional activity in intact cells as previous described with some modifications [229]. Briefly, cells ($10^5$ cells/ml) in growth medium were loaded with 1 µM calcein-AM in the presence or absence of the test inhibitor. After 30 min at 37°C (*uptake period*), cells were washed with PBS and analyzed for cell fluorescence using a Cellometer Vision cell counter fitted with a VB-595-402 (excitation/emission 495/515 nm) filter cube (Nexcelom Bioscience, Lawrence, MA). Cells were then incubated in growth medium with or without test inhibitor for 60 min to allow calcein efflux from the cells (*efflux period*). After washing, cell fluorescence was again determined. The difference in fluorescence after the uptake phase and the efflux phase was taken to be an indicator of MRP1/Mrp1-mediated transport.

The fluorescent substrate glutathionyl bimane (bimane-GS) was used to monitor MRP1 functional activity in inverted membrane vesicles as previously described with

some modifications [84, 230]. Briefly, inside-out Sf9 insect cell plasma membrane vesicles expressing human MRP1 and those transfected with an empty vector were purchased from Sigma-Aldrich (St. Louis, MO). To assess the ability of HN2 to inhibit MRP1-mediated transport of bimane-GS, 20 mg vesicles were incubated with 10 µM bimane-GS, 4 mM adenosine triphosphate, 2 mM reduced glutathione, and 100 nM HN2 in reaction buffer at 37ºC for 10 min according to the manufacturer's protocol. Vesicles were washed, vacuum filtered, and solubilized with 50% methanol. Fluorescence was read at excitation wavelength 430 nm and emission wavelength 538 nm.

E. Cell Growth Inhibition

Cell growth inhibition by HN2, melphalan, chlorambucil, CEES etoposide, methotrexate, and vincristine was determined as previously described with some modifications [231, 232]. Briefly, A549, HEK, or PAM212 cells were seeded at low density (1.8–3.0 × $10^4$ cells/well) in 24-well tissue culture dishes and allowed to adhere overnight. Primary epidermal keratinocytes were seeded (0.8-1.2 x $10^4$) and allowed to grow until their morphology appeared normal (3-4 days). The medium was then replaced with 0.35 mL of serum-free growth medium supplemented with increasing concentrations of HN2, melphalan, chlorambucil, CEES, etoposide, methotrexate, or vincristine. After 30 min, A549 and PAM212 cells were washed twice with HBSS and refed with fresh drug-free medium containing 10% FBS and penicillin/streptomycin. After an additional 72 h, the cells were removed from the dishes with trypsin and counted using a Z1 Coulter Particle Counter (Beckman Coulter). Concentrations of HN2 that caused 50% growth inhibition ($IC_{50}$) were then determined. In experiments using HEK cells, they were not washed after the HN2 treatment because they do not adhere to the wells very well and washing causes significant cell loss. Instead, medium was

supplemented with 10% FBS after 30 min exposure to HN2. In some experiments, cells were treated with sulforaphane, MK-571, or other compounds prior to HN2 as indicated.

F. RNA isolation and real-time quantitative PCR (qPCR)

RNA was isolated and analyzed by qPCR as previously described [84, 181, 224]. Total RNA was isolated using RNABee reagent (Tel-Test Inc, Friendswood, TX) according to the manufacturer's protocol. cDNA was synthesized using M-MLV reverse transcriptase, diluted in RNase-DNase-free water and Mrp1, Mrp2, heme oxygenase 1 (Ho-1), and NAD(P)H quinone oxidoreductase (Nqo1) mRNA quantified by qPCR using Sybr Green for detection of amplified products using a Viia7 qPCR system (Applied Biosystems, Carlsbad, CA). Forward (5'-3') and reverse (3'-5') primer sequences are shown in Table 1. The housekeeping gene Rpl13a was used to normalize all values. Average raw $C_t$ values in control PAM212 cells were 22.4 for Mrp1, 28.5 for Mrp2, 30.3 for Nqo1, 20.4 for Ho-1, and 15.0 for Rpl13a. Average raw $C_t$ values in control WT primary mouse keratinocytes were 19.7 for Mrp1, 28.2 for Mrp2, 24.2 for Nqo1, 20.4 for Ho-1, and 15.6 for Rpl13a. While differences in primer efficiency can account for some variability, the differences in raw $C_t$ values seem to indicate that Mrp1 is much more highly expressed than Mrp2 or Mrp3.

## RESULTS

### A. Characterization of MRP1 and MRP2 in A549 cells

A549 cells express MRP1 and MRP2 as measured by western blotting (Figure 1, Panel A). Using a calcein efflux assay, these transporters were found to be functionally active in the cells. Efflux was inhibited by treatment of the cells with MK-571 (25 μM), a selective MRP1/MRP2 inhibitor [233] (Figure 1, Panel B).

A549 cells were found to be highly sensitive to HN2; the $IC_{50}$ for growth inhibition was 840 nM (Figure 2, Panel A). Treatment of A549 cells with the concentration of MK-571 above had no effect on cell growth. MK-571 (25 μM) was found to increase their sensitivity to HN2 ($IC_{50}$ = 150 nM). Growth of A549 cells was also inhibited by chlorambucil, melphalan, and the half mustard CEES (Figure 2 and Table 2, $IC_{50}$ = 30 μM, 65 μM, and 400 μM for chlorambucil, melphalan, and CEES, respectively). MK-571 (25 μM) increased sensitivity to chlorambucil, and melphalan, but not CEES ($IC_{50}$ = 1.8 μM, 7 μM, and 510 μM for chlorambucil, melphalan, and CEES, respectively).

### B. Effect of nitrogen mustards in HEK cells overexpressing MRP1 and MRP2

To assess whether MRP1 or MRP2 plays the predominant role mediating resistance to HN2 and other mustards, we examined effects on HEK cells overexpressing each transporter. First we confirmed the genotype of cells showing that the HEK MRP1 cells expressed MRP1 protein (Figure 3, Panel A) and had the ability to export fluorescent MRP substrate calcein (Figure 3, Panel C). We also confirmed that the HEK MRP2 cells likewise expressed MRP2 protein and possessed the ability to efflux calcein (Figure 3, Panels B and D). We also confirmed that both sets of HEK control cells possessed neither MRP1 or MRP2 protein expression or MRP functional activity (Figure 3, Panels A-D).

HEK MRP1 cells were more resistant to HN2-induced cell growth inhibition than HEK control cells (Figure 3, Panels E and G and Tables 3 and 4, $IC_{50}$ = 200 nM and 3.3 µM for HEK control cells and HEK MRP1 cells, respectively). This resistance is eliminated by pre-treatment with MK-571 ($IC_{50}$ = 200 nM), while inhibition has little effect on HEK Control cells ($IC_{50}$ = 0.12 µM).

HEK MRP2 cells are not more resistant than HEK control cells against HN2-induced growth inhibition (Figure 3, Panels F and H and Table 4, $IC_{50}$ = 73 nM and 82 nM for HEK control and HEK MRP2 cells, respectively). MK-571 also had no effect on either cell type ($IC_{50}$ = 110 nM for each cell type).

HEK MRP1 cells are also more resistant to melphalan and chlorambucil than HEK control cells (Figure 4 and Table 3, melphalan $IC_{50}$ = 1.2 µM and 20 µM for HEK control and HEK MRP1 cells, respectively; chlorambucil $IC_{50}$ = 0.91 µM and 11 µM for HEK control and HEK MRP1 cells, respectively). For each alkylating agent, treatment with MK-571 eliminated the protection provided by MRP1 ($IC_{50}$ = 2.0 µM and 1.2 µM for HEK MRP1 cells treated with melphalan and chlorambucil, respectively).

C. Effects of HN2, melphalan, and chlorambucil on MRP1 functional activity

Treatment of A549 cells with HN2 inhibited the efflux of the MRP1 substrate calcein (Figure 5, Panel A) with an $IC_{50}$ of 5.5 nM. Similar effects were seen following exposure to melphalan (Figure 5, Panel B, $IC_{50}$ = 63 nM) and chlorambucil (Figure 6, Panel C, $IC_{50}$ = 77 nM).

A similar effect was seen in HEK MRP1 cells, where significant inhibition of MRP1 functional activity was observed after treatment with 2 nM and 4 nM HN2 (Figure 6, Panel A). The efflux of calcein in the HEK control cells was significantly lower than

that of HEK MRP1 cells, and this background efflux was not inhibited by HN2. HN2 also inhibited efflux of calcein in HEK MRP2 cells, but 30 nM HN2 was required for significant inhibition of MRP2 function (Figure 6, Panel B).

HN2 (100 nM) also inhibits uptake of the fluorescent MRP1 substrate bimane-GS in inverted membrane vesicles prepared from Sf9 cells that overexpress the human MRP1 gene (Figure 7).

D. Effects of vesicants on etoposide-, methotrexate-, and vincristine-induced growth inhibition

Treatment of A549 cells with MRP1 ligand etoposide inhibited growth of A549 cells (Figure 8 and Table 6, $IC_{50}$ = 880 nM). Co-treatment with 10 nM, a concentration previously determined not to induce any growth inhibition in A549 cells, sensitized the cells to etoposide ($IC_{50}$ = 78 nM). This increase in sensitivity was saturated at 10 nM, and 30 nM HN2 did not cause any further increase in etoposide-induced cytotoxicity ($IC_{50}$ = 75 nM). Smaller effects were seen following co-treatment with 1 nM and 3 nM HN2 ($IC_{50}$= 440 nM and 230 nM for 1 nM and 3 nM HN2, respectively). An analogous increase was seen in methotrexate- and vincristine-induced cytotoxicity.

Similar effects were seen when cells were exposed to 1 µM melphalan or chlorambucil concurrent to treatment with etoposide (Figure 9, $IC_{50}$ = 730 nM for etoposide alone and 44 nM and 14 nM for cells co-treated melphalan and chlorambucil, respectively).

HN2 (3 nM and 10 nM) were also effective in sensitizing HEK cells overexpressing MRP1 to etoposide-induced growth inhibition (Figure 10, Panel A and Table 2, $IC_{50}$ = 960 nM for etoposide alone and 42 nM and 26 nM for cells co-treated with 3 nM and 10 nM HN2, respectively). HEK control cells were much more sensitive to

etoposide (Figure 10, Panel B and Table 2, $IC_{50}$ = 55 nM), and co-treatment with 3 nM and 10 nM HN2 caused no change to etoposide-induced growth inhibition ($IC_{50}$ = 47 nM and 48 nM for HEK control cells co-treated with 3 nM and 10 nM HN2, respectively).

We also investigated the effects of a 1 hr pre-treatment with 10 nM HN2 on etoposide-induced growth inhibition. In this experiment, the pre-treatment only caused a minimal increase in sensitivity to etoposide (Figure 11, Panel A, $IC_{50}$ = 460 nM and 370 nM without and with 10 nM HN2 pre-treatment). In this experiment, co-treatment with etoposide caused a similar change compared with the previous experiment ($IC_{50}$ = 77 nM). Because pre-treatment with HN2 was ineffective at sensitizing A549 cells, we investigated whether inhibition of calcein efflux by HN2 was reversible, and found that this inhibition is transient (Figure 11, Panel B).

F. Expression of Mrp1 in mouse keratinocytes

PAM212 cells and primary mouse keratinocytes seem to express Mrp1 mRNA at a much higher level than Mrp2 or Mrp3, as demonstrated by examination of the raw $C_t$ values following qPCR (Figure 12). While there may be differences in the efficiency of each primer, the differences are overwhelming. PAM212 cells and primary mouse keratinocytes were found to express Mrp1 mRNA and protein (Figures 13 and 14). Mrp1 was functionally active in both cell types as indicated by their ability to export calcein, a substrate for Mrp transporters (Figures 13 and 14). Mrp2 mRNA was also expressed in the cells, but at much lower levels than Mrp1. Both PAM212 cells and primary keratinocytes were highly sensitive to growth inhibition by HN2 ($IC_{50}$ = 1.4 µM and 1.0 µM for PAM212 cells and primary keratinocytes, respectively). Treatment with the Mrp1 specific inhibitor, MK-571, was found to completely inhibit calcein export in primary keratinocytes and somewhat inhibit this activity in PAM212 cells (Figures 13 and 14).

These data indicate that Mrp1 is active in exporting calcein in primary keratinocytes. Treatment of these cells with MK-571 markedly enhanced growth inhibition in primary keratinocytes ($IC_{50}$ = 0.48 µM), but had minimal effects on growth inhibition in PAM212 cells. These data are consistent with the idea that primary keratinocytes have the ability to export HN2 or an HN2 metabolite and thus limit toxicity.

G. Effects of sulforaphane on Mrp1 in mouse keratinocytes

We next analyzed the effects of sulforaphane on Mrp1 and HN2-induced growth inhibition in the keratinocytes. In PAM212 cells, treatment with 3 µM sulforaphane increased expression of total cellular Nrf2 and Nrf2 localized in the nuclei (Figure 15). That Nrf2 was functionally active was indicated by the fact that sulforaphane increased expression of two downstream gene products known to be regulated by Nrf2, Nqo-1 and Ho-1 (Figure 15). Nqo1 and Ho-1 were also upregulated in primary keratinocytes treated with sulforaphane (Figure 16).

Sulforaphane was also found to upregulate Mrp1 mRNA in both primary keratinocytes and PAM212 cells (Figures 13 and 14). Mrp2 mRNA was constitutively expressed at low levels in both cell types and was not altered by sulforaphane. Increased expression of Mrp1 protein was also evident following treatment of the cells with sulforaphane (Figures 13 and 14). This resulted in an increase in the rate of calcein export in both cell types which was inhibited by MK-571 (Figures 13 and 14). In primary keratinocytes and PAM212 cells, increases in Mrp1 by sulforaphane were associated with decreased sensitivity of the cells to HN2-induced growth inhibition (Figure 17 and Table 9). These increases were blocked by MK-571.

The ability of Nrf2 to mediate Mrp1 upregulation and sensitivity to HN2 was confirmed using primary keratinocytes from $Nrf2^{-/-}$ mice. In these cells, sulforaphane does not increase Mrp1 mRNA or protein and it does not increase calcein export

(Figures 13 and 14). However, calcein export is sensitive to MK-571 in Nrf2$^{-/-}$ cells (Figure 14). These cells are more sensitive than wild type cells to HN2 ($IC_{50}$ = 1.4 µM vs. 0.31 µM, respectively, Table 2). Like the wild type cells, treatment of Nrf2$^{-/-}$ cells with MK-571 also increased their sensitivity to HN2 ($IC_{50}$ = 0.080 µM, Table 9). However, in contrast to wild type cells, sulforaphane increased the sensitivity of Nrf2$^{-/-}$ cells to HN2 ($IC_{50}$ = 0.14 µM). A further increase in sensitivity of the cells to HN2 was evident after treatment with MK-571 and sulforaphane ($IC_{50}$ = 0.074 µM).

H. Role of glutathione *S*-transferases in HN2-induced growth inhibition in PAM212 cells

Since many Mrp1/MRP1 ligands are glutathione conjugates [57, 234-236], we sought to determine whether or not glutathione *S*-transferase enzymes are involved in protection of mouse keratinocytes. Inhibition of glutathione *S*-transferases using 3 µM ethacrynic acid does not alter the effects of sulforaphane on HN2 toxicity by sulforaphane (Figure 17, Panel B, $IC_{50}$ = 13 µM with sulforaphane and 14 µM with both sulforaphane and ethacrynic acid). In fact, pre-treatment with ethacrynic acid alone protects PAM212 cells from HN2 ($IC_{50}$ = 3.6 µM). This indicates that HN2 is likely conjugated by GSH in a non-enzymatic reaction. Inhibition of glutathione *S*-transferases can reduce the amount of competing reactions that involve GSH, making more GSH available to react with HN2.

I. Effects of HN2 on Mrp1 functional activity in PAM212 cells

Treatment with HN2 inhibited the efflux of Mrp1 substrate calcein and also eliminated the induction of Mrp1 functional activity by sulforaphane at concentrations ranging from 1 to 10 nM (Figure 18).

# DISCUSSION

MRP1/Mrp1 is a membrane-bound transport protein implicated in limiting the cytotoxicity and thereby decreasing efficacy of a number of cancer chemotherapeutic agents mainly via its ability to mediate the ATP-dependent cellular efflux of glutathione-, glucuronide-, and other amphiphatic conjugates [45]. Earlier studies reported that glutathione *S*-conjugates of several HN2-derived compounds including those of chlorambucil and melphalan are mediated by MRP1/Mrp1 [2-4]. Indeed both activity of MRP1/Mrp1 and glutathione *S*-conjugation reactions are thought to be important in mediating toxicity of nitrogen mustards [2-4]. This is due, in part, to the ability of MRP1/Mrp1 to eliminate nitrogen mustard monoglutathionyl conjugates that may be potent inhibitors of cytosolic glutathione *S*-transferase enzymes [4].

## A. Modulation of MRP activity in A549 human alveolar epithelial cells

MRP functional activity plays an important role in mediating HN2-induced growth inhibition in A549 human lung epithelial cells. These cells express both MRP1 and MRP2 proteins and readily export the fluorescent MRP substrate calcein. HN2 and related nitrogen mustards chlorambucil and melphalan inhibit cell growth, and pharmacological inhibition of MRP transporters by MK-571 caused a marked increase in their sensitivity to HN2. It has previously been demonstrated that MK-571 inhibits efflux of the endogenous glutathione-conjugated MRP1/Mrp1 ligand leukotriene $C_4$ [81], as well as glutathione-conjugated xenobiotics such as cisplatin [237], doxorubicin [238], and vincristine [239]. It is notable that a similar effect is seen in several other human tumor cell lines, where pharmacological inhibition of MRP transporters by MK-571 is effective in increasing HN2-induced growth inhibition. These cell lines are MCF7 (breast cancer), HeLa (cervical cancer), HepG2 (liver cancer), and BeWo (placental cancer), and as

sensitization is observed for HN2, melphalan, and chlorambucil in each cell line (Supplemental Figures 1, 2, 3, and 4).

HN2 also inhibited export of the fluorescent MRP substrate calcein in A549 cells with an $IC_{50}$ of 5.5 nM. Melphalan and chlorambucil also inhibited efflux of calcein at non-cytotoxic concentrations.

B. Relative roles of MRP1 and MRP2 mediating resistance of HEK cells to HN2

To further characterize the relative roles of MRP1 and MRP2 mediating resistance to HN2, we examined the effects on HN2-induced growth inhibition in HEK cells that overexpress functionally active MRP1 and MRP2. Cells that express MRP1 showed an order of magnitude decrease in their sensitivity to HN2, and this resistance to HN2 was inhibited by MK-571, whereas HEK MRP2 cells were equally sensitive to HEK control cells. This finding is consistent with an earlier work that demonstrated that a monoglutathionyl conjugate of HN2 analog chlorambucil is more readily taken up by inside-out membrane vesicles prepared from MCF7 cells overexpressing MRP1 than those overexpressing MRP2 [168]. We also confirmed that MRP1 expression also confers resistance to chlorambucil and melphalan and that resistance to these compounds was inhibited by MK-571.

HN2 inhibited functional activity in the nanomolar range in two MRP1 overexpressing systems. HN2 was shown to significantly inhibit the efflux of the fluorescent MRP1 substrate calcein in intact HEK cells overexpressing MRP1 at concentrations as low as 2 nM and 4 nM. HN2 also inhibited uptake of the fluorescent MRP1 substrate bimane-GS in inverted membrane vesicles from Sf9 cells transfected with the human MRP1 gene.

HN2 also inhibited functional activity in HEK cells overexpressing MRP2. However, an order of magnitude higher concentration, 30 nM HN2, was required to

observe significant inhibition of calcein efflux. These are far from the first MRP1/Mrp1 substrates that have been shown to also be inhibitors of MRP1/Mrp1-mediated, as glutathione conjugates of doxorubicin and duanorubicin, two other MRP1/Mrp1 substrates have been shown inhibit the uptake of leukotriene $C_4$ in inverted membrane vesicles prepared from SR3A cells, but this inhibition occurs in the micromolar range [240]. These data demonstrate that MRP1 is the more appropriate pathway to target to increase cells' sensitivity to HN2 when compared with MRP2.

