

EFFECT OF POTENTIAL VESICANT ANTAGONIST DRUGS ON WHITE BLOOD CELL METABOLIC ACTIVITY IN HUMAN WHOLE BLOOD EXPOSED TO 2-CHLOROETHYL ETHYL SULFIDE

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ABSTRACT

Mustard agent affects several pathways that contribute to its toxicity. Some of the pathways involve calcium poisoning and protein degradation, but it is not clear how these pathways affect vesicant-induced toxicity. This makes it difficult to develop a good screening test to evaluate chemicals which might be used to antagonize or mitigate mustard's actions in cells. We examined here a screening test that uses live human tissue (human whole blood) and describe how monitoring the tissue after exposure to potential drugs and a vesicant can be used to estimate the overall effect of a drug or combination of drugs on vesicant exposure. Antagonizing cell death pathways of calcium poisoning (zaldaride maleate [CGS9343B]; C) or protein degradation (Leupeptin; L) does not satisfactorily resolve vesicant-induced toxicity. However, antagonizing pathways simultaneously might be successful. Moreover, when used with an oxidizing agent (copper-based polyoxometalate; P) that may reduce vesicant toxicity, the effectiveness of these antagonists might be improved. This approach was evaluated using a simple human whole blood (HWB) *in vitro* system. Prior treatment (30 min) of heparinized (40 U/mL) HWB (n=9) without (buffer vehicle) or with C (13.6 µg/mL), L (0.5 mg/mL) or P (5.0 µg/mL) singly (N=3) or in combination (N=4) was tested for white blood cell metabolic activity (WBCMA; Intergen ProCheck[®]) post (24 h of *in vitro* culture) carrier (air) gas or vesicant (2-chloroethyl ethyl sulfide; CEES; 18mg at 1.5 mg/L/min) exposure. With carrier gas, L and/or C, significantly enhanced the measured WBCMA, while P sustained WBCMA, when compared to HWB without drug(s). This salutary, *in vitro* effect explained why L significantly improved or C, P, C+L, C+P or L+P sustained WBCMA with CEES, when compared to HWB without drug(s). However, when drug(s) was present in both the CEES and carrier gas conditions, the WBCMA was significantly reduced by CEES exposure. Thus, antagonizing multiple cell death pathways simultaneously along with CEES oxidation was not protective.

INTRODUCTION

The vesicant dichloroethyl sulfide (sulfur mustard; HD) is a strong alkylating agent that induces injury similar to that incurred by exposure to ionizing radiation (Papirmeister et al., 1991). These radiomimetic-like actions include direct lethal effects on leukocytes, bone marrow, and rapidly dividing cells. Such actions are likely attributable to the alkylation of deoxyribonucleic

acid (DNA) which leads to cross-links and strand breaks in this macromolecule. Sulfur mustard exposure also causes inhibition of glucose metabolism and lactate production, as well as depletion of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide.

The extremely harmful and lethal effects of HD agents preclude their study in human subjects. This laboratory has used artificial human skin models (Blaha et al., 2001; Blaha et al. 2000a) to explore several intracellular interventions, such as zaldaride maleate [(CGS-9343B), C] or Leupeptin (L) as a means of providing vesicant protection. Previously proposed modes of HD toxic action provided the basis for the selection of these particular interventions, which is described in detail by Blaha et al., 2000. Briefly, L, a cysteine protease inhibitor, counteracts proteolytic enzymes that degrade critical cell proteins after HD exposure, while C antagonizes calmodulin to reduce calcium-mediated toxic events resulting from HD inhibition of calcium-ATPase pumps. Each potential HD antagonist was demonstrated to be non-toxic to the artificial human skin systems studied. Unfortunately, neither antagonist appeared to offer significant protection from the detrimental effects of 2-chloroethyl ethyl sulfide (CEES; half-mustard) exposure (Blaha et al., 2000). However, since each antagonist was studied singly, it suggests blocking only one potential injury pathway is insufficient to prevent or reduce mustard-induced injury. Perhaps use of these intracellular mustard antagonists in combination to block simultaneously multiple injury pathways would be more effective than use of a single antagonist. Moreover, an extracellular intervention through vesicant oxidation might also reduce the level of induction of these intracellular cell death pathways and assist the actions of intracellular vesicant antagonists. Polyoxometalate (P) is an early transition metal oxygen anion cluster that oxidizes HD (Hill et al., 1998). While relatively non-toxic and soluble (Rhule et al., 1998), the oxidative action of P may reduce HD toxicity. The present study used an *in vitro* approach employing human whole blood (HWB) to assess the efficacy of simultaneously blocking multiple cell death pathways in conjunction with vesicant oxidation. Efficacy screening employing such a readily available and inexpensive human tissue contributes to resource and animal use conservation since it can be used to prioritize those promising approaches for further study in more expensive and complex *in vitro* or *in vivo* model systems.

