DEVELOPMENT OF MEDICAL COUNTERMEASURES TO SULFUR MUSTARD VESICATION

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ABSTRACT

Sulfur mustard (HD) is an alkylating agent with cytotoxic, mutagenic and vesicating properties. Its use on the battlefield results in debilitating injuries to skin, eyes and the respiratory system (1, 2). To elucidate the toxic sequelae that follow cutaneous exposure to HD, the United States Army Medical Research Institute of Chemical Defense (USAMRICD) has undertaken a broad-based research program encompassing both intramural and extramural research. This report summarizes our current understanding of the toxicology of human exposure to HD based on *in vitro* and *in vivo* experimental models.

INTRODUCTION

While many of the toxic manifestations that follow HD exposure have been defined, the actual mechanisms of pathology remain elusive. Much of the research in this area has been conducted in the Pharmacology and Drug Assessment Divisions of USAMRICD, the laboratories of our NATO allies and laboratories funded through the Medical Research and Materiel Command extramural contract program. Based on the technological database developed, through this program, we have been able to generate a unifying hypothesis for cellular and tissue events that explains the formation of cutaneous blisters following exposure to HD. Studies of individual toxic events, such as alkylation of cellular macromolecules, formation of DNA strand breaks, activation of poly(ADP-ribose) polymerase (PARP or PADPRP), disruption of calcium regulation, proteolytic activation and tissue inflammation, have together led to the formulation of six strategies for therapeutic intervention (3, 4). The proposed pharmaceutical strategies are intracellular scavengers, DNA cell cycle modulators, PARP inhibitors, calcium modulators, protease inhibitors and anti-inflammatory compounds.

These compound classes are currently being evaluated as medical countermeasures against HD dermatotoxicity. We have validated four *in vitro* testing modules for compound screening: solubility, direct toxicity, protection against HD-induced cytotoxicity and protection against HD-induced depletion of cellular nicotinamide adenine dinucleotide (NAD+) levels. Two additional *in vitro* modules, preservation of cellular adenosine triphosphate (ATP) levels and inhibition of proteolysis, are in the final stages of validation. For *in vivo* screening, we have utilized the mouse ear vesicant model (MEVM) with associated histopathological evaluation (5) and cutaneous vapor exposure in hairless guinea pigs (6). For systemic drug therapy, we are validating a hairless mouse cutaneous vapor exposure model.

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EXPERIMENTAL DESIGN

DECISION TREE NETWORK (DTN)

A DTN has been devised to outline the selection process used to evaluate candidate pretreatment or treatment compounds. This DTN consists of pathways through *in vitro* and *in vivo* compound screening modules based on known characteristics of the compounds being evaluated.

IN VITRO SCREENING MODULES

As compounds are placed in the Drug Assessment Compound Tracking System, they are assigned to specific functional categories. Based on their categorization, they are evaluated through a series of assays such as aqueous solubility, direct cytotoxicity in human lymphocytes (PBL), protection of PBL against the cytotoxicity of HD, depletion of metabolic factors (NAD+ or ATP), and inhibition of HD-induced proteolysis. Results from these assays are used to prioritize movement of candidate compounds into the *in vivo* screening modules.

IN VIVO SCREENING MODULES

Compounds passing the *in vitro* modules or compounds from classes not applicable to *in vitro* screening (i.e., anti-inflammatory compounds) are tested in the MEVM for edema and histopathologic (i.e., epidermal-dermal separation and epidermal necrosis) evaluation. Other *in vivo* assays available for additional testing as required include cutaneous HD vapor exposure in the hairless guinea pig or the domestic swine and cutaneous HD vapor and liquid exposures in the hairless mouse. These modules usually employ topical treatment with candidate compounds, but new modules are being designed for systemic treatment regimes.

RESULTS AND DISCUSSION

BASIC RESEARCH

After its introduction onto the battlefield in World War I and through the 1940's, most of the research efforts directed toward HD focused on defining the histopathological sequeale of exposure in humans. Attempts were also made to establish relevant animal model systems. Beginning in the 1950's, research turned more toward the biochemical effects of HD and empirical studies were conducted with the aim of identifying therapeutic modalities. While the biochemical studies led to significant inroads for our understanding of the toxic mechanisms, the therapeutic approaches were futile. In the 1960's and 70's, HD research focused mostly on DNA damage and repair, cytotoxic mechanisms and mutagenesis.