C. Combination treatment of HN2 with etoposide, methotrexate, and vincristine

It has long been suggested and observed that multiple drugs are more effective than just one compound when treating tumors. In fact, treatment with etoposide and cisplatin or chlorambucil has been the standard of care to treat B cell lymphoma for many years [241]. Another common "cocktail" of anticancer agents is the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone, also known as CHOP, which is commonly used and effective in combatting non-Hodgkins lymphoma [242]. Interestingly, cyclophosphamide is a nitrogen mustard-derived compound that has, similar to chlorambucil and melphalan, been hypothesized to be a potential substrate for MRP1/Mrp1 once conjugated to glutathione [243]. In fact, a correlation was observed between MRP1 expression and poor response to cyclophosphamide for breast cancer patients using this drug [244]. Vincristine and doxorubicin, two other compounds that are included in CHOP, are also known substrates for MRP1/Mrp1 [103, 148, 153, 245]. Despite the fact that it has been known for some time that treatment with multiple cancer drugs is more efficacious than treatment with just one drug, and the impact can often be greater than an additive effect, the mechanism for such an interaction between treatments has remained elusive [246-248]. It has long been thought that HN2 can

sensitize tumors to other cancer chemotherapeutic agents simply by causing DNA damage [22].

Non-cytotoxic concentrations of HN2 may have the ability to sensitize tumors to another anticancer drug that can act as a substrate for MRP1. This explanation could also prove useful in limiting the concentration used for various cancer chemotherapeutic compounds to thereby limit side effects. Because of this finding and potential implication, we investigated whether 1 nM, 3nM, 10 nM, and 30 nM HN2 could be employed to sensitize cells to etoposide, methotrexate, or vincristine and propose a novel mechanism mediating the efficacy of combination therapy in cancer treatment. Etoposide is an anticancer agent that causes cytotoxicity through inhibition of topoisomerase II, leading to DNA strand breaks [249] that has long been thought to be exported from tumor cells by MRP1/Mrp1 [145, 146, 250-255]. Methotrexate is an inhibitor of dihydrofolate reductase that also is implicated as a substrate for MRP1/Mrp1 [216, 221, 256-258]. Vincristine is a microtubule-disrupting anti-mitotic compound also transported by MRP1/Mrp1 [103, 148, 259-262]. Co-treatment with 3 nM, 10 nM, and 30 nM HN2 caused significant decreases in the $IC_{50}$ for A549 cell growth inhibition, as compared with treatment with etoposide alone, and these sensitizations were similar to the six-fold increase in sensitivity when treating with 25 μM MK-571. Co-treatment with etoposide and either chlorambucil or melphalan also is effective in increasing the sensitivity of A549 cells to etoposide-induced growth inhibition; however, the concentration of each required to observe significant increases in etoposide-induced growth inhibition is much higher, 1 μM. This concentration is not cytotoxic for either chlorambucil or melphalan.

HN2 (3 nM and 10 nM) also enhanced etoposide-induced growth inhibition in HEK cells that overexpress MRP1 but not in HEK control cells, indicating that MRP1

expression is necessary in order for these sub-cytotoxic concentrations of HN2 to sensitize cells to etoposide.

We further determined that pre-treatment with HN2 does not increase the sensitivity of A549 cells to etoposide and that the inhibition of calcein export by HN2 in A549 cells is reversible. Taken together and combined with previous work suggesting that glutathione *S*-conjugates of chlorambucil and melphalan serve as substrates for MRP1/Mrp1 [4, 168, 203, 263], these data suggest that HN2 is acting as a reversible inhibitor of MRP1 by forming a conjugate to glutathione which also acts as a substrate for the transporter (Scheme 7). Because the human MRP1 protein contains twenty-five cysteine residues [131], we cannot completely eliminate the possibility that HN2, a highly electrophilic compound, is forming *S*-conjugates with some of these cysteine residues, rather than glutathione. In fact, there are seven cysteine residues near the extracellular amino terminus of the protein, and two of these, cysteine-7 and cysteine-32 have been determined to be required for MRP1 functional activity [133]. Because of the remarkably low concentration of HN2 required to observe inhibition in functional activity, a direct alkylation, and especially one in an extracellular region of the protein that eliminates the need for HN2 to cross the cell membrane to interact with the protein seems compelling. However, the fact that the inhibition of MRP1 functional activity by HN2 is reversible and that pre-treatment with HN2 is ineffective enhancing cytotoxicity while co-treatment causes a large sensitization of A549 cells to etoposide suggests that HN2 does not inhibit MRP1 functional activity by directly alkylating the transporter. Combination therapy with HN2 and other MRP1 substrates with antitumor activity represents a promising pathway to enhance the efficacy of many of these compounds when they are used to treat tumors.

Clinically, combination therapy does not actually involve concurrent treatment between different anticancer drugs like we used in our experiments. Instead, the drugs are administered sequentially over a period of hours. For example, in CHOP, cyclophosphamide, doxorubicin, and vincristine are administered over a period of four hours on day 1, and prednisone is administered orally on days 1 through 5 [264]. This regimen is conducted this way because administered these cytotoxic drugs all at once has the potential to create significant side effects. However, when considering the ability of MRP1 substrates to inhibit the transport of other substrates, it would be worth considering treating with lower doses of one or more of these compounds but administering them concurrently rather than sequentially. This is especially true when these anticancer agents are reversible inhibitors of MRP1.

D. Induction of Mrp1 Activity through the Nrf2 Pathway

It has previously been shown that sulforaphane, an isothiocyanate found in cruciferous vegetables such as broccoli that activates the Nrf2 pathway, can lead to increases in MRP1 mRNA and protein expression in HepG2 human liver cancer and A549 human lung cancer cells [198]. It is worth noting that despite the fact that another group showed induction of MRP1 in A549 cells through the Nrf2 pathway by sulforaphane, we found that sulforaphane did not protect A549 cells against HN2-induced cell growth inhibition (Supplemental Figure 11). This finding is not extremely surprising because A549 cells are known to express high constitutive Nrf2 transcriptional activity [265]. Therefore, it is likely that the reason that these data show that sulforaphane does not protect A549 cells from HN2-induced growth inhibition is that the transcriptional activity of Nrf2 is already saturated and cannot be further agonized. For this reason, we decided to shift our focus to a different cell type to assess the transcription of Mrp1 through the Nrf2 pathway.

When considering the potential use of vesicants in chemical warfare, the other important target tissues besides the lungs are the skin and the eyes. Little work has been done to date to study transport proteins in either of these tissues. It is known that Mrp1 is expressed in mouse skin; however, little characterization of the substrate specificity and methods to regulate Mrp1 expression have been determined [266, 267]. MRP1 is also thought to be the predominant transport protein present on the epidermal layer in human keratinocytes [110, 268]. MRP1 has also been shown to be expressed in A375 human melanoma cells [269].

In the eye, even less work has been done to investigate drug transport. One group conducted qPCR and functional analysis to determine that MRP1, MRP2, and BCRP but not MDR1 are present with limited functional activity in transformed human corneal epithelial cells [270]. Some other groups have confirmed that MRP1 is present in human corneal epithelial cells as somewhat higher levels than other transporters [271-273]. It has also been demonstrated that these expression profiles are similar in rabbit [274], but the MRP1 protein was not found using immunohistochemical staining in the entire rat cornea [275]. Mrp1 has also been observed in rabbit conjunctival epithelial cells and confirmed via immunohistochemical staining to be localized on the basolateral membrane [276]. Similar to the skin, only the most basic characterization of transporter genes and proteins has been investigated in the eye. Examination of how these transporters affect toxins and toxicants in this tissue remains uninvestigated.

Because little is known about the interaction between Mrp1 and HN2 toxicity in the skin, we studied the impact of induction of Mrp1 in PAM212 cells, a murine keratinocyte cell line, as well as in primary mouse keratinocytes. Our studies demonstrate that sulforaphane treatment increases Mrp1 mRNA, protein expression, and catalytic activity in PAM212 cells and primary mouse keratinocytes isolated from WT mice. Mrp2 and Mrp3, also efflux pumps that can mediate the transport of glutathione-

conjugated electrophiles, were undetectable in PAM212 cells by western blot analysis. We did observe Mrp2 and Mrp3 mRNA expression; however, constitutive expression was much lower than that of Mrp1, and this expression was unaffected by treatment with sulforaphane. Sulforaphane protected PAM212 cells and primary WT cells against cell growth inhibition, and this protection was eliminated by MK-571, an Mrp1 antagonist.

We also showed that Mrp1 mRNA and protein expression and functional activity is unaffected by sulforaphane, as is HN2-induced growth inhibition in keratinocytes isolated from $Nrf2^{-/-}$ mice. Sulforaphane does not protect against HN2-induced toxicity in cells that lack Nrf2.

Mrp1 functional activity plays an important role in mediating HN2-induced growth inhibition in primary mouse keratinocytes. HN2 inhibits cell growth, and MK-571 caused a marked increase in their sensitivity to HN2. We have also demonstrated a similar protection from sulforaphane and suppression of that protection by MK-571 in human corneal epithelial cells (Supplemental Figure 21).

In PAM212 cells, sulforaphane was functionally active in causing a translocation of Nrf2 from the cytosol to the nucleus. Sulforaphane caused upregulation of both mRNA and protein for Nqo1 and Ho-1 in both PAM212 cells and primary keratinocytes from WT mice. There was no change in Nqo1 or Ho-1 in keratinocytes isolated from $Nrf2^{-/-}$ mice.

Inhibition of Mrp1 by MK-571 has minimal impact on HN2 cytotoxicity in PAM212 cells. This may be due to low constitutive Mrp1 expression. We also do not observe any decrease from constitutive Mrp1 function following treatment with MK-571. Constitutive Mrp1 activity may be so low that efflux of the fluorescent Mrp1 ligand calcein in PAM212 cells is either simple diffusion or stimulated by other transporters and therefore not inhibited by MK-571. One group showed a correlation between constitutive MRP1 protein expression and increases in cytotoxicity of the MRP1 ligand etoposide by

MK-571 in three human stomach cancer cell lines with variable expression [277]. Another possibility is that in the absence of an inducer, PAM212 cells have other factors that mediate HN2 toxicity.

Similar compounds, including cisplatin and other nitrogen mustards melphalan and chlorambucil are conjugated by glutathione and then transported by MRP/Mrp transporters [2, 4, 84]. We hypothesize that activation of the Nrf2 pathway protects mouse keratinocytes against HN2-induced cell growth inhibition by increasing the Mrp1-mediated efflux of glutathione conjugates of HN2 from cells. We further confirm previous work [278] that the reaction between HN2 and glutathione may be non-enzymatic because inhibition of glutathione *S*-transferases by ethacrynic acid causes no change to sulforaphane's protection of both PAM212 cells and primary mouse keratinocytes. In fact, ethacrynic acid alone causes a small attenuation of HN2-induced cytotoxicity. One possible explanation is that glutathione cannot conjugate compounds that require enzymatic reaction and therefore the free glutathione concentration is increased. Thus, more glutathione is available to react with HN2. HN2 also inhibits the efflux of calcein by Mrp1. If a glutathione-conjugate of HN2 can act as a substrate for Mrp1, then it follows that it would also act as a competitive inhibitor.

E. Implications in Cancer Pharmacology

Understanding the role that MRP1/Mrp1-mediated efflux transport plays in mustard injury has the potential to lead to exciting new therapy regimens to enhance the sensitivity of mustards in cancer chemotherapy. Reversing multidrug resistance through approaches such as sequence-specific gene silencing by RNA interference (RNAi) is a promising way to target these transporters with great specificity [279]. MRP1/Mrp1 can also be downregulated at the transcriptional level by small interfering RNAs (siRNA), which have been shown to enhance the efficacy of doxorubicin in several different

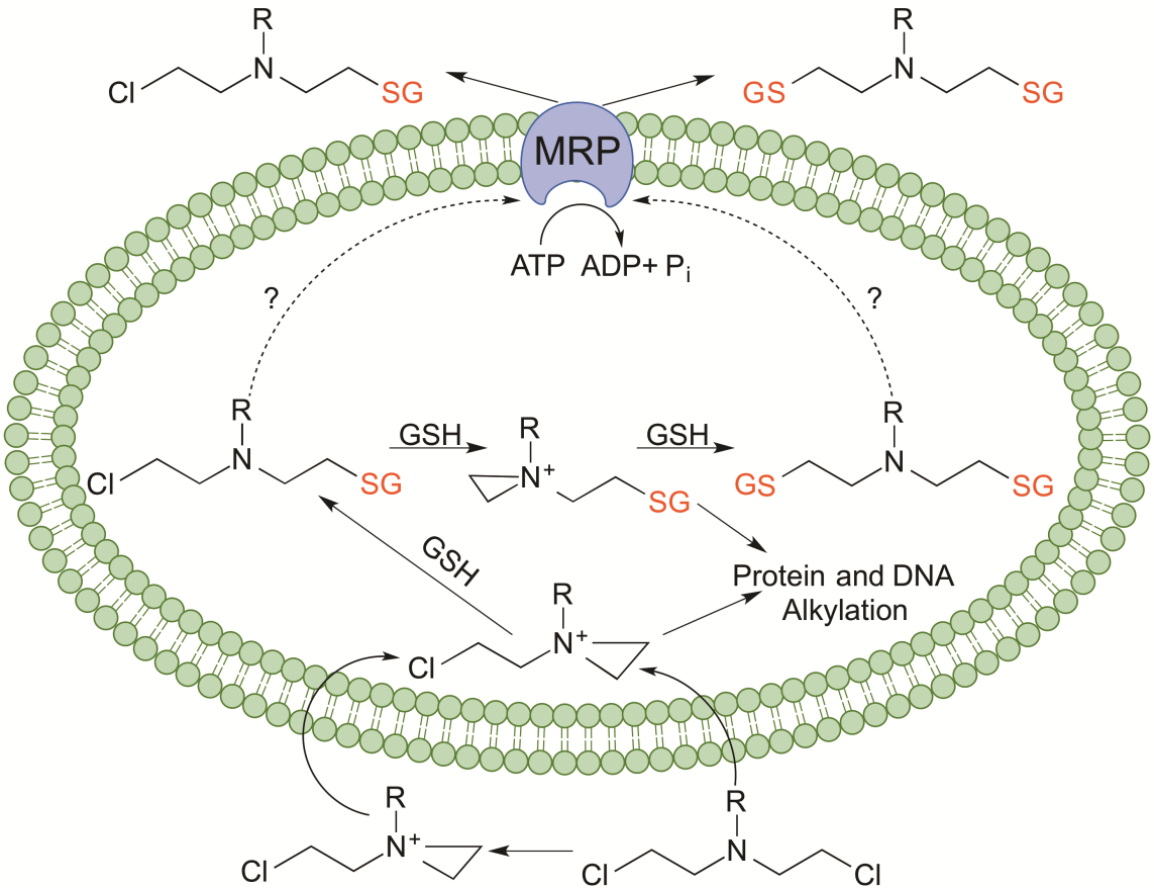
human tumor cell lines in the presence of cationic liposomes, which are used to carry the siRNA into the cell [280]. Another approach recently investigated to modulate MRP1/Mrp1 expression and functional activity is through micro RNAs (miRNAs). There are 463 human mature miRNA probes that have been tested in MCF7 human breast epithelial cells that showed resistance to etoposide [174]. While most of these miRNAs increased the expression of MRP1, treatment with miRNA-326, mi-RNA-429, mi-RNA-187, miRNA-7, and miRNA-92-2 caused decreases in the expression levels of MRP1 [174]. It was further determined that human breast tumors express lower constitutive levels of miRNA-326 than normal breast tissue, and transfection of etoposide-resistant MCF7 cells increased their sensitivity to both etoposide and doxorubicin [174]. One complicating factor of transcriptional approaches to suppress MRP1 expression in human tumors is possible compensation by other MRPs, especially MRP2. One study showed that samples from patients with acute myeloid leukemia who had MRP1 deleted by inversion of chromosome 16 still showed no difference in general MRP functional activity [281]. If the anticancer agents used, however, are more efficient substrates for MRP1 than for MRP2, however, this approach of transcriptional downregulation of MRP1 should still be helpful to patients.

There are also several known single nucleotide polymporphisms (SNP) in human MRP1 that potentially can cause alterations in multidrug resistance. An example of a naturally occurring mutation in the MRP1 protein is replacement of cysteine-43 with serine [282, 283], and this mutation decreases resistance to MRP1 ligands arsenite and vincristine [284]. Another SNP that causes decreases in MRP1 function is replacement of glycine-671 with valine, and this SNP has been shown to cause increased cardiotoxicity in non-Hodgkin's lymphoma patients treated with anthracyclines, another MRP1 ligand [285, 286]. There are many other polymorphisms in the protein sequence of MRP1 that are yet to be well-characterized, such as replacement of threonine-117

with methionine and serine-1512 with leucine [287]. Determination of MRP-mediated efflux of vesicants is also useful in determining susceptible populations to vesicant-induced toxicity [213-217]. Knowledge of individuals with diminished MRP1 function could lead to significant decreases in the appropriate dose when prescribing an MRP1 ligand in cancer chemotherapy or to selection of alternative compounds to treat tumors in these patients.

Characterization of the action of MRP1/Mrp1 mediating mustard toxicity can also help lead to exciting new therapies to combat exposure to HN2 and similar compounds when considering their history in chemical warfare. The Nrf2 pathway explored in this work is just one way to pharmacologically induce expression of these transport proteins. There are many nuclear receptors that also have the ability to increase transcription of MRP1/Mrp1, including the pregnane X receptor [201, 288]. It has also been shown that ultraviolet irradiation used in cancer chemotherapy can cause increased expression of both MRP1 and MDR1 [289]. Induction of these transporters may give exposed populations an improved recovery when exposed to mustard agents.

**Scheme 7. Proposed molecular mechanism by which HN2 interacts with MRP1/Mrp1.** HN2 reacts rapidly with water to form a highly electrophilic aziridinium ion. Once inside the cells, it reacts rapidly with intracellular nucleophiles such as DNA bases, cysteine residues on proteins, and other thiols such as the protective electrophile scavenging tripeptide glutathione (GSH). These data suggest that GSH-modified HN2 may be a substrate for MRP proteins, which commonly facilitate the efflux of GSH-conjugated electrophiles from cells.


R
N
Cl
SG
GS
MRP
ATP
ADP+ P$_i$
?
GSH
Protein and DNA
Alkylation

## CONCLUDING REMARKS

Sulfur mustard and HN2 are bifunctional alkylating agents with a wide variety of molecular targets relevant in both chemical warfare and cancer chemotherapy. The toxicity of some HN2-derived compounds such as chlorambucil, melphalan, cyclophosphamide, and bendamustine is limited by export from cells of glutathione *S*-conjugates of these compounds by the transport protein MRP1/Mrp1. This presents major problems limiting these compounds' efficacy when they are used as anticancer agents.

We found that pharmacological inhibition of MRPs by MK-571 transporters sensitizes A549 cells to HN2, as well as chlorambucil and melphalan. This inhibitor, however, does not sensitize cells exposed to the monofunctional alkylating agent CEES, likely because monoglutathonyl-CEES is no longer biological active once stably conjugated to glutathione. Bifunctional alkylating agents, on the contrary, can still react with biomolecules even after bound to one glutathione molecule.

We then further determined the relative roles of the two main membrane bound GS-X pumps, MRP1 and MRP2, in limiting mustard toxicity using HEK cells that overexpress each of these proteins. We determined that cells that express MRP1 are resistant to HN2, while cells that express MRP2 do not possess such resistance.

In both A549 cells and overexpressing systems, HN2 inhibited MRP1 functional activity in the low nanomolar range. This led to the conclusion that HN2 might be effective at these concentrations in combination with another anticancer drug that is also limited in efficacy by MRP1-catalyzed efflux. Indeed, we determined that co-treatment with 3 nM, 10 nM and 30 nM HN2 does cause significant increases in the sensitivity of A549 cells to etoposide-, methotrexate-, and vincristine-induced cell growth inhibition. Because of the remarkably low concentration at which HN2 inhibits MRP1 functional

activity and increases the cytotoxicity of etoposide, we hypothesized that HN2 may be acting through a direct alkylation of one of the twenty-five cysteine residues in the human MRP1 protein, especially one of the seven in the extracellular amino terminus. Modification of one of these amino terminal cysteine residues seemed likely because interaction between HN2 and an extracellular amino acid residue would eliminate the need for HN2 to penetrate the cell's plasma membrane at all; however, we also observed that pre-treatment with 10 nM HN2 causes very little sensitization of A549 cells to etoposide-induced growth inhibition, and that pre-treating A549 cells also has no effect on MRP-mediated efflux of fluorescent substrate calcein. A direct alkylation would almost certainly be irreversible, so this finding makes it more likely that competitive inhibition is taking place. Regardless of the exact mechanism of MRP1 inhibition, this finding represents a promising pathway to explore in terms of enhancing the activity of antitumor agents and also provides a novel explanation for why combination therapy is effective in cancer treatment.