METHODS

Human Volunteers. Two (54 ± 3.0 years [y]) or nine (24.6 ± 3.6 y) male volunteers provided blood samples for development of CEES delivery parameters or drug screening, respectively. Informed consent was obtained prior to blood collection.

Potential Vesicant Antagonists. Table 1 provides a list of the potential vesicant antagonistic drugs tested, their symbols, and modes of antagonistic action. Zaldaride maleate [(CGS-9343B), C] was acquired from Novartis, Inc., Nyon, Switzerland. Leupeptin, L, was obtained from Sigma Chemical Co., St. Louis, MO. C. Hill, Ph.D. of Emory University, Atlanta, GA provided the copper-based P and determined the CEES-oxidizing dose.

Human Whole Blood Collection/Dilution. Using syringes (Becton Dickinson, Rutherford, NJ), each containing heparin (Sigma Chemical Co., St. Louis, MO; 40U/mL blood), HWB was drawn by venipuncture using a 19–21-gauge butterfly needles (Terumo Medical Corp., Elkton, MD).

Vesicant Exposure Procedure. After calibrating the gas flows of the exposure apparatus (Blaha et al., 2002), 3 mL of HWB were dispensed into the wells of Costar trays (Allegiance Health

Care, Lee, MA). In duplicate trays for CEES or carrier gas exposure, a 0.5 mL volume of a 10x concentration in RPMI 1640 medium (Gibco/BRL, Grand Island, NY) of each individual drug (N=3) or multiple 0.5 mL volumes to create the various drug combinations (N=4) was added to the tray wells. To one well in each tray, 2 mL of RPMI 1640 medium was added, which served as a control for HWB without drug. Where necessary, wells were adjusted to a total volume of 5 mL by addition of RPMI 1640 medium to produce a 40 percent dilution of the HWB in all wells. The final working drug concentrations when used singly or in combination were as follows: C=13.6 microgram (μg)/mL, L=0.5 milligram (mg)/mL, and P=5.0 μg /mL. These HWB mixtures were then incubated for 30 minutes (min) with rocking at 37°C. Following incubation, trays were placed in one of two chambers of the exposure apparatus. The CEES exposure length and the HWB rotation rate were adjusted to achieve an approximate (~) 50 percent reduction in the white blood cell metabolic activity (WBCMA), when compared to that of carrier gas (humidified air) exposure. To achieve this, one chamber received humidified CEES vapor for 12 min (1.5 mg/liters/min or 18 mg total), the other received only carrier gas (humidified air). During exposure, the chambers were rotated at 15 revolutions per min (rpm) to ensure blood homogeneity and uniform interaction with the respective treatment gas. Following exposure, the trays were removed from the chambers. The samples in the trays were allowed to outgas for 5 min within a chemical hood before their incubation with rocking, at 37°C and 5 percent CO₂ for a total of 24 hours (h) of *in vitro* culture.

White Blood Cell Isolation Procedure. From HWB samples prior to and post experimental procedures, 400 microliters (μL) of blood was transferred to 15 mL polypropylene conical tubes. To each tube, 8 mL of red blood cell lysing solution (Easy-Lyse™, Leinco Technologies, St. Louis, MO) was added, immediately mixed, and incubated at room temperature (RT) for 10 min. The tubes were centrifuged at 1,300 rpm (300–500 gravities) for 5 min at RT; the supernatants were decanted, and then the tubes were gently vortexed to resuspend the cells in residual supernatant. The suspended cells in each tube were washed by mixing with 4 mL of Easy-Lyse™ buffer and then centrifuged, decanted, and re-suspended, as before. After a repeat of this washing procedure, the samples were reconstituted with 500 mL of RPMI 1640 medium (no phenol red) and mixed.