Around 1990, the US Army decided to focus it efforts for developing medical intervention strategies for HD injury through the formulation of an Army Science and Technology Objective (STO) titled *Medical Countermeasures Against Vesicant Agents*. This STO presented three technical milestones: by 1996, define technological and pathophysiological databases and establish pharmacological intervention strategies for the HD injury; by 1997, show efficacy of a candidate medical countermeasure in an animal model; and by 2000, prepare a Milestone 0 drug development decision.

The first technical milestone for 1996 was met through the research efforts of the USAMRICD, the extramural contract program of the US Army Medical Research and Materiel Command (USAMRMC), and the medical research programs of our allied nations. From this research, we were able to construct a schema of the major events of the pathological processes documented in cells and tissues exposed to HD (Figure 1). This schema was presented at numerous Department of Defense and professional scientific forums, including the 20th Army Science Conference (3). The research findings of this program served as the core of a NATO sponsored monograph on HD research (7).

The second part of the 1996 milestone, i.e., define strategies for pharmacological protection against the vesicant injury, was met by utilizing the information developed for the pathology schema. We identified 6 specific areas of the pathologic mechanism that could serve as points of pharmacological intervention into the HD injury. These were presented along with the pathology schema at numerous meetings and are presented in Table 1 along with prototypic compounds, in each area, that have been shown to be efficacious against HD toxicity in various model systems.

The 1997 technical milestone called for the demonstration of efficacy by a candidate countermeasure in an animal model. This was first met by research in hairless guinea pigs by Yourick et al. (6) and subsequently confirmed in the MEVM (5, 8).

CANDIDATE COMPOUND SCREENING

In FY97, the program was converted from an Army STO to a Defense Technology Objective (DTO),CB.22, and while the technical milestones remained intact, a new metric was imposed on the drug development effort. Rather than identifying compounds that just significantly reduced our pathological endpoints, we were required to attain at least 50% reduction of indicators of morbidity.

Over 500 candidate prophylactic or therapeutic compounds have been evaluated through the antivesicant DTN. Sixty-two compounds have demonstrated an ability to provide significant modulation of edema and/or histopathology caused by HD *in vivo*. Of these 62 compounds, nineteen have demonstrated at least 50% protection against the pathological indicators of mustard injury (Table 2). All of these 19 successful candidates fall into four of our six original proposed strategies: anti-inflammatories (7), antiproteases (3), scavengers (6), or PARP inhibitors (3).

With these compounds as proof of concept we received approval for transition to Concept Development in November 2000. A new DTO (CB.30) has been approved and work has been initiated to drive the drug development process through Concept Exploration toward a transition to Advanced Development in the FY03/04 time period.

FUTURE

Having established proof of concept for the potential development of a medical countermeasure against vesicant agents, we will move the 19 most successful candidate compounds from Basic Research into the Concept Exploration phase of the drug development process. Through a downselection process currently underway we will present to the US Army Medical Materiel Development Activity the optimal candidate, route of administration and timing of dosage for transition to advanced drug development within 3 years.