Another way to mediate MRP1/Mrp1 functional activity is to activate transcription factors that will initiate transcription of its mRNA. An example of such a transcription factor is Nrf2. For these experiments, we used mouse keratinocytes because it was necessary to investigate a cell line, which unlike A549 cells, has a low level on constitutively active Nrf2 transcription and basal MRP1/Mrp1 expression. In both PAM212 cells and primary mouse keratinocytes, we observed an increase in Mrp1 mRNA and protein expression, as well as functional activity following treatment with sulforaphane, an isothiocyanate known to activate Nrf2. This increase in Mrp1 following sulforaphane treatment is associated with an attenuation of HN2-induced growth inhibition, both in PAM212 cells and wild-type primary mouse keratinocytes, and this protection is completely reversed by Mrp1 inhibitor MK-571. Enhancing MRP1/Mrp1 functional activity through Nrf2 or other transcription factors represents a possible

pathway to target to combat exposure to mustards as chemical warfare agents. It also may be an important way to mitigate side effects in non-target tissues when using mustards in cancer chemotherapy, or antagonism of MRP1/Mrp1 transcription could be effective in increasing the efficacy of these alkylating agents in cancer chemotherapy.

Taken together, these results suggest a mechanism by which HN2 and other mustard agents are exported from cells, as well as providing a novel explanation for the effectiveness of combination therapy in cancer treatment.

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# FIGURES AND TABLES

**Table 1. qPCR primer sequences**

| Gene | Forward 5'-->3' | Reverse 5'-->3' |
|---|---|---|
| Mrp1/Abcc1 | GCTGTGGTGGGCGCTGTCTA | CCCAGGCTCAGCCACAGGAA |
| Mrp2/Abcc2 | AGCAGGTGTTCGTTGTGTGT | AGCCAAGTGCATAGGTAGAGAAT |
| Mrp3/Abcc3 | CTGGGTCCCCTGCATCTAC | GCCGTCTTGAGCCTGGATAAC |
| Nqo1 | CTCAACATCTGGAGCCATGG | CAGCTCACCTGTGATGTCATT |
| Ho-1 | GGTGACAGAAGAGGCTAAGACCGC | GCAGTATCTTGCACCAGGCTAGCA |
| Rpl13a | GGGCAGGTTCTGGTATTGGAT | GGCTCGGAAATGGTAGGGG |

**Table 2. Effects of inhibition of MRP1 and MRP2 on the sensitivity of A549 cells to vesicant-induced growth inhibition.**

| | control | + 25 µM MK-571 |
|---|---|---|
| | $IC_{50}$ (µM) | |
| HN2 | 0.84 | 0.15 |
| melphalan | 65 | 1.8 |
| chlorambucil | 30 | 7 |
| CEES | 400 | 510 |

A549 were treated with 25 µM MK-571 or control in serum-free medium. After 1 hr, increasing concentrations of HN2, chlorambucil, melphalan, or CEES were added to the medium. After an additional 30 min, cells were washed free of the drugs and returned to complete growth medium. Seventy-two hr later, cells were washed and counted using a Coulter counter (*See Figure 2)*.

**Table 3. HEK293 cells overexpressing MRP1 are resistant to HN2-, melphalan-, and chlorambucil- induced growth inhibition.**

| | HEK control | | HEK MRP1 | |
|---|---|---|---|---|
| | No MK-571 | + 25 µM MK-571 | No MK-571 | + 25 µM MK-571 |
| | $IC_{50}$ (nM) | | | |
| HN2 | 200 | 200 | 3300 | 120 |
| melphalan | 1200 | 2000 | 20000 | 2000 |
| chlorambucil | 590 | 950 | 19000 | 1200 |

HEK MRP1 or HEK control cells were incubated in the absence or presence of 25 µM MK-571. After 1 hr, increasing concentrations of HN2, melphalan, or chlorambucil were added to the medium. After an additional 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter (*See Figures 3 and 4)*.

**Table 4. HEK293 cells overexpressing MRP1 but not those overexpressing MRP2 are resistant to HN2-induced growth inhibition.**

| | HEK control | | HEK MRP1 or MRP2 | |
|---|---|---|---|---|
| | No MK-571 | + 25 µM MK-571 | No MK-571 | + 25 µM MK-571 |
| | $IC_{50}$ (nM) | | | |
| MRP1 | 200 | 200 | 3300 | 120 |
| MRP2 | 73 | 110 | 82 | 110 |

HEK MRP1 or HEK MRP2 cells or HEK control cells were incubated in the absence or presence of 25 µM MK-571. After 1 hr, increasing concentrations of HN2 were added to the medium. After an additional 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter (*See Figure 3)*.

**Table 5. Effects of HN2 on etoposide-, methotrexate-, and vincristine-induced growth inhibition in A549 cells.**

| | Etoposide | Methotrexate | Vincristine |
|---|---|---|---|
| | | $IC_{50}$ (nM) | |
| control | 880 | 230 | 360 |
| + 1 nM HN2 | 440 | 210 | 170 |
| + 3 nM HN2 | 230 | 88 | 82 |
| + 10 nM HN2 | 78 | 48 | 45 |
| + 30 nM HN2 | 75 | 48 | 42 |
| + 25 µM MK-571 | 230 | 70 | 67 |

A549 cells were treated with increasing concentrations of etoposide, methotrexate, or vincristine in the absence or presence of HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter (*See Figure 8 and Supplemental Figures 6, 7, and 8)*.

**Table 6. Effects of chlorambucil and melphalan on etoposide-induced growth inhibition in A549 cells.**

| | $IC_{50}$ (nM) | Fold Sensitization |
|---|---|---|
| control | 730 | |
| + 300 nM melphalan | 420 | 1.7 |
| + 1 µM melphalan | 44 | 17 |
| + 300 nM chlorambucil | 450 | 1.6 |
| + 1 µM chlorambucil | 14 | 53 |
| + 25 µM MK-571 | 57 | 13 |

A549 cells were treated with increasing concentrations of etoposide in the absence or presence of melphalan or chlorambucil in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter (*See Figure 9)*.

**Table 7. Effects of HN2 on etoposide-induced growth inhibition in HEK MRP1 cells.**

| | HEK MRP1 | HEK control |
|---|---|---|
| | $IC_{50}$ (nM) | |
| Etoposide only | 960 | 55 |
| + 3 nM HN2 | 42 | 47 |
| + 10 nM HN2 | 26 | 48 |
| + 25 µM MK-571 | 68 | 53 |

HEK MRP1 and HEK control cells were treated with increasing concentrations of etoposide absence or presence of HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. After 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter (*See Figure 10)*.

**Table 8. Effects of MK-571 on HN2-induced growth inhibition in PAM212 cells.**

| | $IC_{50}$ (μM) |
|---|---|
| control | 1.0 |
| + 3 μM SFN | 13 |
| + 25 μM MK-571 | 0.73 |
| + 3 μM SFN + 25 μM MK-571 | 0.63 |

Cells were treated with 3 μM sulforaphane or vehicle control in serum-free medium. After 3 hr, cells were treated with increasing concentrations of HN2. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter Counter. Some cells were treated with 25 μM MK-571 for 1 hr prior to sulforaphane treatment (*See Figure 17)*.

**Table 9. Effects of HN2 on growth inhibition in primary mouse keratinocytes**

| | Wild-type | $Nrf2^{-/-}$ |
|---|---|---|
| | $IC_{50}$ (μM) | |
| Control | 1.4 | 0.31 |
| + 3 μM SFN | 4.8 | 0.14 |
| + 25 μM MK-571 | 0.48 | 0.080 |
| + 3 μM MK-571 + 25 μM SFN | 0.27 | 0.074 |

Wild-type and $Nrf2^{-/-}$ cells were treated with 3 μM sulforaphane or vehicle control in serum-free medium. After 3 hr, cells were treated with increasing concentrations of HN2. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter Counter. Some cells were treated with 25 μM MK-571 in serum-free medium 1 hr prior to sulforaphane treatment.

**Figure 1. Protein expression and functional activity of MRP1 and MRP2 in A549 cells.** *Panel A*, Lysates prepared from A549 cells were analyzed for MRP1 and MRP2 protein expression by Western blotting. Lysates were obtained from two different cultures. *Panel B,* MK-571 inhibits efflux of the MRP substrate calcein. Cells were incubated with 1 μM calcein-AM and analyzed for MRP functional activity by measuring efflux of calcein in the absence or presence of 25 μM MK-571. Each data point represents the mean ± SEM (*n = 3). ***P < 0.001* compared with control.

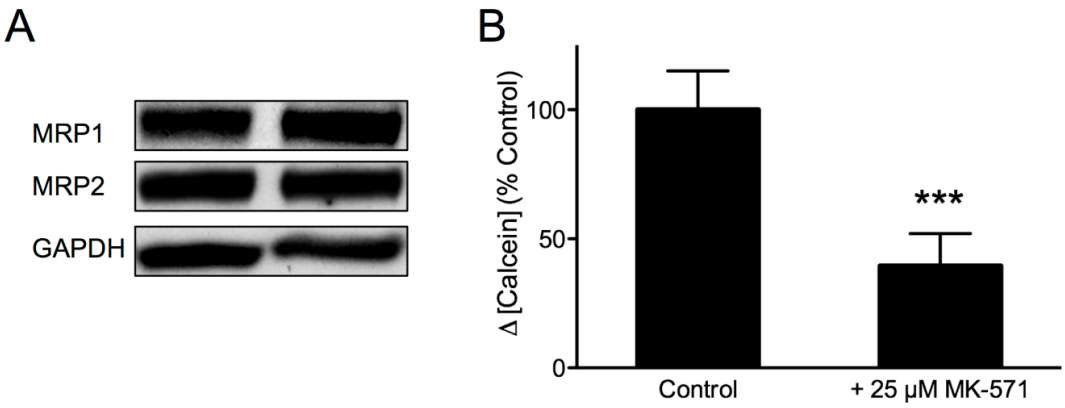

A
MRP1
MRP2
GAPDH
B
Δ [Calcein] (% Control)
100
50
0
***
Control
+ 25 μM MK-571

**Figure 2. Effects of inhibition of MRP1 and MRP2 on the sensitivity of A549 cells to vesicant-induced growth inhibition.** A549 were treated with 25 μM MK-571 or control in serum-free medium. After 1 hr, increasing concentrations of HN2 (*Panel A*), chlorambucil (*Panel B)*, melphalan (*Panel C)* or CEES (*Panel D*) were added to the medium. After an additional 30 min, cells were washed free of the drugs and returned to complete growth medium. Seventy-two hr later, cells were washed and counted using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*

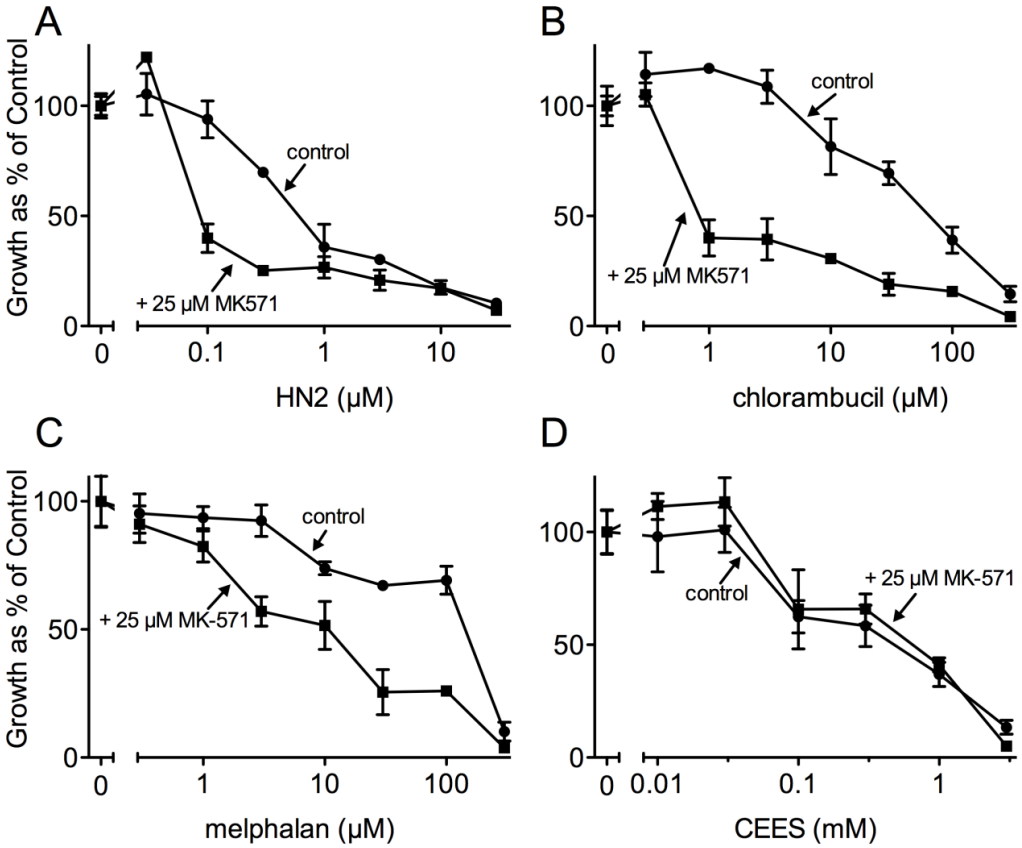

A
Growth as % of Control
100
50
0
control
+ 25 μM MK571
0
0.1
1
10
HN2 (μM)
B
100
50
0
control
+ 25 μM MK571
0
1
10
100
chlorambucil (μM)
C
Growth as % of Control
100
50
0
control
+ 25 μM MK-571
0
1
10
100
melphalan (μM)
D
100
50
0
control
+ 25 μM MK-571
0
0.01
0.1
1
CEES (mM)

**Figure 3. HEK293 cells overexpressing MRP1 are resistant to HN2-induced growth inhibition.** *Panels A and B,* Western blot analysis showing MRP1 (*Panel A)* and MRP2 (*Panel B)* protein expression in HEK control cells and those overexpressing MRP1 or MRP2. *Panels C and D,* Effects of overexpression of MRP transporters on efflux of the fluorescent substrate calcein. HEK MRP1 (*Panel C)* or HEK MRP2 (*Panel D)* cells were incubated in the absence and presence of 25 µM MK-571 and 1 µM calcein-AM. MRP1 or MRP2 functional activity was measured by the efflux of calcein. Each data point represents the mean ± SEM (*n = 3). *P < 0.05* compared with untreated HEK MRP1 or HEK MRP2 cells. HEK MRP1 (*Panel E)* or HEK MRP2 *(Panel G*) cells or HEK control cells (*Panels F and H)* were incubated in the absence or presence of 25 µM MK-571. After 1 hr, increasing concentrations of HN2 were added to the medium. After an additional 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter. Each data point represents the mean ± SEM (*n = 3).*

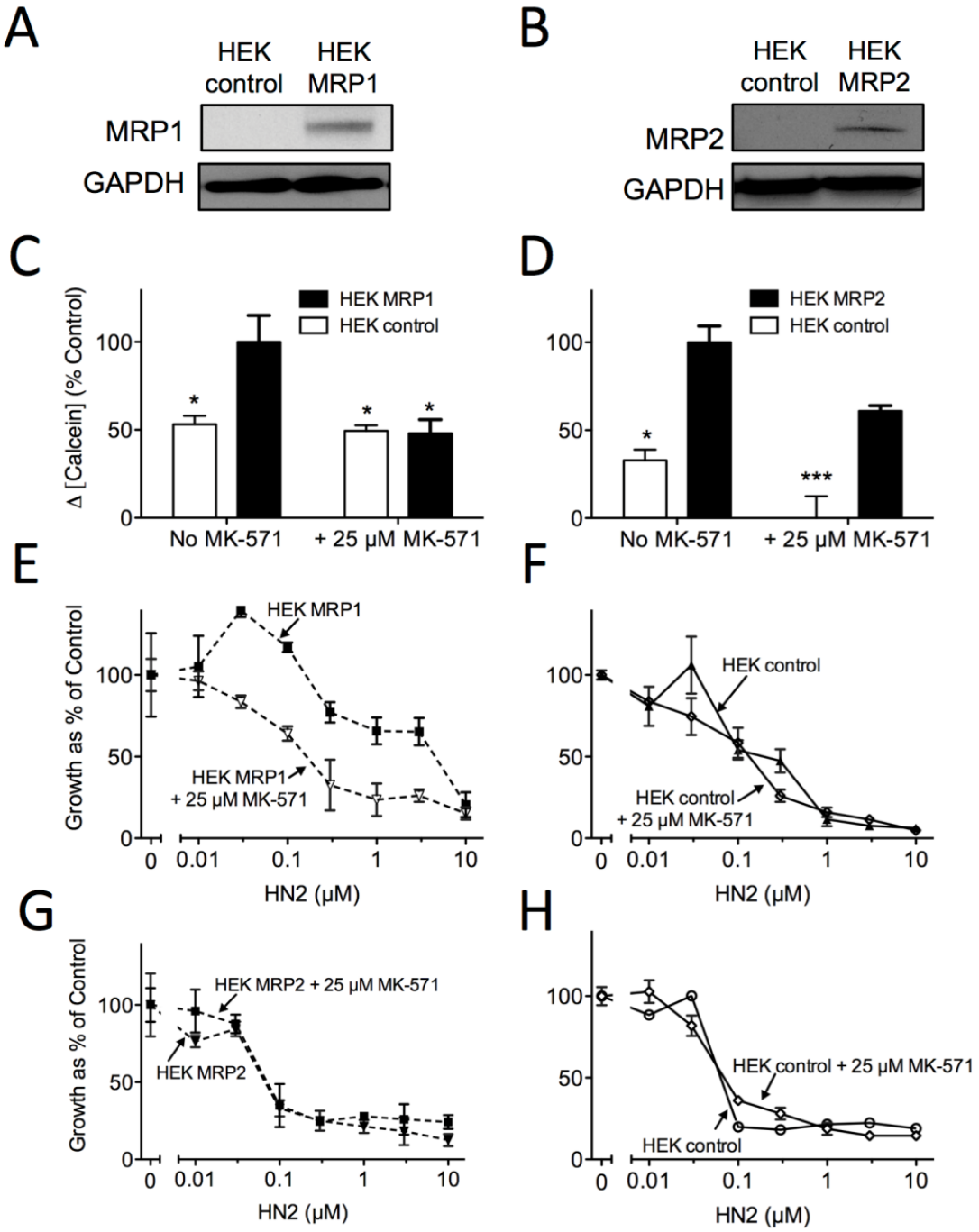

A
HEK control
HEK MRP1
MRP1
GAPDH
B
HEK control
HEK MRP2
MRP2
GAPDH
C
HEK MRP1
HEK control
Δ [Calcein] (% Control)
100
50
0
*
*
*
No MK-571
+ 25 μM MK-571
D
HEK MRP2
HEK control
100
50
0
*
***
No MK-571
+ 25 μM MK-571
E
Growth as % of Control
HEK MRP1
HEK MRP1
+ 25 μM MK-571
100
50
0
0
0.01
0.1
1
10
HN2 (μM)
F
HEK control
HEK control
+ 25 μM MK-571
100
50
0
0
0.01
0.1
1
10
HN2 (μM)
G
Growth as % of Control
HEK MRP2 + 25 μM MK-571
HEK MRP2
100
50
0
0
0.01
0.1
1
10
HN2 (μM)
H
HEK control + 25 μM MK-571
HEK control
100
50
0
0
0.01
0.1
1
10
HN2 (μM)