White Blood Cell Metabolic Activity. The WBCMA was measured colorimetrically using the ProCheck™ Cell Viability Assay (Intergen Co., Purchase, NY). The assay was based on the enzymatic conversion of the tetrazolium salt XTT from an oxidized tetrazole to a reduced formazan compound. The degree of color change from yellow to an orange/red as determined by optical density (O.D.) was proportional to the number of metabolically active or viable white blood cells (WBCs). The procedure was as follows: Three, 100 μL aliquots of cell suspension (test required 10^4 – 10^6 cells/mL for optimal results after 4 h incubation) were added to 3 wells of a 96-well plate. Each well then received 20 μL of ProCheck™ viability reagent. The plate was covered and incubated for 4 h at 37°C, 5 percent CO₂. Following incubation, O.D. of the solution in each well was measured at 480 nm on a Dynatech 7000 plate reader (Thermo Labystems, Franklin, MA).

Cell Counts. The WBC counts were performed on a Coulter Z-1 particle counter (Beckman Coulter, Inc., Miami, FL) by mixing 200 μL of HWB with 10 mL of Coulter Z-1 diluent and counting the cells in the resulting mixture (cells > 4 microns in size).

Statistics. The WBCMA comparisons between HWB with or without drug(s) after carrier gas or CEES exposure employed a one-way analysis of variance followed by a Holm-Sidak post-hoc test for significance in the pair-wise multiple comparisons. When drug(s) was present in both the carrier gas and CEES condition, a paired t-test was employed. Values are reported as means \pm S.E. Significance was set at $p < 0.05$.

RESULTS

White blood cell counts ranged between 2.3 and 7.8×10^3 cells/ μL , with a mean of $5.03 \pm 0.57 \times 10^3$ cells/ μL . WBC counts pre- and post- CEES or carrier gas exposure were not significantly different (data not shown).

Influence of WBC count on WBCMA after carrier gas (air) or CEES exposure and 24 h of *in vitro* culture is described in table 2. The WBC count for Subject A was significantly less than that of Subject B, and the O.D. readings measuring the WBCMA were also significantly lower after either carrier gas or CEES exposure and 24 h of *in vitro* culture. However, the ratio of WBCMA for blood from Subjects A and B, as measured by O.D., was approximately 50 percent for exposure to carrier gas compared to exposure to CEES. This is expected if the decrease in WBCMA is independent of the initial WBC. Increasing the CEES exposure time to 15, 20, or 30 min and the HWB rotation to 24 rpm further reduced the WBCMA to a measured O.D. of 33 ± 2.8 percent, which was significantly different than the ~ 50 percent obtained when the CEES exposure was for 12 min and the HWB rotation rate was 15 rpm.

Table 3 shows the influence of the air or CEES exposure on the WBC as expressed by the change in the WBCMA after 24 h of *in vitro* culture. The O.D. measure of the WBCMA after carrier gas exposure and culture was ~ 20 percent of the value at the time of collection (T_0). But after exposure to CEES, this was ~ 9 percent. Thus, the exposure to CEES reduced the O.D. significantly more than carrier gas (air) did; the carrier gas (air) exposure reduced the WBCMA by ~ 80 percent and the CEES reduced it by ~ 91 percent. Since O.D. readings were within the dynamic range for spectrophotometric detection of reduced formazan when the O.D. (WBCMA) was ~ 9 percent of the T_0 value, these measurements are considered reliable.

Figure 1 illustrates the influence of potential vesicant antagonist drugs (C, P, and L, and combinations) on the WBCMA. As noted in the methods section, the drugs were added to the HWB samples and incubated for 30 minutes. The reductions in WBCMA compared with the controls (no drug) are indicative of the antagonistic effects of the drug; if the drug causes a smaller reduction in WBCMA than the controls, the drug has potential for anti-vesicant activities. From figure 1, it appears that the drugs L, C+L, and C+P offer some protection from CEES. However, it should be noted that there were increases in O.D., relative to the controls, even for the carrier gas (air) exposures. In addition, the drug combination C+L+P appeared to decrease the O.D. relative to both controls (carrier gas and CEES) and C, P, L+P appeared to have either no effect or further reduced the O.D. for CEES-exposed samples.