Proposed Mechanism of HD Action

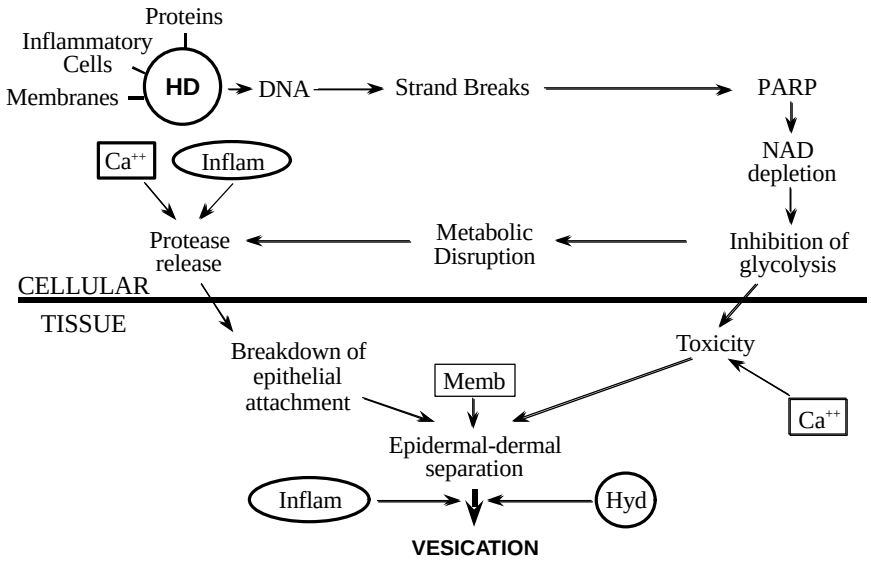

Figure 1. The cellular and tissue alterations induced by HD that are proposed to result in blister formation. HD can have many direct effects such as alkylation of proteins and membrane components (Memb) as well as activation of inflammatory cells. One of the main macromolecular targets is DNA with subsequent activation of poly(ADP-ribose) polymerase (PARP). Activation of PARP can initiate a series of metabolic changes culminating in protease activation. Within the tissue, the penultimate event is the epidermal-dermal separation that occurs in the lamina lucida of the basement membrane zone. Accompanied by a major inflammatory response and changes in the tissue hydrodynamics (Hyd), fluid fills the cavity formed at this cleavage plane and presents as a blister.

TABLE 1. Strategies for Pharmacologic Intervention of the HD Lesion.

Biochemical Event	Pharmacologic Strategy	Example
DNA Alkylation	Intracellular Scavengers	N-acetyl cysteine
DNA Strand Breaks	Cell Cycle Inhibitors	Mimosine
PARP Activation	PARP Inhibitors	Niacinamide
Disruption of Calcium	Calcium Modulators	BAPTA*
Proteolytic Activation	Protease Inhibitors	AEBSF*
Inflammation	Anti-inflammatories	Indomethacin; Olvanil

*BAPTA is a calcium chelator; AEBSF is a sulfonyl fluoride compound

TABLE 2. Candidate Countermeasures With Greater Than 50% Efficacy In Mouse Ear Model.

Total # of Positive Compounds = 19

Anti-inflammatory drugs	ICD #	% reduction in pathology
fluphenazine dihydrochloride	2040	50
Indomethacin	2086	96
olvanil	2723	91
olvanil (saturated)	2974	53
retro olvanil	2976	84
olvanil (urea analog)	2977	81
octyl homovanillamide	2980	100
Scavenger drugs		
2-Mercaptopyridine-1-oxide	1304	66
6-Methyl-2-Mercaptopyridine-1-oxide	1307	56
4-Methyl-2-Mercaptopyridine-1-oxide	1308	94
dimercaprol	2525	78
Na 3-sulfonatopropyl glutathionyl disulfide	3195	64
Hydrogen Peroxide gel, 3%	2828	58
Protease Inhibitors		
1-(40-aminophenyl)-3-(4-chlorophenyl) urea	1883	54
N-(0-P)-L-Ala-L-Ala-benzy ester hydrate	2780	62
Ethyl p-Guanidino Benzoate Hydrochloride	1579	62
PARP Inhibitors		
3-(4'-Bromophenyl)ureidobenzamide	1548	74
Benzoylene Urea	1796	54
4-amino-1-naphthol hydrochloride tech	2059	80

CONCLUSIONS

This research has been directed at meeting the Medical Chemical Defense DTO *Medical Countermeasures Against Vesicant Agents*. Based on results to date, we have met every milestone of the DTO, i.e., "to develop a technological database and define therapeutic strategies that protect against the vesicant injury," and "demonstrate efficacy in an animal model." Having identified at least 19 compounds that are capable of protecting against the *in vivo* pathology of HD, we now have the means to move from the research phase of pharmaceutical investigation into the Concept Exploration phase of drug development. This work, the combined efforts of Army, academic, industrial and contracted research laboratories, has set the stage for development of a fielded medical countermeasure against HD. For the first time since HD's introduction onto the battlefield more than 80 years ago, we have the true potential to protect our warfighters against this insidious weapon through pharmacological therapy.

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