**Figure 4: Effects of overexpression of MRP1 on sensitivity of HEK cells to growth inhibition induced by chlorambucil and melphalan.** HEK MRP1 (*Panels A and B)* and HEK control cells (*Panels C and D)* were incubated in the absence or presence of 25 µM MK-571. After 1 hr, increasing concentrations of melphalan (*Panels A and C)* or chlorambucil (*Panels B and D)* were added to the culture medium. After an additional 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter. Each data point represents the mean ± SEM (*n = 3).*

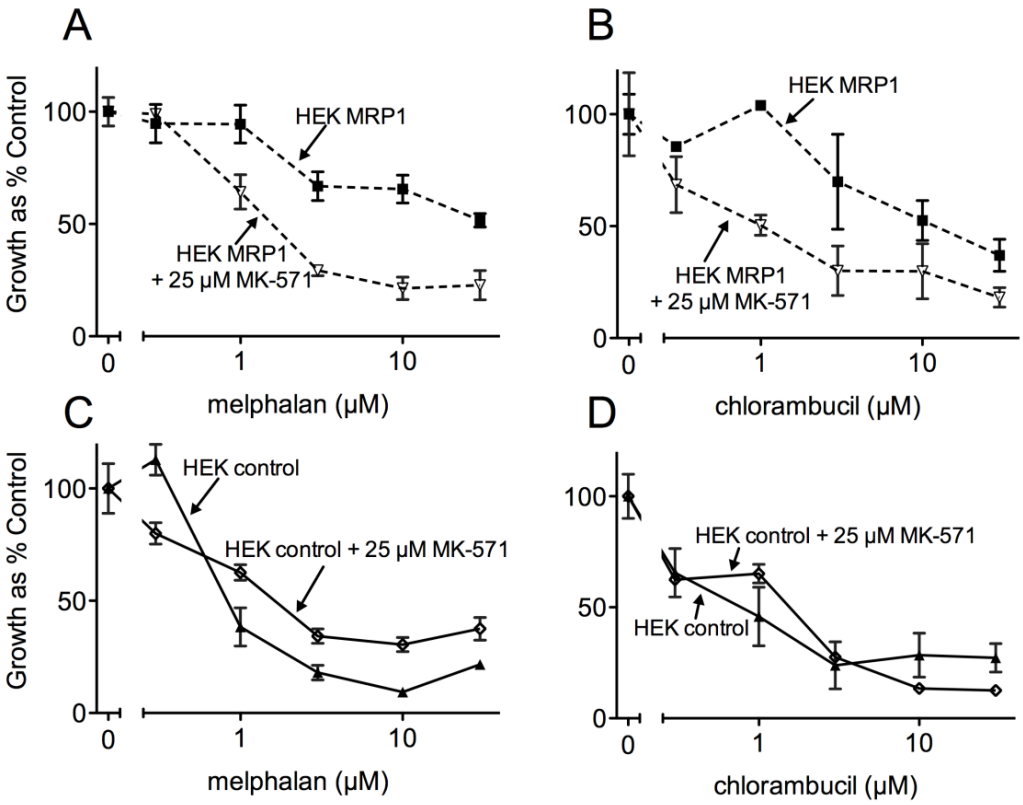

A
B
C
D
Growth as % Control
100
50
0
0
1
10
HEK MRP1
HEK MRP1
+ 25 μM MK-571
melphalan (μM)
chlorambucil (μM)
HEK control
HEK control + 25 μM MK-571

**Figure 5. Effects of HN2 on calcein efflux in A549 cells.** A549 cells were incubated with increasing concentrations HN2 (*Panel A)*, melphalan (*Panel B)*, and chlorambucil (*Panel C)* and 1 μM calcein-AM in serum-free medium. MRP functional activity was assayed by measuring the efflux of the fluorescent substrate calcein. Each data point represents the mean ± SEM (*n = 3).*

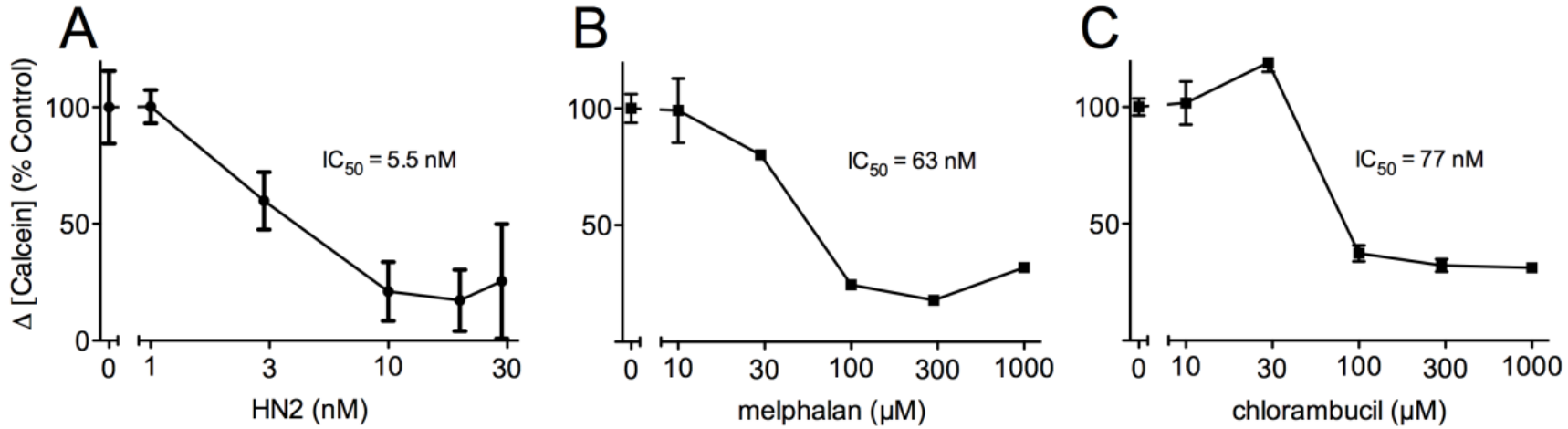

A
B
C
Δ [Calcein] (% Control)
100
50
0
IC50 = 5.5 nM
IC50 = 63 nM
IC50 = 77 nM
0 1 3 10 30
HN2 (nM)
0 10 30 100 300 1000
melphalan (μM)
0 10 30 100 300 1000
chlorambucil (μM)

**Figure 6. Effects of HN2 on calcein efflux in HEK MRP1 and HEK MRP2 cells.** HEK cells overexpressing MRP1 and vector control cells (*Panel A)* or cells overexpressing MRP2 and vector control cells (*Panel B)* were incubated with increasing concentrations HN2 and 1 μM calcein-AM in serum-free medium and efflux of fluorescent substrate calcein was measured. Each data point represents the mean ± SEM (*n = 3). ***P < 0.001, **P < 0.01 or *P < 0.05* for HEK MRP1 exposed to HN2 compared with untreated HEK MRP1. ●●●*P < 0.001,* ●*P < 0.05* for untreated HEK control cells compared with untreated HEK MRP1 cells.

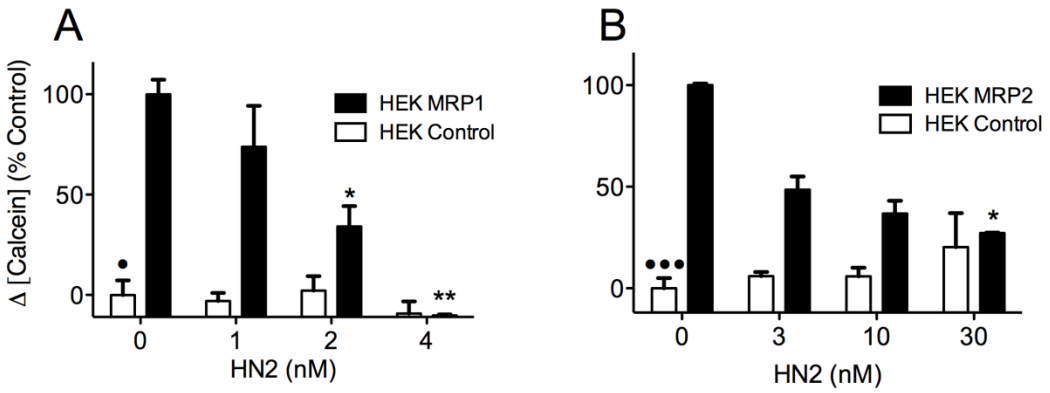

A
Δ [Calcein] (% Control)
100
50
0
HEK MRP1
HEK Control
*
**
0
1
2
4
HN2 (nM)
B
100
50
0
HEK MRP2
HEK Control
*
0
3
10
30
HN2 (nM)

**Figure 7. Effects of HN2 on the MRP1 transporter.** Bimane-GS, a fluorescent MRP1 substrate, is incorporated into inside out membrane vesicles isolated from insect cells overexpressing the MRP1 transporter. Treatment with 100 nM HN2 inhibited ATP-dependent transporter activity at 37ºC. Reaction mixtures containing vesicles were supplemented with 5 mM GSH. Each data point represents the mean ± SEM (*n = 6). *P < 0.05* compared with untreated MRP1 vesicles.

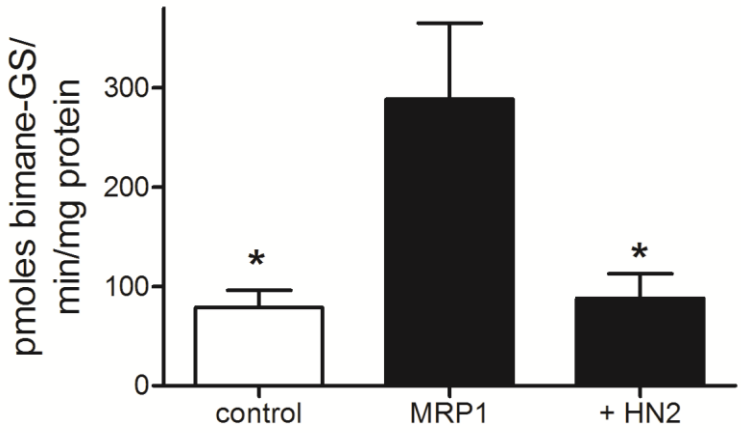

pmoles bimane-GS/
min/mg protein
300
200
100
0
*
*
control
MRP1
+ HN2

**Figure 8. Effects of HN2 on etoposide-, methotrexate-, and vincristine-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of etoposide (*Panel A)*, methotrexate (*Panel B)*, and vincristine (*Panel C)* in the absence or presence of HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*

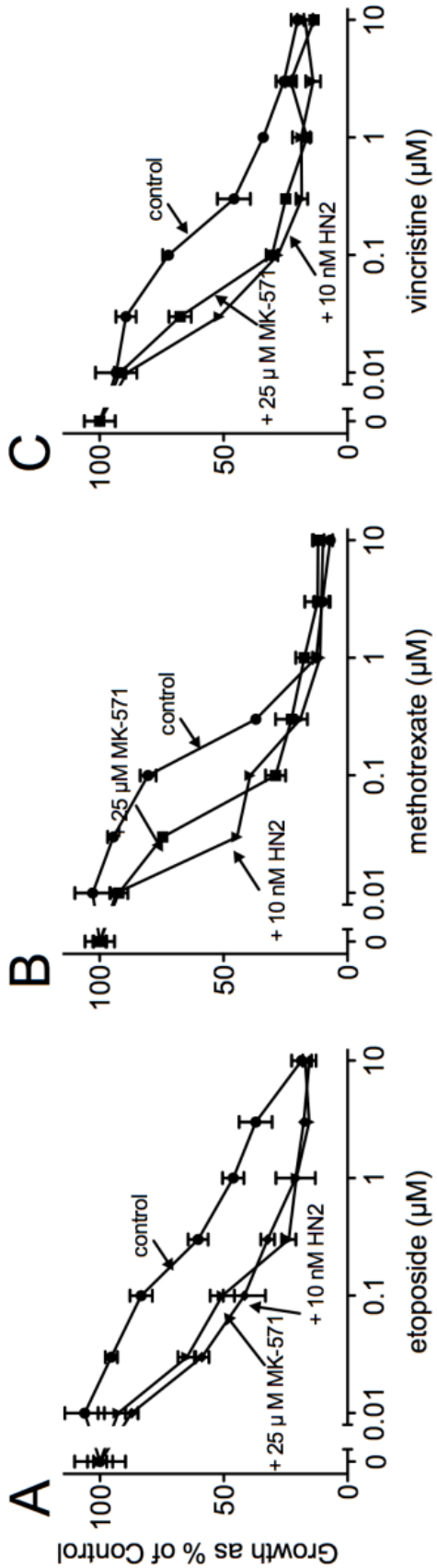

A
B
C
Growth as % of Control
100
50
0
0 0.01 0.1 1 10
etoposide (μM)
methotrexate (μM)
vincristine (μM)
control
+ 25 μ M MK-571
+ 10 nM HN2
+ 25 μM MK-571

**Figure 9. Effects of melphalan and chlorambucil on etoposide-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of etoposide in the absence or presence of melphalan (*Panel A*, or chlorambucil (*Panel B)* in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*

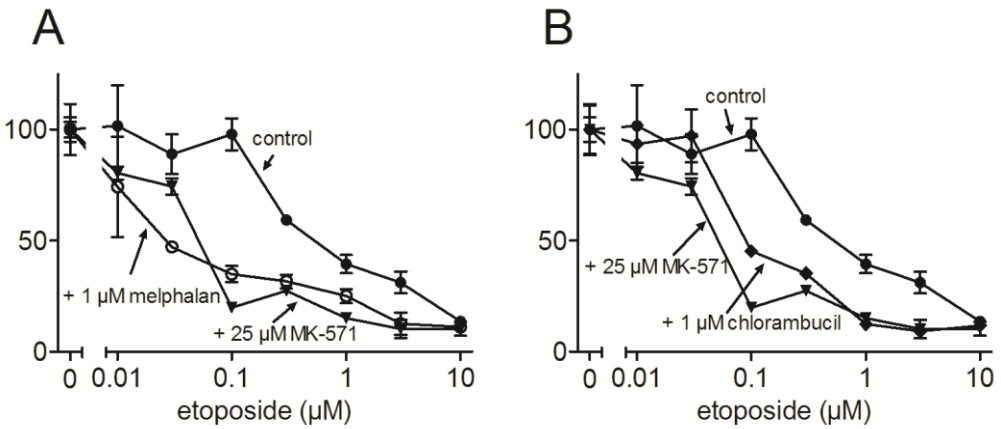

A
100
50
0
control
+ 1 μM melphalan
+ 25 μM MK-571
0
0.01
0.1
1
10
etoposide (μM)
B
100
50
0
control
+ 25 μM MK-571
+ 1 μM chlorambucil
0
0.01
0.1
1
10
etoposide (μM)

**Figure 10. Effects of HN2 on etoposide-induced growth inhibition in HEK MRP1 cells.** HEK MRP1 (*Panel A)* and HEK control *(Panel B)* cells were treated with increasing concentrations of etoposide absence or presence of HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. After 30 min, fetal bovine serum was added to cells (final concentration 10%). Seventy-two hr later, cells number was determined using a Coulter counter. Each data point represents the mean ± SEM (*n = 3).*

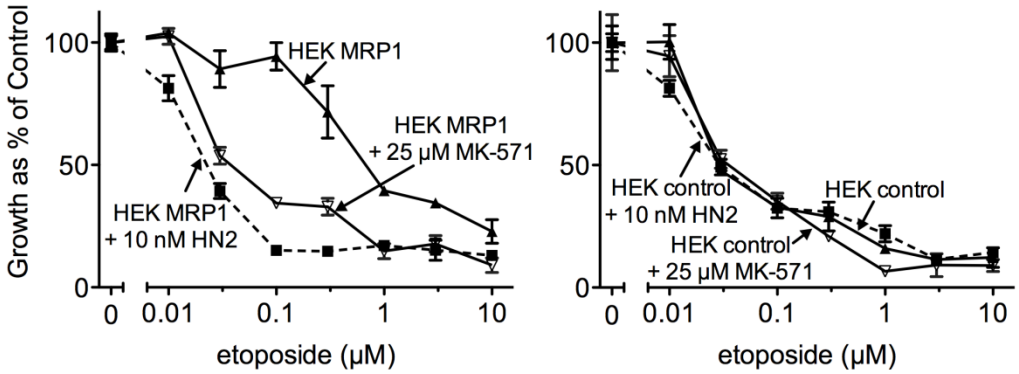

Growth as % of Control
100
50
0
HEK MRP1
HEK MRP1
+ 25 μM MK-571
HEK MRP1
+ 10 nM HN2
0
0.01
0.1
1
10
etoposide (μM)
100
50
0
HEK control
+ 10 nM HN2
HEK control
HEK control
+ 25 μM MK-571
0
0.01
0.1
1
10
etoposide (μM)

**Figure 11. Reversibility of HN2-induced increases in sensitivity of A549 cells to etoposide.** *Panel A*, A549 cells were treated with increasing concentrations of etoposide in serum-free medium. After 30 min, cells were washed and returned to complete growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Cells in the group labeled "pre-treatment" were incubated with 10 nM HN2 for 1 hr in serum-free medium prior to washing and treatment with etoposide alone. Cells in the group labeled co-treatment were exposed to 10 nM HN2 during the 30 min etoposide treatment. Each data point is the mean ± SEM (*n = 3). Panel B*, Reversibility of HN2 inhibition of calcein efflux from A549 cells. Cells were incubated with 1 μM calcein-AM and analyzed for calcein efflux. Cells in the group labeled "pre-treatment" were treated with 0, 10, or 30 nM HN2 for 1 hr in serum free medium, washed and incubated for 2 hr in growth medium, then incubated with 1 μM calcein-AM and analyzed for calcein efflux. Cells in the group labeled "co-treatment" were also exposed to 0, 10, or 30 nM HN2 during their incubation with calcein-AM. Each data point is the mean ± SEM (*n = 3). **P < 0.01* compared to no HN2 point within each group.

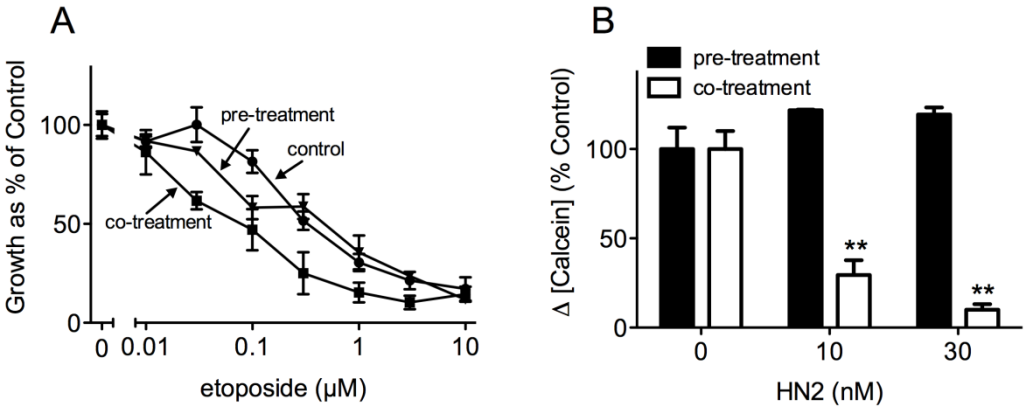

A
Growth as % of Control
100
50
0
pre-treatment
control
co-treatment
0
0.01
0.1
1
10
etoposide (μM)
B
pre-treatment
co-treatment
Δ [Calcein] (% Control)
100
50
0
**
**
0
10
30
HN2 (nM)

**Figure 12. Raw $C_t$ values for Mrp transporters in mouse keratinocytes.** PAM212 cells and primary mouse keratinocytes were analyzed for expression of Mrp1, Mrp2, and Mrp3 mRNA by qPCR. Data are means ± SEM. (*n = 6)*.