DISCUSSION

As illustrated by the long-term effects of mustard exposure on military and civilian victims of the Iraq-Iran war (Bijani et al., 2002), developing effective antagonists of this chemical warfare agent is of paramount importance. An HWB model was employed to screen potential vesicant

antagonists singly and in combinations. This approach employed a readily available, complete human tissue that involved only simple *in vitro* culturing methods. As such, it provided a relatively inexpensive and rapid test of efficacy to screen for the most beneficial vesicant antagonist or combination of antagonists (table 1).

To establish a level of CEES exposure that would result in an ~50 percent WBCMA reduction when compared to carrier gas exposure followed by 24 h of culture, CEES exposure time and HWB rotation rate were adjusted. As illustrated in table 2, a CEES exposure at 1.5 mg/L/min for 12 min with 15 rotations/min produced an ~50 percent reduction in WBCMA, independent of the WBC count. This was important to establish since, while all WBC counts were within the normal range, those at the high or low end of the range had significantly different counts. Interestingly, WBC counts were not significantly altered by CEES exposure to indicate metabolic function was influenced more by CEES exposure than was cell integrity.

It is important to note that since increasing the exposure time and HWB rotation rate further reduced WBCMA, the selected CEES exposure parameters did not induce a maximal reduction in WBCMA. Therefore, if a drug or drug combinations were protective, then the level of CEES exposure was not so severe as to preclude the demonstration of an efficacious result.

White blood cell metabolic activity post-CEES exposure reflected effects on those WBCs surviving 24 h of *in vitro* culture since with carrier gas (air) exposure, the WBCMA at T₂₄ was reduced to ~20 percent of the T₀ value (table 3). That CEES exposure reduced WBCMA to a greater extent than the stress of *in vitro* culture itself was demonstrated by the significant reduction of WBCMA to ~9 percent (table 3). Thus, the use of a 24 h *in vitro* culture allowed us to measure effects of CEES on the hardiest HWB. Employing a shorter *in vitro* incubation time may have produced the same effect since the more fragile cell types that succumb during *in vitro* culture are also likely to be those most rapidly affected by CEES. Unless the incubation times were very short, the more fragile cell types would not have contributed to the post-CEES WBCMA.

Vapor exposure of HWB by CEES was employed since it reflected the nature of delivery in most mustard chemical weapons. The consistency with which a near 50 percent reduction in WBCMA was achieved suggested exposures were administered with repeatable precision. The use of liquid exposure might further improve precision to promote fine adjustment of the sensitivity of the HWB model.

Comparisons of carrier gas exposure in the presence or absence of drug(s) demonstrated that while C or L did influence WBCMA, under the conditions of *in vitro* culture, P did not (figure 1). Polyoxometalate was selected because it oxidizes mustard and CEES and, if the oxidized product was less toxic than the vesicant, P might offer some protection of cells from the vesicant. The P concentration (5 µg/mL) selected, which was non-toxic to HWB (figure 1), was based on tests that determined its CEES-oxidizing dose (data not shown). Higher P concentrations (50 or 500 µg/mL) reduced WBCMA with *in vitro* culture and did not afford CEES protection (data not shown). The fact that the exposures to both CEES and carrier gas in the presence of P reduced the WBCMA suggests that the oxidation product of CEES is not less toxic than CEES. Both C and L are expected to offer protection via other mechanisms.

Both C (Beitner et al., 1991) and L (Sarin et al., 1993) are known blockers of cell death pathways. As such, their enhancement of *in vitro* WBCMA was not surprising because WBCs

proceed through cell death pathways as a result of *in vitro* culture (Herbert et al., 2001). The drug concentrations used for C (Beitner et al., 1991) and L (Cowan et al., 1992) were consistent with those previously employed to block their respective cell death pathway and were non-toxic for HWB cells (figure 1). However, titrating each drug and drug combination for the concentration that supported the most robust WBCMA after 24 h of *in vitro* HWB culture might optimize the conditions to test for drug antagonistic actions. Such optimization might influence what appeared to be a synergistic interaction of C+L+P with CEES on the reduction of WBCMA. Although not necessarily optimized for concentration, the fact that C or L increased WBCMA, with 24 h of *in vitro* culture, perhaps explained the maintenance or increase in WBCMA despite exposure to carrier gas or CEES when the WBCMA with combinations of these drugs was compared to the WBCMA in HWB without drugs. However, as expected, when drug(s) influences on *in vitro* culture were examined using paired comparisons between the CEES and carrier gas conditions, in which drug(s) was present, exposure to CEES, in all cases, significantly reduced the O.D. readings for WBCMA (figure 1).