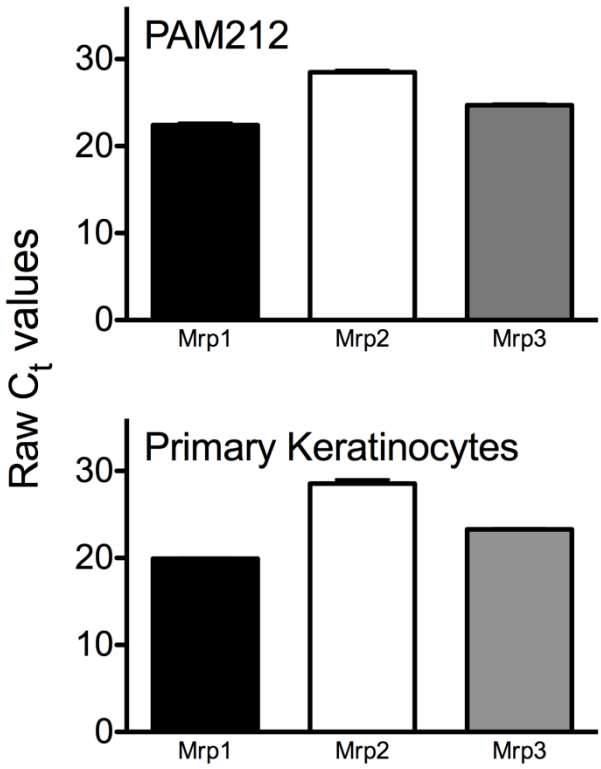

PAM212
30
20
10
0
Mrp1
Mrp2
Mrp3
Raw $C_t$ values
Primary Keratinocytes
30
20
10
0
Mrp1
Mrp2
Mrp3

**Figure 13. Effects of sulforaphane on Mrp expression in PAM212 cells.** *Panel A,* PAM212 cells were treated with 3 µM sufloraphane (SFN) or vehicle control and mRNA for Mrp1, Mrp2, and Mrp3 was analyzed using qPCR. Data are means ± SEM (*n = 4-6)*. **P< 0.01 compared with control. *Panel B,* Cells were treated with 3 µM sulforaphane or control, and cell homogenates were prepared and analyzed for Mrp1 protein expression by Western blotting. *Panel C*, Cells were incubated with 1 µM calcein-AM and analyzed for Mrp1 functional activity. Some were also treated with 25 µM MK-571. Other cells were pre-treated for 3 hr with 3 µM sulforaphane. Data are means ± SEM (*n = 5*). **P < 0.01, *P < 0.05 compared with untreated control within each cell type.

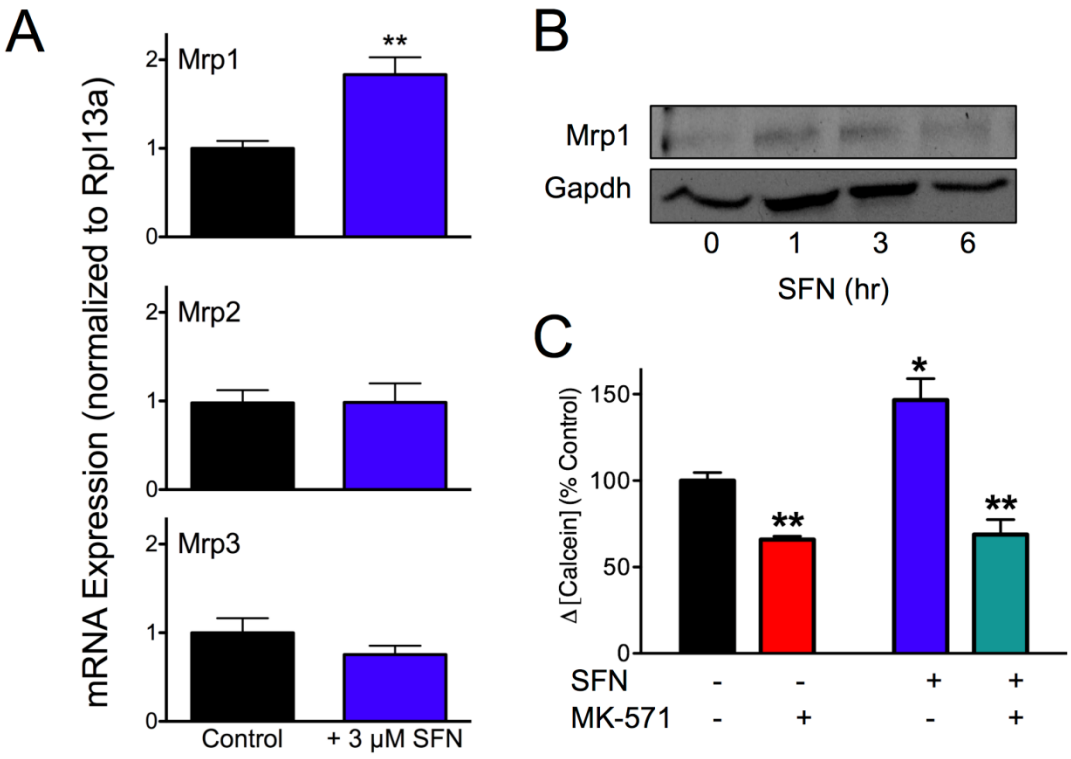

A
mRNA Expression (normalized to Rpl13a)
Mrp1
Mrp2
Mrp3
**
Control
+ 3 μM SFN
B
Mrp1
Gapdh
0
1
3
6
SFN (hr)
C
Δ [Calcein] (% Control)
150
100
50
0
*
**
**
SFN - - + +
MK-571 - + - +

**Figure 14. Effects of sulforaphane on Mrp transporters in primary mouse keratinocytes.** *Panel A,* Primary mouse keratinocytes from wild type and Nrf2$^{-/-}$ mice were treated with 3 µM sulforaphane (SFN) or control. After 3 hr, mRNA for Mrp1, Mrp2, and Mrp3 was analyzed using qPCR. Data are means ± SEM (*n = 4-6)*. **P< 0.01 compared with control. *Panel B,* Keratinocytes were treated with 3 µM sulforaphane or control for 0, 1, 3 or 6 hr. After the indicated times, cells were refed with growth medium. After an additional 24 hr, cell homogenates were prepared and analyzed for Mrp1 by Western blotting. *Panel C*, Cells were incubated with 1 µM calcein-AM and analyzed for Mrp1 functional activity. Some were also treated with 25 µM MK-571. Other cells were pre-treated for 3 hr with 3 µM sulforaphane. Data are means ± SEM (*n = 5*). *P < 0.05 compared with untreated control within each cell type.

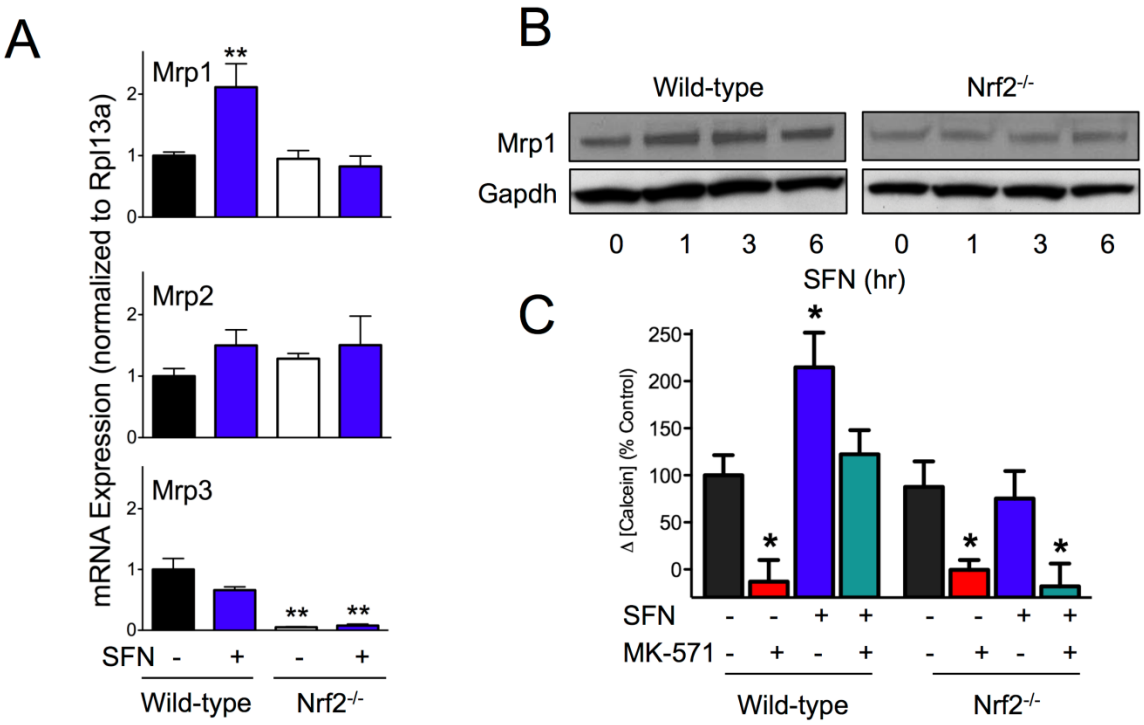

A
Mrp1
Mrp2
Mrp3
mRNA Expression (normalized to Rpl13a)
SFN
Wild-type
Nrf2-/-
B
Wild-type
Nrf2-/-
Mrp1
Gapdh
SFN (hr)
C
Δ [Calcein] (% Control)
SFN
MK-571
Wild-type
Nrf2-/-

**Figure 15. Activation of Nrf2 and downstream targets by sulforaphane in PAM212 cells.** *Panel A,* Cells were treated for 0, 3 or 6 hr with 3 μM sulforaphane in serum-free medium, washed, and refed with growth medium. After an additional 24 hr, lysates and nuclear fractions were prepared and analyzed for Nrf2 expression by Western blotting. *Panel B,* Cells were treated with 3 μM SFN or control. After 3 hr, RNA was extracted and analyzed for Nqo1 and Ho-1 mRNA expression by qPCR. Data are means ± SEM (*n = 4-6)*. ***P < 0.001 compared with control. *Panel C,* Cells were treated for 0, 3, or 6 hr with 3 μM sulforaphane in serum-free medium, washed, and refed with growth medium. After an addition 24 hr, cell homogenates were analyzed for Nqo1 and Ho-1 protein expression by Western blotting. Gapdh was used as a loading control.

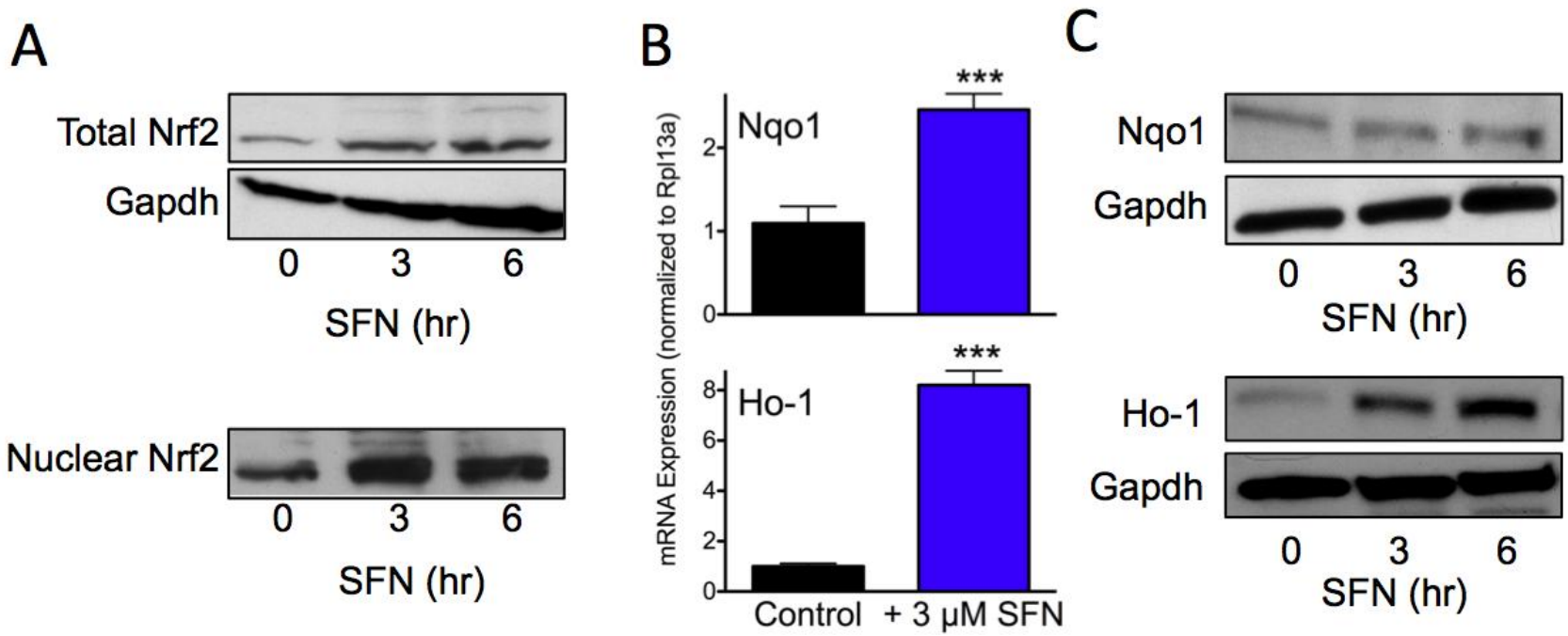

A
Total Nrf2
Gapdh
0
3
6
SFN (hr)
Nuclear Nrf2
0
3
6
SFN (hr)
B
mRNA Expression (normalized to Rpl13a)
Nqo1
***
2
1
0
Ho-1
***
8
6
4
2
0
Control
+ 3 μM SFN
C
Nqo1
Gapdh
0
3
6
SFN (hr)
Ho-1
Gapdh
0
3
6
SFN (hr)

**Figure 16. Effects of sulforaphane on Nqo1 and Ho-1 expression in primary mouse keratinocytes.** *Panel A,* Primary keratinocytes from wild type and Nrf2$^{-/-}$ mice were treated with 3 µM SFN or vehicle control in serum-free medium. After 3 hr, RNA was extracted and analyzed for Nqo1 and Ho-1 mRNA expression using qPCR. Data are means ± SEM (*n = 4-6)*. ***P < 0.001 compared with control. *Panels B and C,* Cells were treated with 3 µM SFN for 0, 1, 3, or 6 hr, washed, and refed with growth medium. After 24 hr, total cell lysates were prepared and analyzed for Nqo1 (*Panel B)* and Ho-1 (*Panel C)* protein expression.

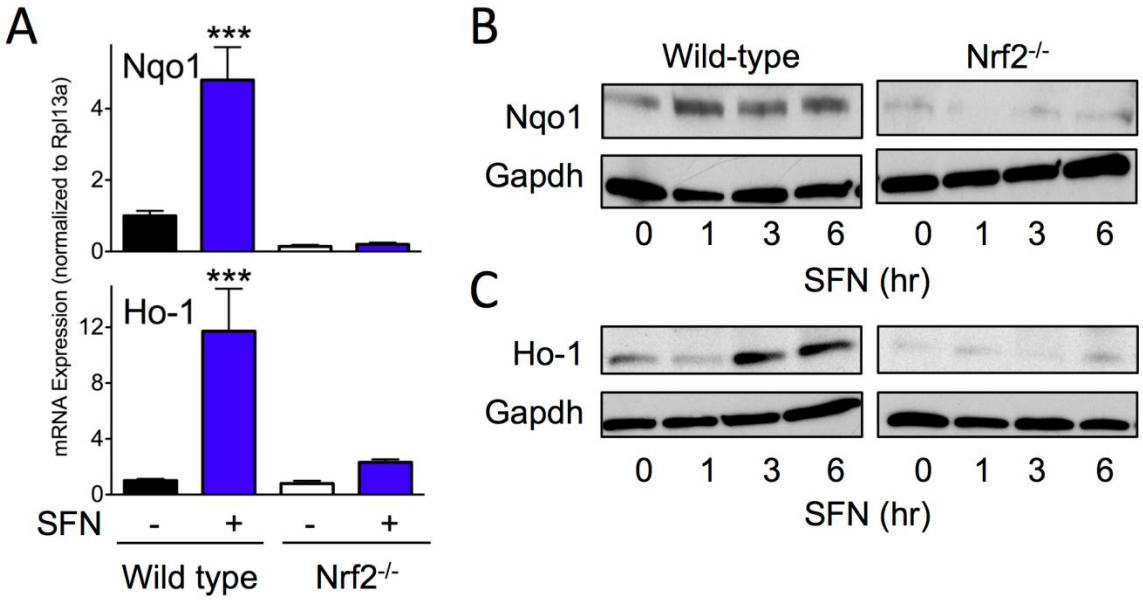

A
Nqo1
***
Ho-1
***
mRNA Expression (normalized to Rpl13a)
0
2
4
0
4
8
12
SFN
-
+
-
+
Wild type
Nrf2-/-
B
Wild-type
Nrf2-/-
Nqo1
Gapdh
0 1 3 6 0 1 3 6
SFN (hr)
C
Ho-1
Gapdh
0 1 3 6 0 1 3 6
SFN (hr)

**Figure 17. Effects of inhibitors on HN2-induced growth inhibition in PAM212 cells.** Cells were treated with 3 µM sulforaphane or vehicle control in serum-free medium. After 3 hr, cells were treated with increasing concentrations of HN2. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter Counter. Some cells were treated with 25 µM MK-571 (*Panel A*) or 3 µM ethacrynic acid (*Panel B*) for 1 hr prior to sulforaphane treatment. Each data point is the mean ± SEM (*n = 3)*.

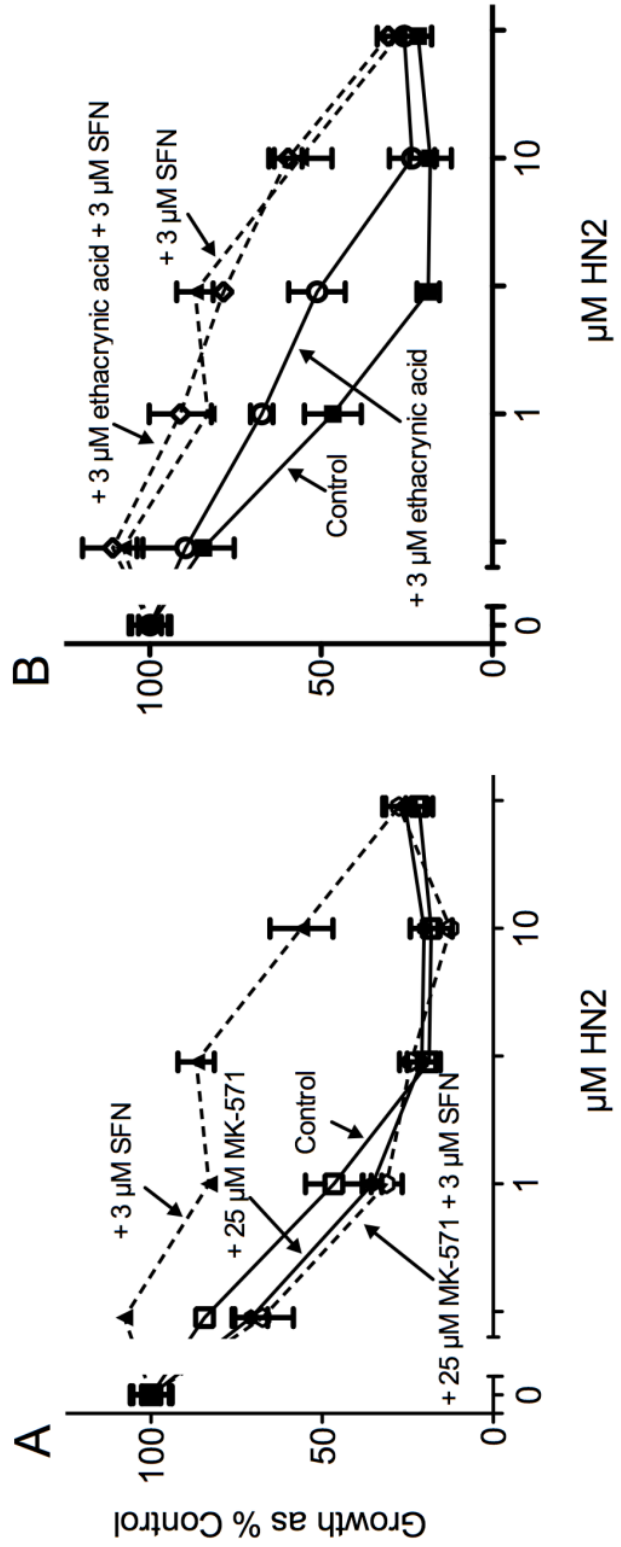
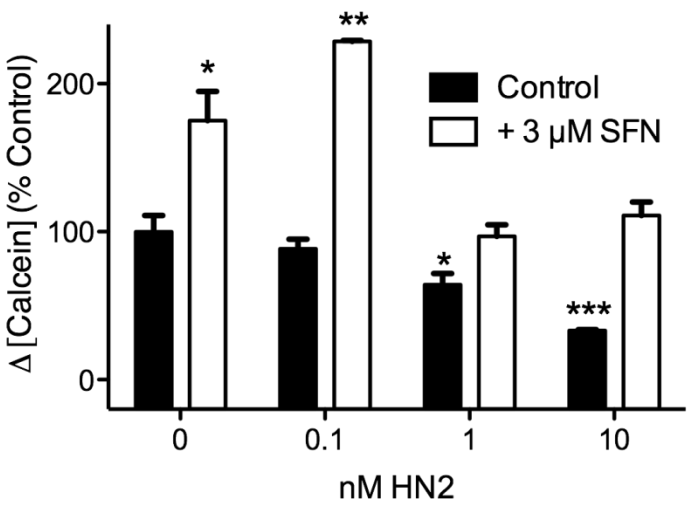
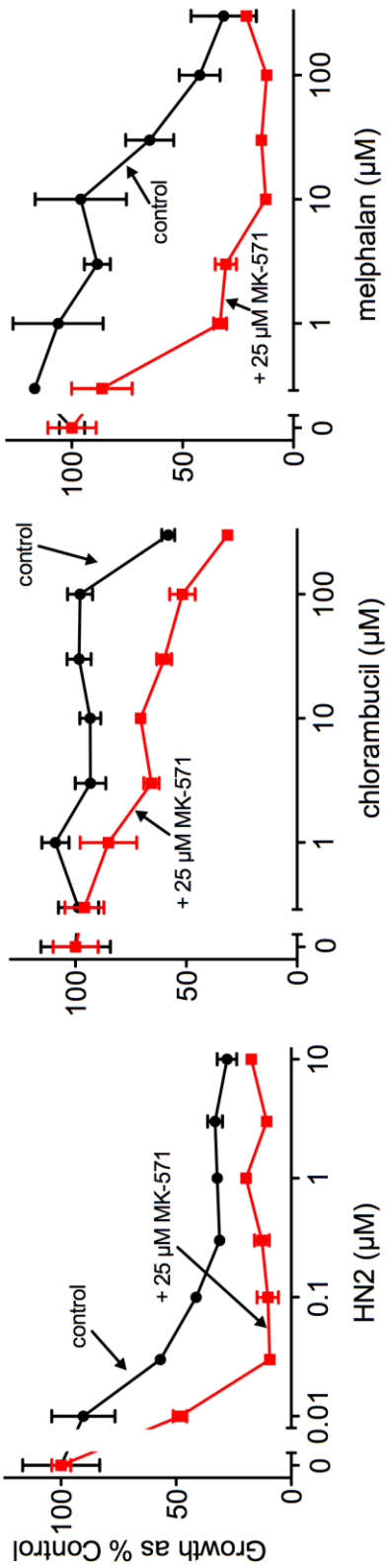

A
B
Growth as % Control
100
50
0
0
1
10
μM HN2
+ 3 μM SFN
+ 25 μM MK-571
Control
+ 25 μM MK-571 + 3 μM SFN
+ 3 μM ethacrynic acid + 3 μM SFN
+ 3 μM SFN
Control
+ 3 μM ethacrynic acid
μM HN2

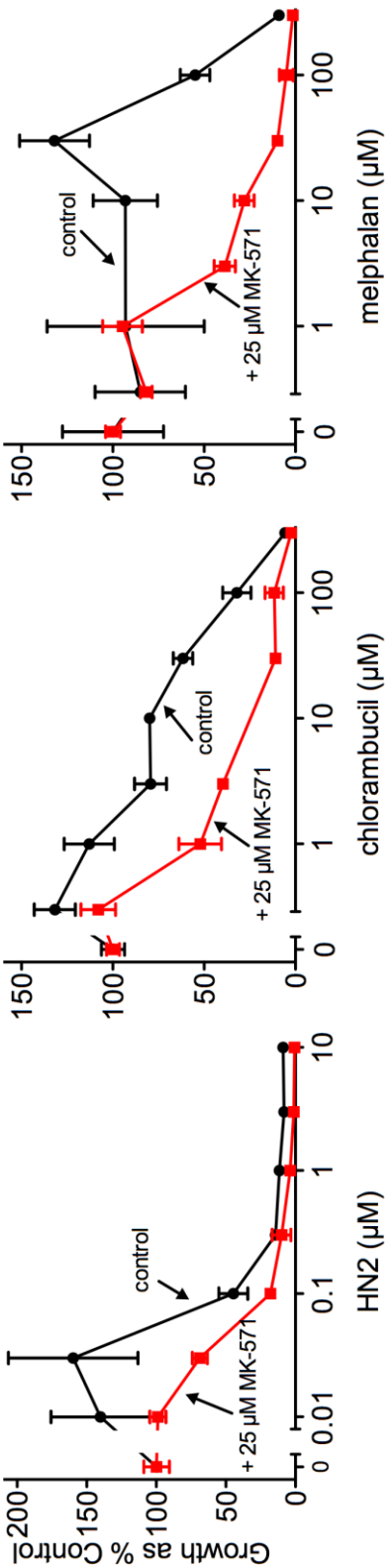
**Figure 18. Effects of HN2 on Mrp1 functional activity in PAM212 cells.** PAM212 cells were pre-treated with 3 μM sulforaphane or vehicle control. After 3 hr, cells were incubated with increasing concentrations HN2 and 1 μM calcein-AM and Mrp functional activity was measured. Each data point represents the mean ± SEM (*n = 3).* $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with untreated control.


Δ [Calcein] (% Control)
200
100
0
*
**
*
***
Control
+ 3 μM SFN
0
0.1
1
10
nM HN2

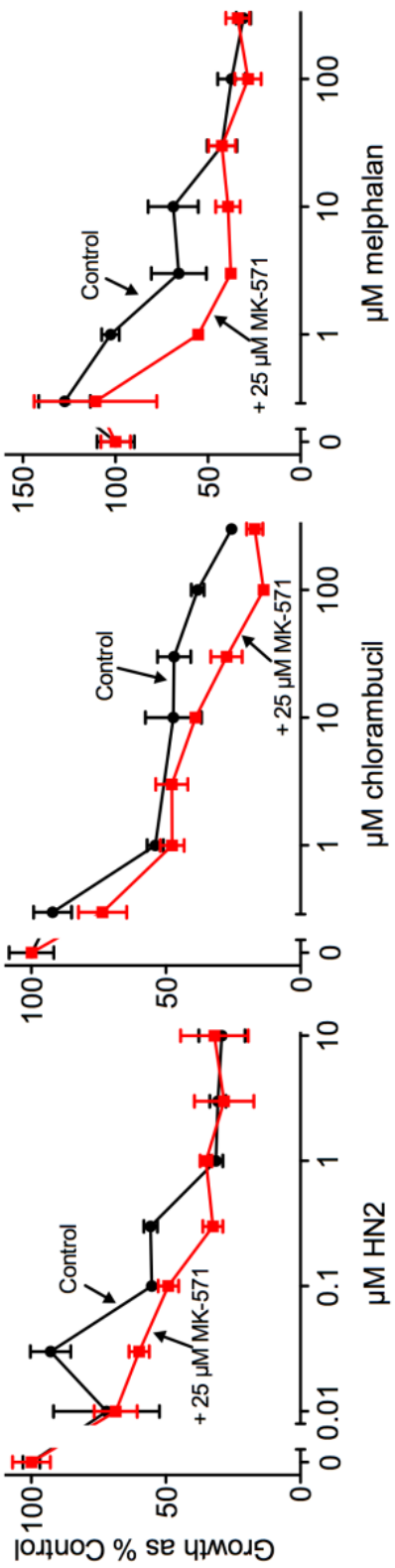
**Supplemental Figure 1. Effects of inhibition of MRP1 and MRP2 on the sensitivity of MCF7 cells to vesicant-induced growth inhibition.** MCF7 (human breast cancer) cells were treated with 25 µM MK-571 or vehicle control in serum-free medium. After 1 hr, increasing concentrations of HN2 (*left panel),* chlorambucil (*center panel)*, or melphalan (*right panel)* were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM.


Growth as % Control
100
50
0
0 0.01 0.1 1 10
HN2 (μM)
control
+ 25 μM MK-571
100
50
0
0 1 10 100
chlorambucil (μM)
control
+ 25 μM MK-571
100
50
0
0 1 10 100
melphalan (μM)
control
+ 25 μM MK-571

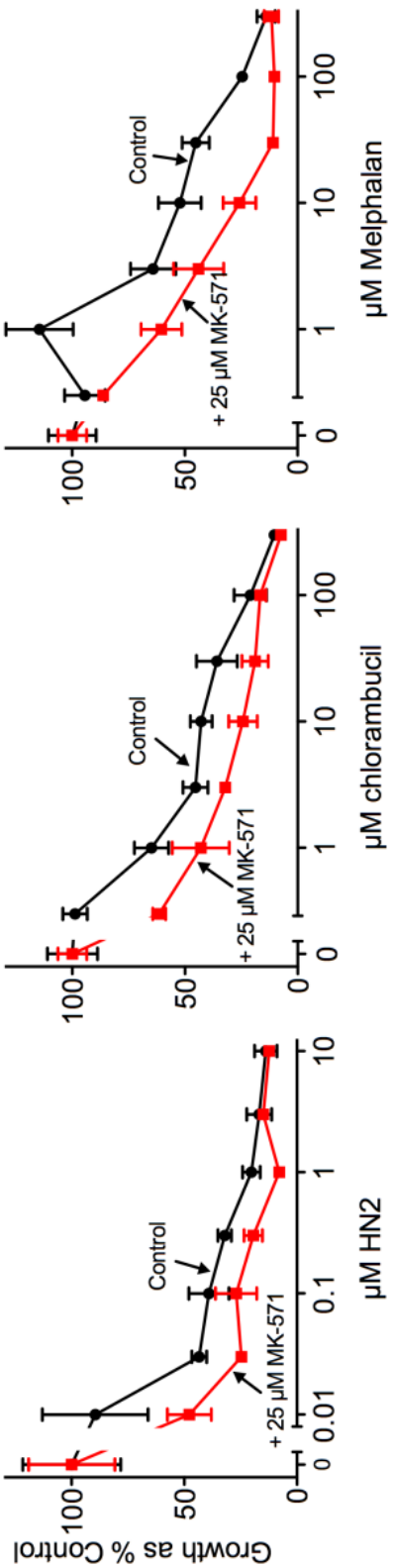
**Supplemental Figure 2. Effects of inhibition of MRP1 and MRP2 on the sensitivity of HeLa cells to vesicant-induced growth inhibition.** HeLa (human cervical adenocarcinoma) were treated with 25 µM MK-571 or vehicle control in serum-free medium. After 1 hr, increasing concentrations of HN2 (*left panel),* chlorambucil (*center panel)*, or melphalan (*right panel)* were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
200
150
100
50
0
0
0.01
0.1
1
10
HN2 (μM)
control
+ 25 μM MK-571
150
100
50
0
0
1
10
100
chlorambucil (μM)
control
+ 25 μM MK-571
150
100
50
0
0
1
10
100
melphalan (μM)
control
+ 25 μM MK-571

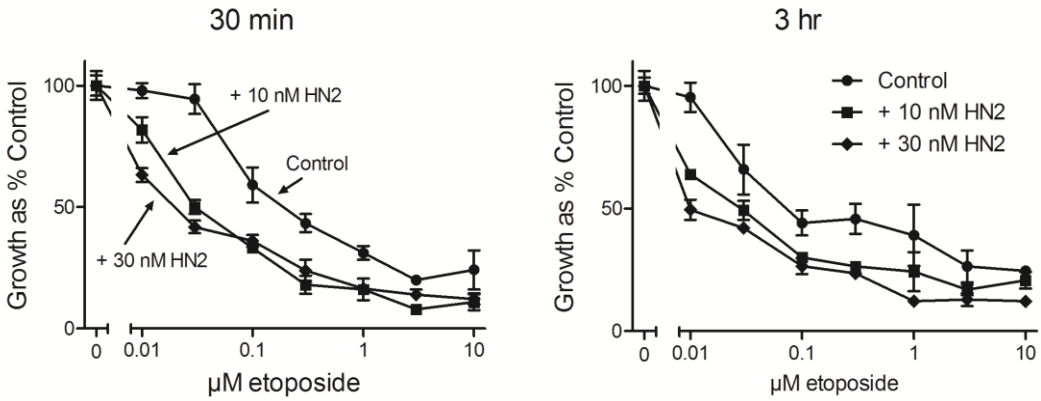
**Supplemental Figure 3. Effects of inhibition of MRP1 and MRP2 on the sensitivity of HepG2 cells to vesicant-induced growth inhibition.** HepG2 (human hepatocellular carcinoma) cells were treated with 25 µM MK-571 or vehicle control in serum-free medium. After 1 hr, increasing concentrations of HN2 (*left panel),* chlorambucil (*center panel)*, or melphalan (*right panel)* were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
Control
+ 25 µM MK-571
µM HN2
µM chlorambucil
µM melphalan

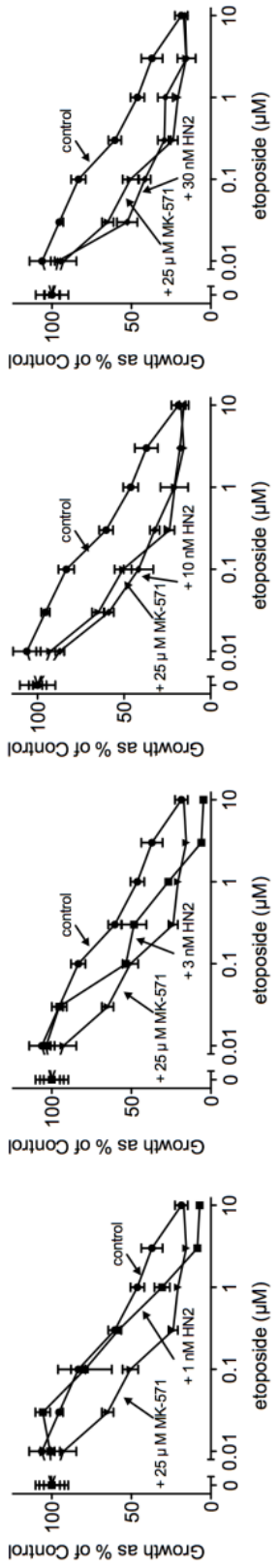
**Supplemental Figure 4. Effects of inhibition of MRP1 and MRP2 on the sensitivity of BeWo cells to vesicant-induced growth inhibition.** BeWo (human placenta, choriocarcinoma) cells were treated with 25 μM MK-571 or vehicle control in serum-free medium. After 1 hr, increasing concentrations of HN2 (*left panel),* chlorambucil (*center panel)*, or melphalan (*right panel)* were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
0.01
0.1
1
10
Control
+ 25 µM MK-571
µM HN2
100
50
0
0
1
10
100
Control
+ 25 µM MK-571
µM chlorambucil
100
50
0
0
1
10
100
Control
+ 25 µM MK-571
µM Melphalan

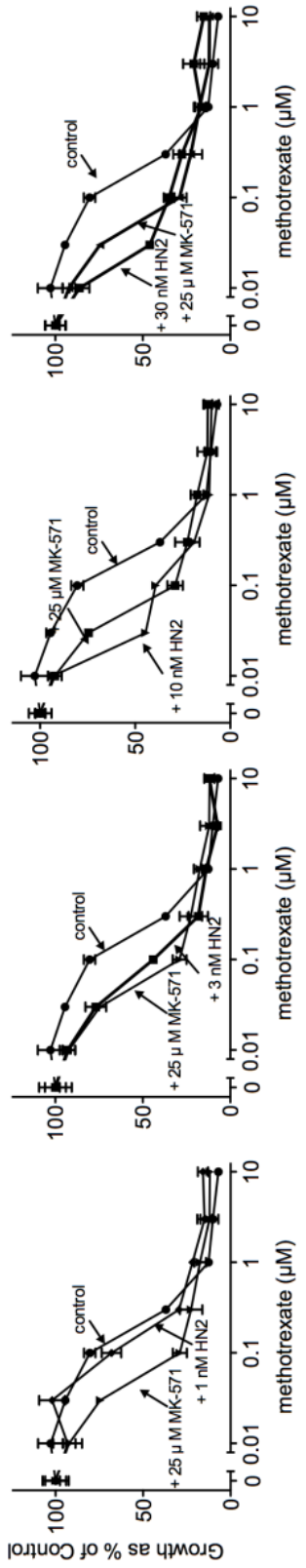
**Supplemental Figure 5. Effects of HN2 etoposide-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of etoposide in the absence or presence of HN2 in serum-free medium. After 30 min (*left panel)* or 3 hr (*right panel)*, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*


30 min
3 hr
Growth as % Control
100
50
0
+ 10 nM HN2
Control
+ 30 nM HN2
0
0.01
0.1
1
10
µM etoposide
Control
+ 10 nM HN2
+ 30 nM HN2

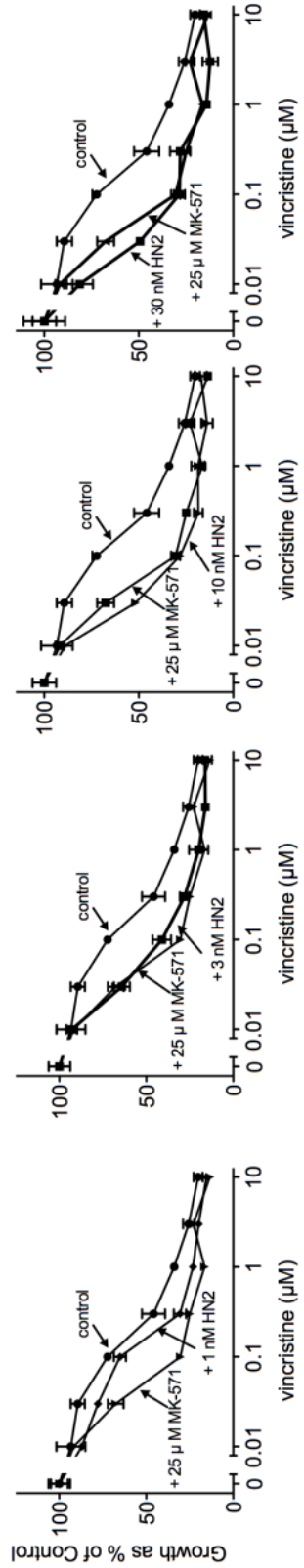
**Supplemental Figure 6. Effects of HN2 etoposide-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of etoposide in the absence or presence of 0, 1, 3, or 10 nM HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*


Growth as % of Control
100
50
0
0 0.01 0.1 1 10
etoposide (μM)
control
+ 25 μ M MK-571
+ 1 nM HN2
+ 3 nM HN2
+ 10 nM HN2
+ 30 nM HN2

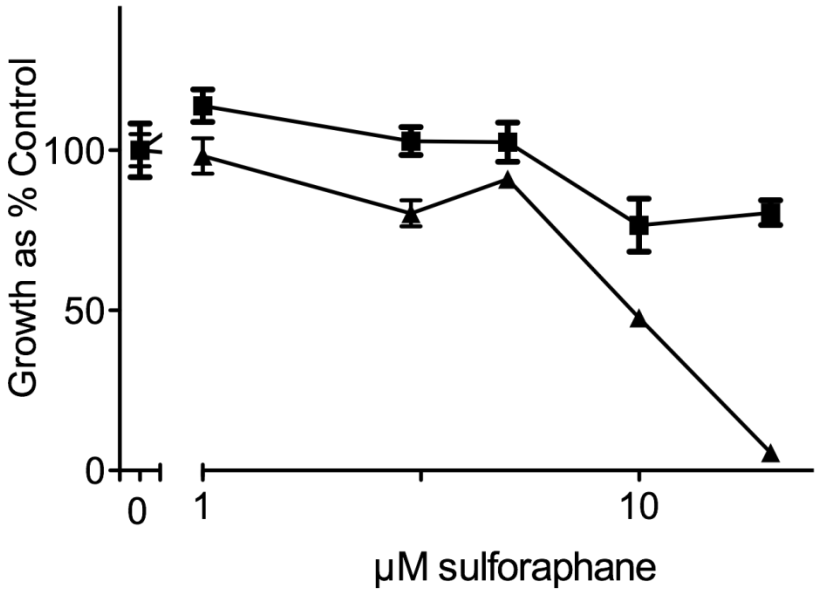
**Supplemental Figure 7. Effects of HN2 methotrexate-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of methotrexate in the absence or presence of 0, 1, 3, or 10 nM HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*