We have demonstrated that the use of a readily available, complete human tissue, with minimal culturing requirements such as HWB, permitted a rapid screening for potential vesicant antidote or antagonist mixtures. This screening of three drugs singly or in all possible combinations did not demonstrate that any of these three drugs or drug combinations alleviated the CEES-mediated reductions in cellular metabolism. However, the dynamics of the HWB model system suggested that each drug and drug combination does have an effect on the WBCMA and that testing concentrations that most optimized WBCMA after 24 h of *in vitro* culture might improve the assessments. The effect of drug(s) on the *in vitro* culturing is perhaps an inherent confounder in other vesicant models, which employ metabolic activity as a marker of cellular health. Finally, considering the fragility of HWB cells relative to other tissues, such as keratinocytes, a mild vesicant exposure perhaps delivered in liquid form might offer another type of test that is less sensitive to *in vitro* culture. If the drug in HWB was exposed to a measured liquid amount of liquid agent and the WBCMA did not decrease significantly, further tests with more complex *in vitro* or laboratory animals could be used. This type of screening would conserve resources and animal use.

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Table 1. Drugs tested singly and in all possible combinations for potential vesicant antagonistic activity

Drug	CAS	Symbol	Mode of Vesicant Antagonistic Action
Leupeptin	24365-47-7	L	Inhibits proteases to reduce critical protein degradation
CGS 9343B (zaldaride maleate)	109826-27-9	C	Blocks calmodulin to limit calcium-mediated toxic events
Polyoxometalate	NA	P	Oxidizes vesicant, which may lower vesicant toxicity

Table 2. Effect of white blood cell (WBC) count on white blood cell metabolic activity (WBCMA)

Subject	N	WBC ($\times 10^3$)	Carrier Gas O.D.	CEES O.D.	% = (Carrier gas O.D./CEES O.D.) x 100
A	3	4.05 \pm 0.29*	0.283 \pm 0.022*	0.149 \pm 0.016* [†]	52.4 \pm 3.6
B	3	5.93 \pm 0.24	0.380 \pm 0.026	0.185 \pm 0.026 [†]	48.6 \pm 2.3

* = p<0.05 compared with Subject B

[†] = p<0.05 compared to carrier gas condition

Note: The reduction in WBCMA after exposure to 2-chloroethyl ethyl sulfide (CEES) or air is independent of the WBCs. Both sets of samples were exposed to either CEES (18 mg at 1.5 mg/L/min) or carrier gas (air) for 12 minutes at a rotation of 15 rpm and then cultured for 24 hours. The changes in WBCMA are reflected by changes in optical density (O.D.).

Table 3. The reduction in white blood cell metabolic activity (WBCMA) after exposure to 2-chloroethyl ethyl sulfide (CEES) or carrier gas (air) and 24 hour *in vitro* cell culture

Exposure	WBCMA (T ₀), O.D.	WBCMA (T ₂₄), O.D.	T ₂₄ /T ₀ x 100
Carrier gas	1.52±0.24	0.303±0.13*	19.9
CEES	1.52±0.24	0.135±0.06 [†]	8.9 [‡]

* = p<0.05 compared to T₀

[†] = p<0.05 compared to T₀ or T₂₄ Carrier gas

[‡] = p<0.05 compared to Carrier gas %

Note: WBCMA is measured at T₀, the time the human whole blood was collected, and at T₂₄, after 24 hours of *in vitro* cell culture.

Figure 1. The effect of various drug combinations on white blood cell metabolic activity (WBCMA) optical density (O.D.) after exposure to 2-chloroethyl ethyl sulfide (CEES) or carrier gas (air)