Growth as % of Control
100
50
0
0 0.01 0.1 1 10
methotrexate (μM)
control
+ 25 μ M MK-571
+ 1 nM HN2
+ 3 nM HN2
+ 10 nM HN2
+ 30 nM HN2
25 μM MK-571

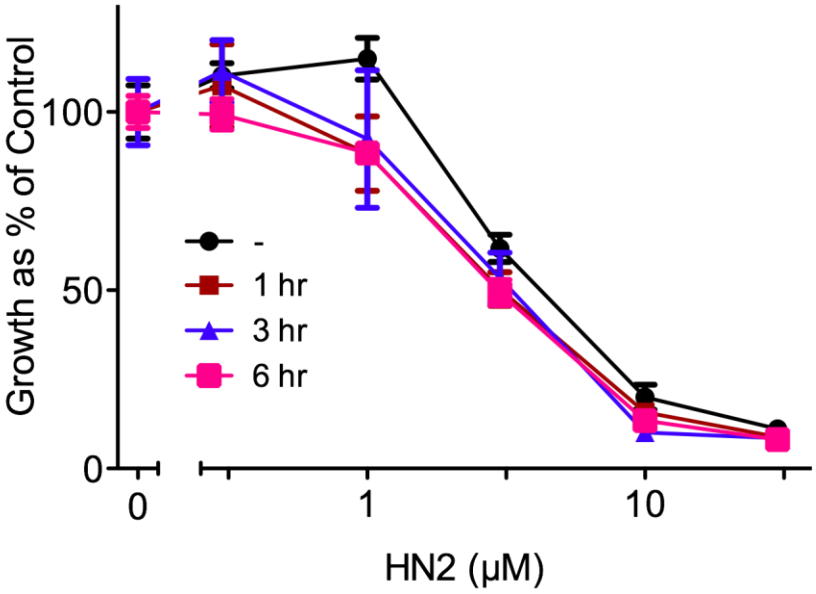
**Supplemental Figure 8. Effects of HN2 vincristine-induced growth inhibition in A549 cells.** A549 cells were treated with increasing concentrations of vincristine in the absence or presence of 0, 1, 3, or 10 nM HN2 in serum-free medium. After 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3).*


Growth as % of Control
100
50
0
control
+ 25 µ M MK-571
+ 1 nM HN2
+ 3 nM HN2
+ 10 nM HN2
+ 30 nM HN2
0 0.01 0.1 1 10
vincristine (µM)

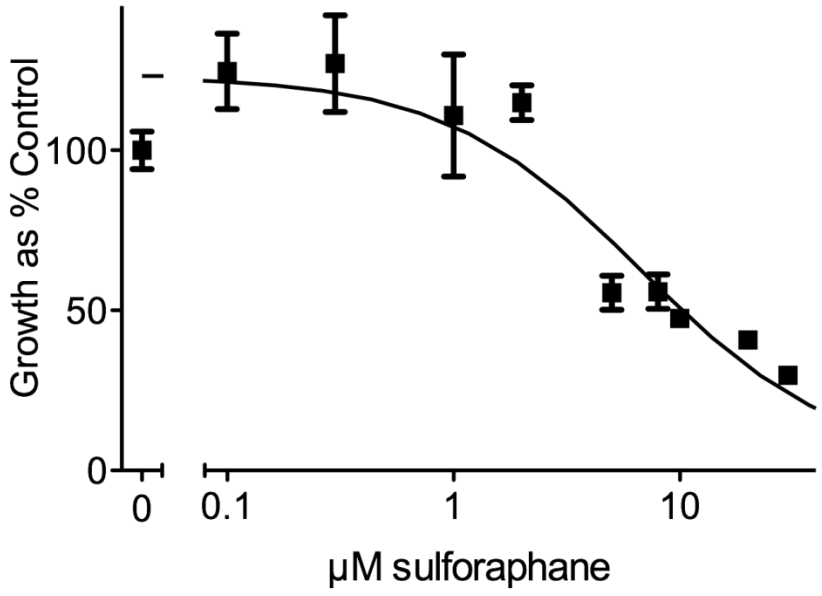
**Supplemental Figure 9. Sulforaphane inhibits growth in A549 cells.** Cells were treated with increasing concentrations of sulforaphane in serum-free medium. After 6 hr (squares) or 24 hr (triangles), cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
1
10
μM sulforaphane

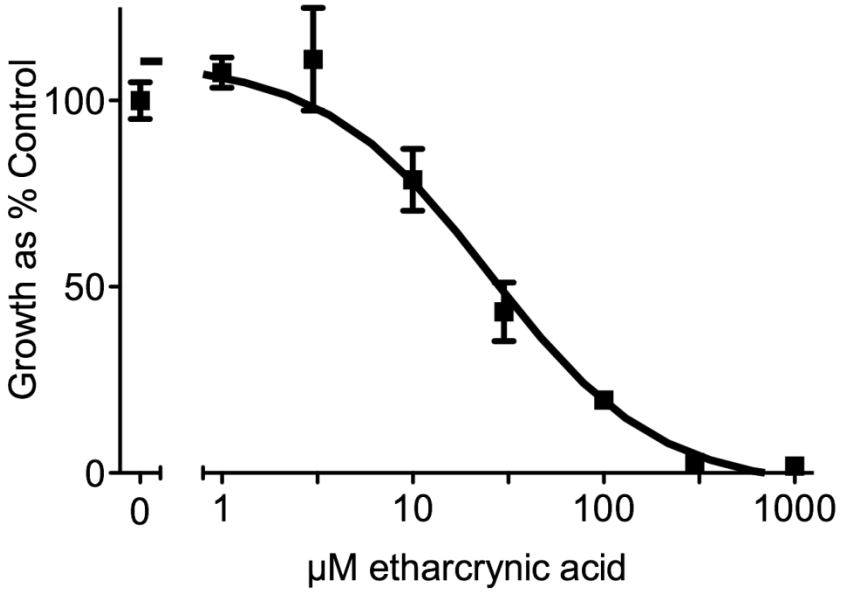
**Supplemental Figure 10. Sulforaphane does not protect A549 cells against HN2-induced growth inhibition.** Cells were treated with 3 µM sulforaphane in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an addition 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % of Control
100
50
0
-
1 hr
3 hr
6 hr
0
1
10
HN2 (μM)

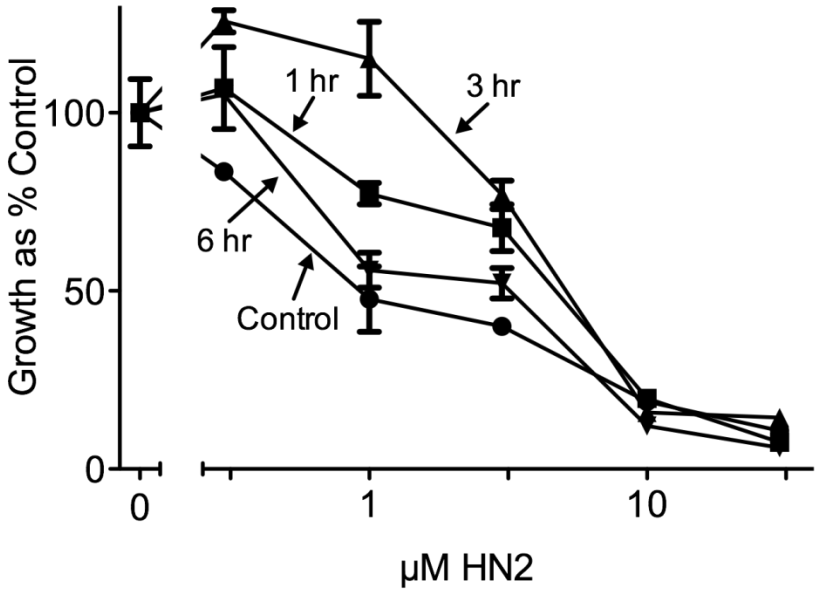
**Supplemental Figure 11. Sulforaphane inhibits growth in PAM212 cells.** Cells were treated with increasing concentrations of sulforaphane in serum-free medium. After 6 hr, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
0.1
1
10
μM sulforaphane

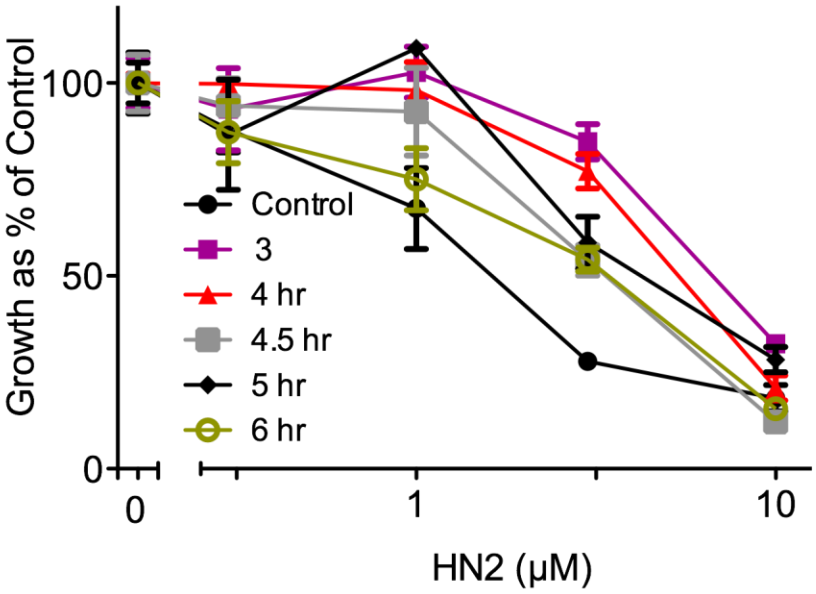
**Supplemental Figure 12. Ethacrynic acid inhibits growth in PAM212 cells.** Cells were treated with increasing concentrations of ethacrynic acid in serum-free medium. After 4 hr, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
1
10
100
1000
μM etharcrynic acid

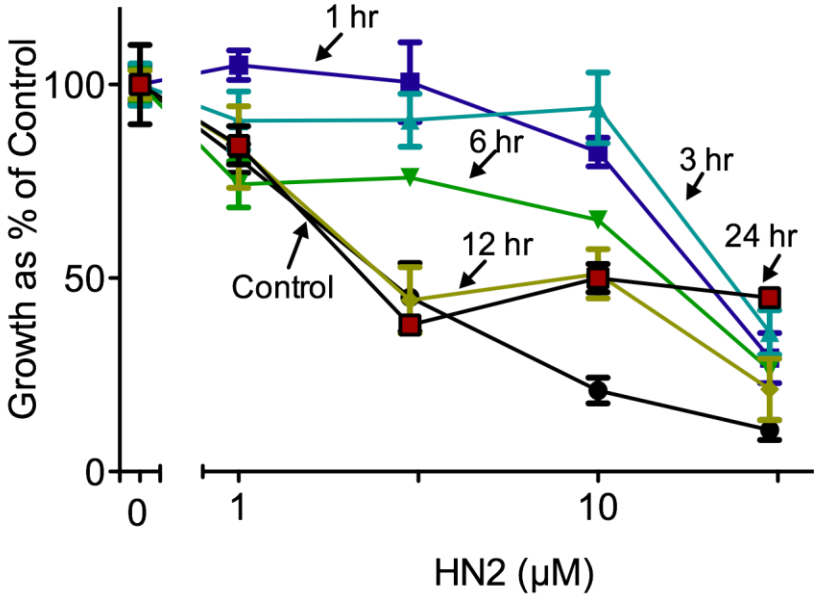
**Supplemental Figure 13. Sulforaphane protects PAM212 cells against HN2-induced growth inhibition.** Cells were treated with 3 µM sulforaphane in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an addition 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
1 hr
3 hr
6 hr
Control
0
1
10
μM HN2

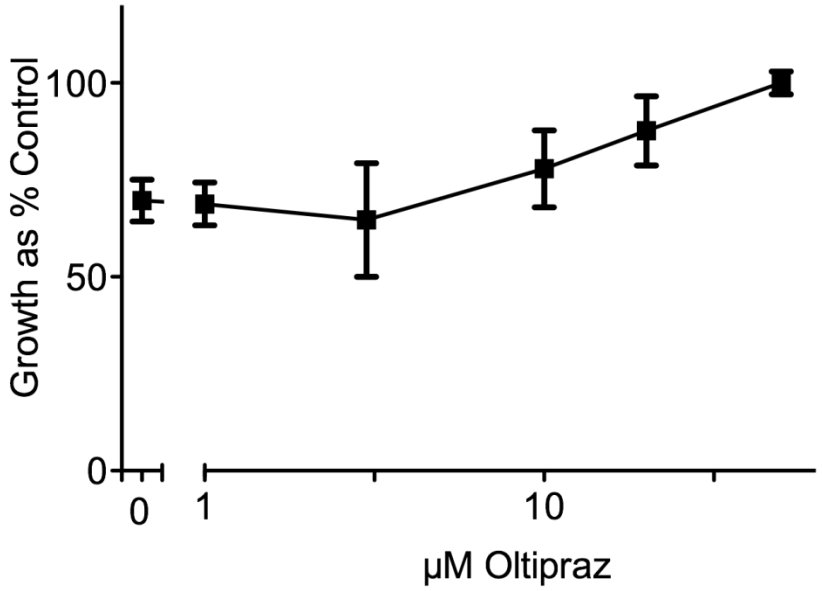
**Supplemental Figure 14. Sulforaphane protects PAM212 cells against HN2-induced growth inhibition.** Cells were treated with 3 µM sulforaphane in serum-free medium. After the indicated times (3-6 hr), increasing concentrations of HN2 were added to the cells. After an addition 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % of Control
100
50
0
0
1
10
HN2 (μM)
Control
3
4 hr
4.5 hr
5 hr
6 hr

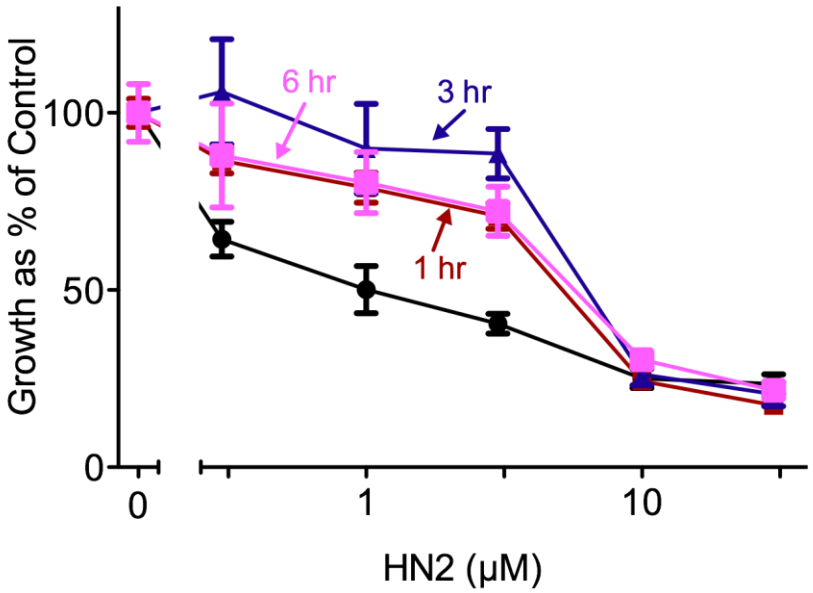
**Supplemental Figure 15. Sulforaphane protects primary mouse epidermal keratinocytes against HN2-induced growth inhibition.** Cells were treated with 3 µM sulforaphane in serum-free medium. After 0, 1, 3, 6, 12, or 24 hr, increasing concentrations of HN2 were added to the cells. After an addition 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


1 hr
6 hr
3 hr
24 hr
12 hr
Control
Growth as % of Control
100
50
0
0
1
10
HN2 (μM)

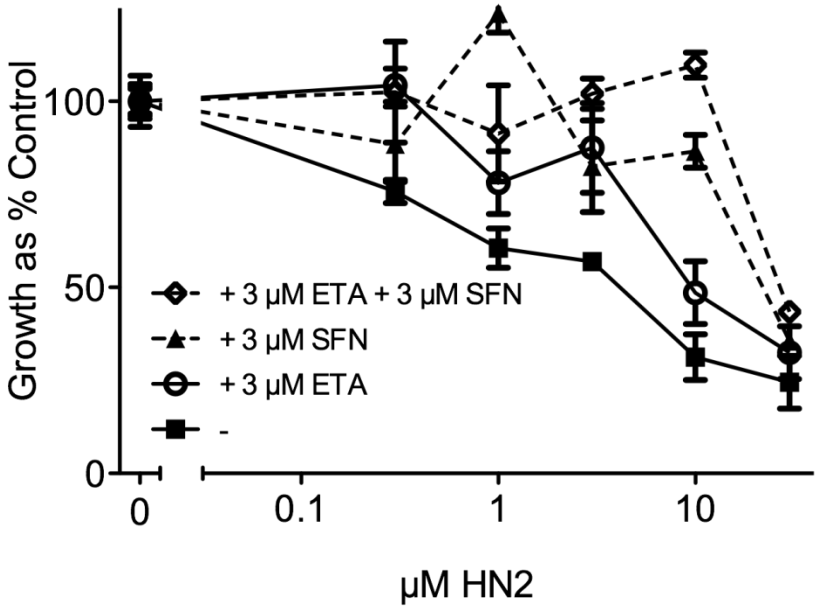
**Supplemental Figure 16. Effects of oltipraz on the growth of PAM212 cells.** Cells were treated with increasing concentrations of oltipraz in serum-free medium. After 6 hr, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
1
10
μM Oltipraz

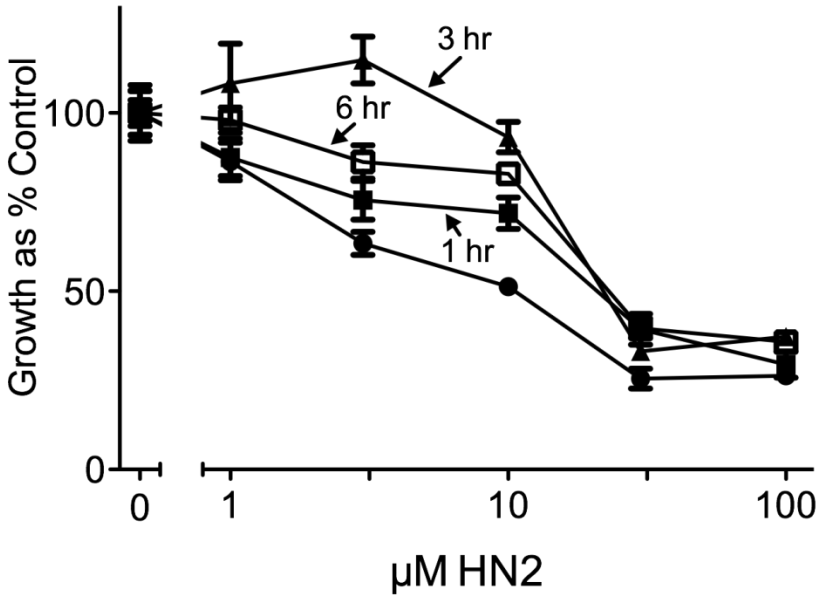
**Supplemental Figure 17. Oltipraz protects PAM212 cells against HN2-induced growth inhibition.** Cells were treated with 10 µM oltipraz in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % of Control
100
50
0
6 hr
3 hr
1 hr
0
1
10
HN2 (μM)

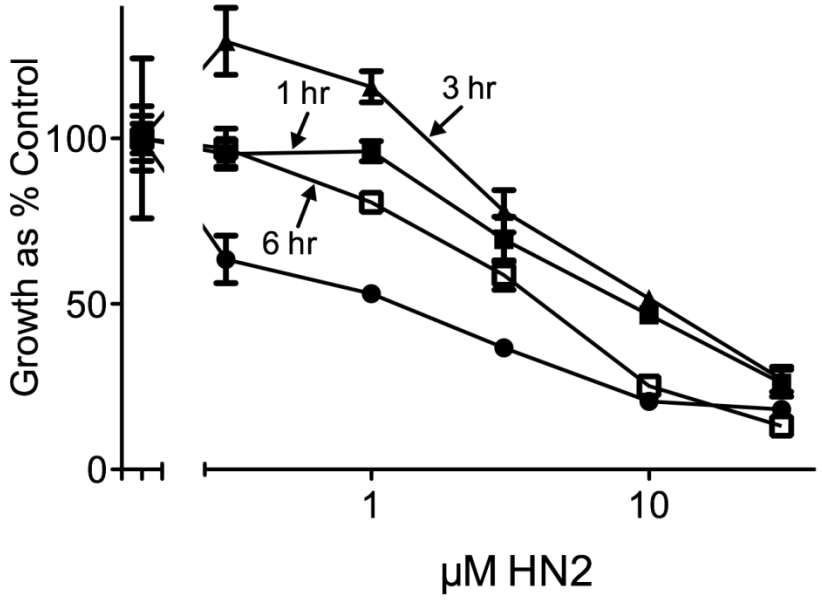
**Supplemental Figure 18. Effect of ethacrynic acid on HN2-induced growth inhibition in primary mouse keratinocytes.** Cells were treated with 3 μM ethacrynic acid or vehicle control in serum free medium. After 1 hr, were treated with 3 μM sulforaphane was added to the cells. After 3 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
+ 3 μM ETA + 3 μM SFN
+ 3 μM SFN
+ 3 μM ETA
-
0
0.1
1
10
μM HN2

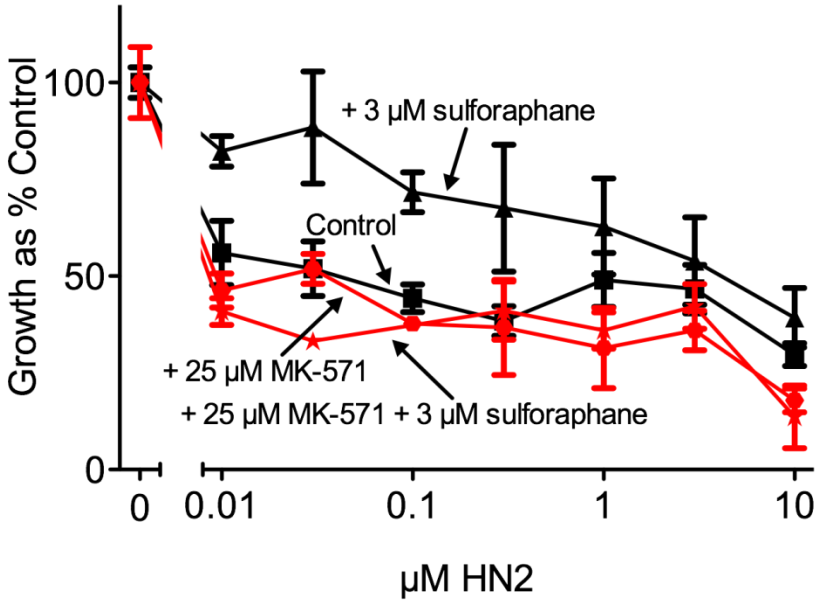
**Supplemental Figure 19. Oltipraz protects primary mouse keratinocytes against HN2-induced growth inhibition.** Cells were treated with 10 µM oltipraz in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
3 hr
6 hr
1 hr
0
1
10
100
μM HN2

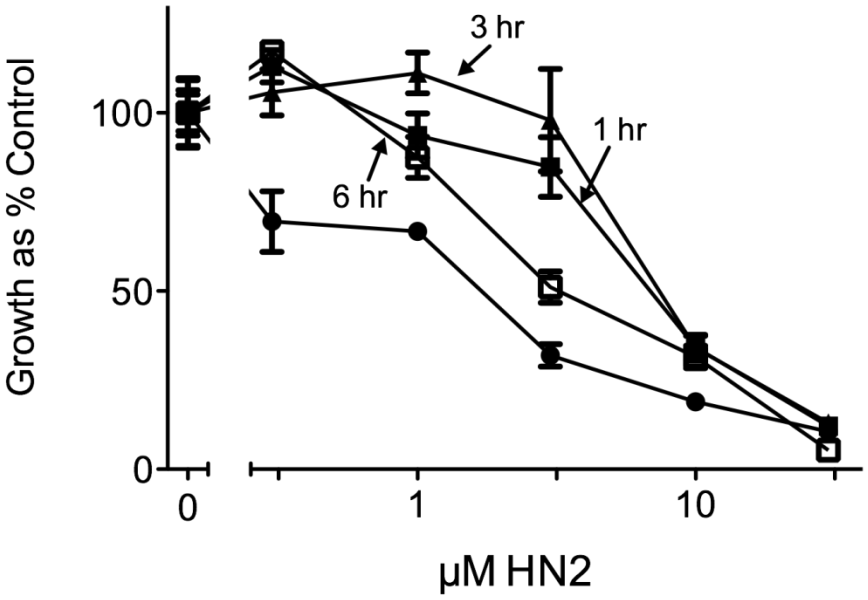
**Supplemental Figure 20. Sulforaphane protects human corneal epithelial cells against HN2-induced growth inhibition.** Cells were treated with 3 µM sulforaphane in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
1 hr
3 hr
6 hr
1
10
μM HN2

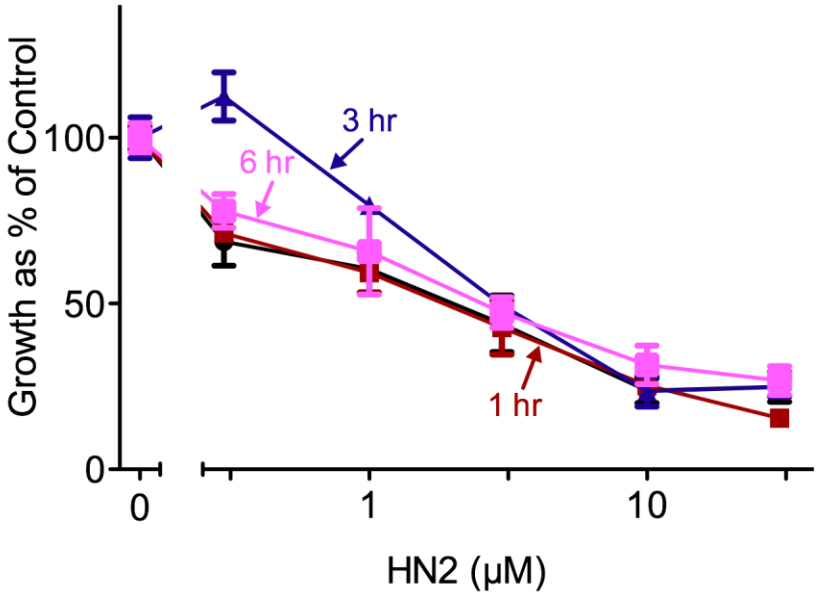
**Supplemental Figure 21. Effects of MK-571 on sulforaphane's protection of human corneal epithelial cells from HN2-induced growth inhibition.** Cells were treated with 25 µM MK-571 or vehicle control in serum free medium. After 1 hr, were treated with 3 µM sulforaphane was added to the cells. After 3 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
0.01
0.1
1
10
μM HN2
+ 3 μM sulforaphane
Control
+ 25 μM MK-571
+ 25 μM MK-571 + 3 μM sulforaphane

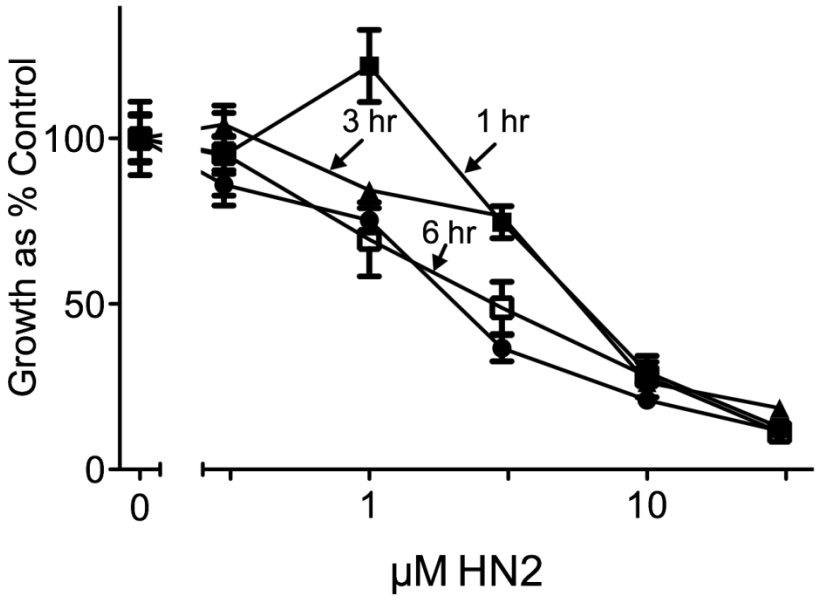
**Supplemental Figure 22. Oltipraz protects human corneal epithelial cells against HN2-induced growth inhibition.** Cells were treated with 10 µM oltipraz in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
0
1
10
μM HN2
3 hr
1 hr
6 hr

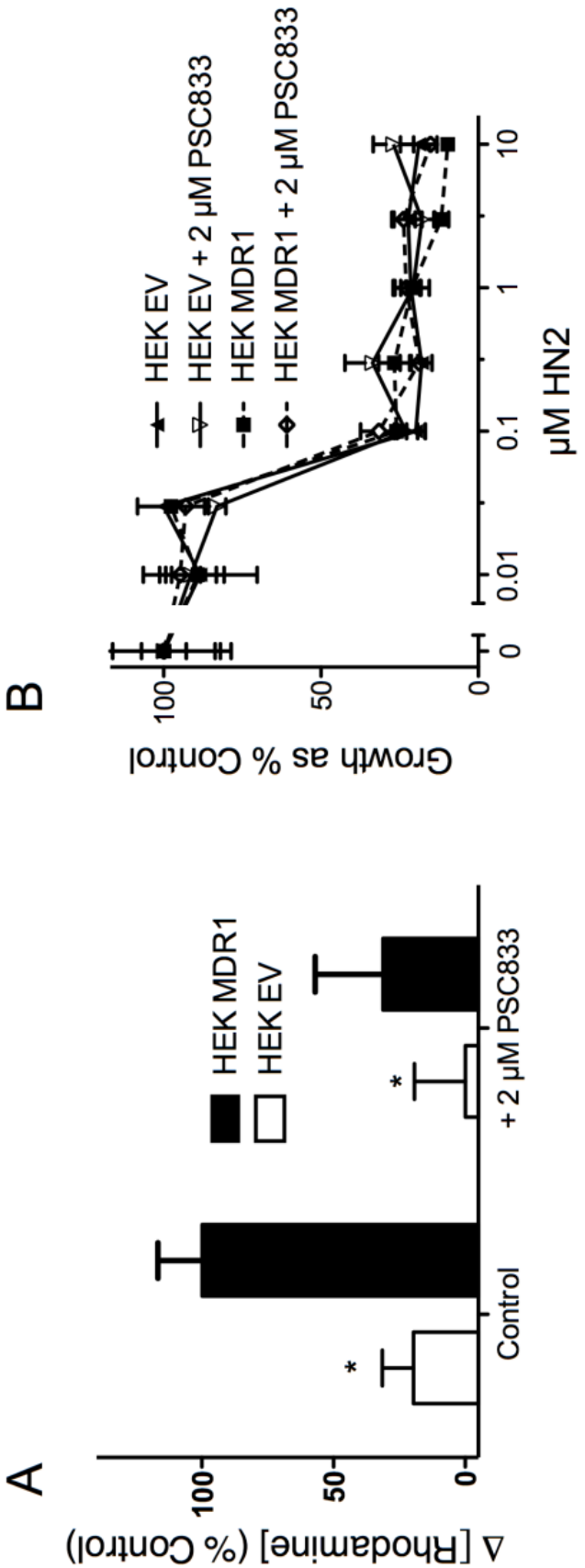
**Supplemental Figure 23. Effects of sulforaphane on HN2-induced growth inhibition in MLE15 cells.** Cells were treated with 3 μM sulforaphane in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % of Control
100
50
0
0
1
10
HN2 (μM)
3 hr
6 hr
1 hr

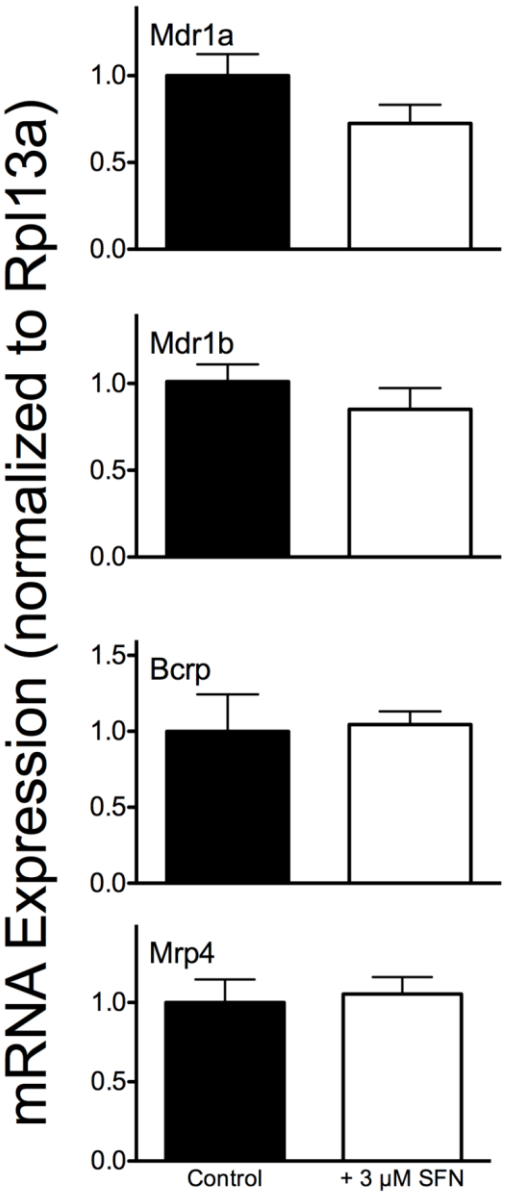
**Supplemental Figure 24. Effects of oltipraz on HN2-induced growth inhibition in MLE15 cells.** Cells were treated with 10 µM oltipraz in serum-free medium. After 0, 1, 3, or 6 hr, increasing concentrations of HN2 were added to the cells. After an additional 30 min, cells were washed and refed with growth medium. Seventy-two hr later, cell number was determined using a Coulter counter. Each data point is the mean ± SEM (*n = 3)*.


Growth as % Control
100
50
0
3 hr
1 hr
6 hr
0
1
10
μM HN2

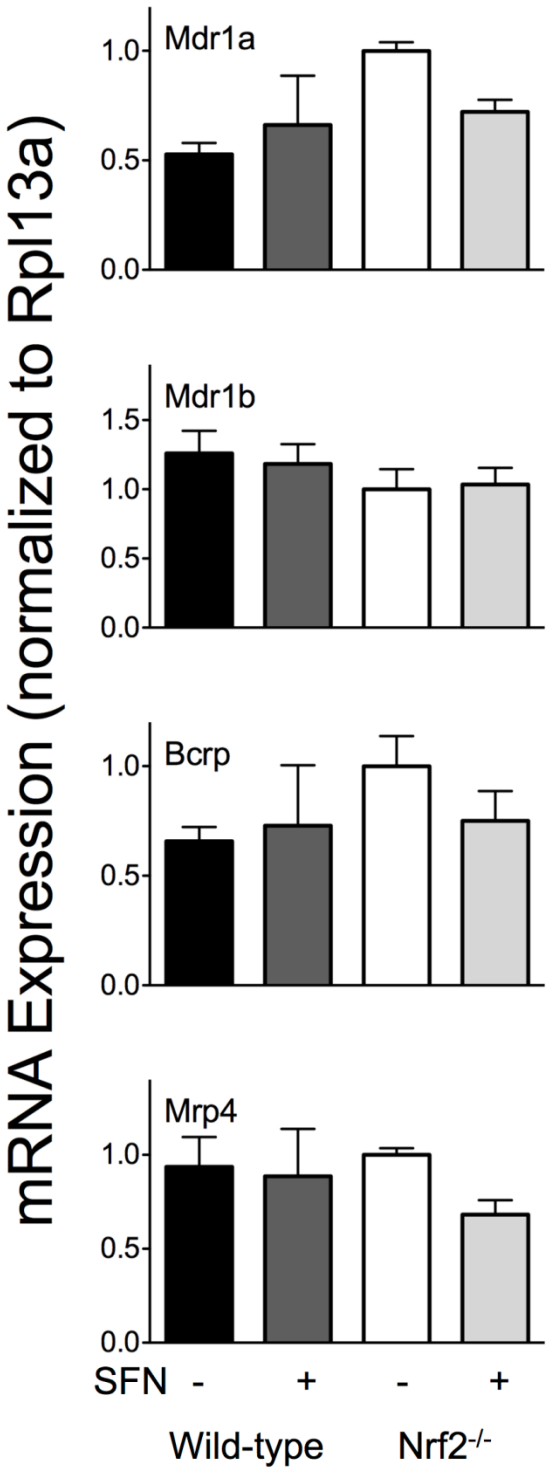
**Supplemental Figure 25. Functional Activity and HN2-induced growth inhibition in HEK cells overexpressing MDR1.** *Panel A,* Effects of overexpression of MDR1 on functional activity. Cells overexpressing MDR1 were incubated with 25 μM MK-571 or vehicle control and 1 μM rhodamine and measured for MDR1 functional activity by examining efflux of fluorescent MDR1 substrate rhodamine. Data are means ± SEM (*n = 3). *P < 0.05 compared with control. Panel B,* HEK MDR1 cells, as well those transfected with the empty vector were treated with 25 μM MK-571 or control. After 1 hr, cells were treated with increasing concentrations of HN2. After an additional 30 min, 10% fetal bovine serum was added to cells. Seventy-two hr later, cells number was determined using a Coulter counter. Each data point represents the mean ± SEM (*n = 3)*


A
Δ [Rhodamine] (% Control)
100
50
0
HEK MDR1
HEK EV
*
*
Control
+ 2 μM PSC833
B
Growth as % Control
100
50
0
HEK EV
HEK EV + 2 μM PSC833
HEK MDR1
HEK MDR1 + 2 μM PSC833
0
0.01
0.1
1
10
μM HN2

**Supplemental Figure 26. Effects of sulforaphane on efflux transporter expression in PAM212 cells.** PAM212 cells were treated with 3 µM sulforaphane or vehicle control and mRNA for Mdr1a, Mdr1b, Bcrp, and Mrp4 was analyzed using qPCR. Data are means ± SEM (*n = 4-6)*.


mRNA Expression (normalized to Rpl13a)
Mdr1a
1.0
0.5
0.0
Mdr1b
1.0
0.5
0.0
Bcrp
1.5
1.0
0.5
0.0
Mrp4
1.0
0.5
0.0
Control
+ 3 μM SFN

**Supplemental Figure 27. Effects of sulforaphane on efflux transporter expression in primary mouse keratinocytes.** Primary mouse keratinocytes from wild-type and $Nrf2^{-/-}$ mice were treated with 3 µM sulforaphane or vehicle control and mRNA for Mdr1a, Mdr1b, Bcrp, and Mrp4 was analyzed using qPCR. Data are means ± SEM (*n = 4-6)*.


mRNA Expression (normalized to Rpl13a)
Mdr1a
1.0
0.5
0.0
Mdr1b
1.5
1.0
0.5
0.0
Bcrp
1.0
0.5
0.0
Mrp4
1.0
0.5
0.0
SFN - + - +
Wild-type
Nrf2-/-