U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-95-01

Drug Assessment Plan for the Evaluation of Antivesicant Pretreatment and Treatment Compounds

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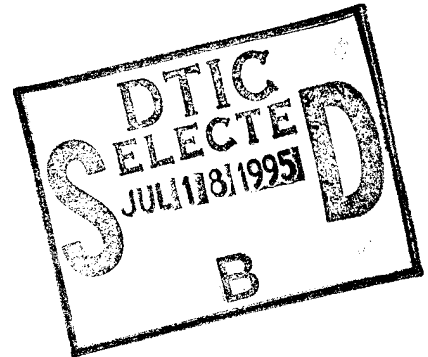

March 1995

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188
Exp. Date: Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

1b. RESTRICTIVE MARKINGS
N/A

2a. SECURITY CLASSIFICATION AUTHORITY
N/A

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE
N/A

3. DISTRIBUTION / AVAILABILITY OF REPORT
DISTRIBUTION A: Approved for public release; Distribution Unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
USAMRICD-TR-95-01

5. MONITORING ORGANIZATION REPORT NUMBER(S)
USAMRICD-TR-95-01

6a. NAME OF PERFORMING ORGANIZATION
U.S. Army Medical Rsch Inst of Cml Def

6b. OFFICE SYMBOL (If applicable)
MCMR-UV-D

7a. NAME OF MONITORING ORGANIZATION
U.S. Army Medical Rsch Inst of Cml Def
ATTN: MCMR-UV-RC

6c. ADDRESS (City, State, and ZIP Code)
Aberdeen Proving Ground, MD 21010-5425

7b. ADDRESS (City, State, and ZIP Code)
Aberdeen Proving Ground, MD 21010-5425

8a. NAME OF FUNDING / SPONSORING ORGANIZATION
N/A

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
N/A

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62787	3M162787A	875BA	

11. TITLE (Include Security Classification)
(U) Drug Assessment Plan for the Evaluation of Antivesicant Pretreatment and Treatment Compounds

12. PERSONAL AUTHOR(S)
Meier, H.L., Clayson, E.T., Romano, J.A., Jr.

13a. TYPE OF REPORT
Final

13b. TIME COVERED
FROM Feb 89 TO Jul 94

14. DATE OF REPORT (Year, Month, Day)
1995 March

15. PAGE COUNT
30

16. SUPPLEMENTARY NOTATION

17. COSATI CODES

FIELD	GROUP	SUB-GROUP
06	15	
06	11	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Blister agents, Decision Tree Networks, in vitro testing, in vivo testing, vesicant agents

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The Antivesicant P/T Plan is designed to rapidly identify compounds which, when given orally or parenterally before or shortly after vesicant exposure, will be effective in the prevention of reduction of vesicant induced cutaneous injury. The following activities are desired of potential compounds: compounds should scavenge vesicants, inhibit poly (ADP-ribose) polymerase, maintain NAD and/or ATP levels, prevent loss in membrane function/integrity, inhibit secretion of relevant mediators and/or proteases, stimulate DNA repair, or slow or arrest cell cycling. While the Plan is designed with these classes of compounds in mind, it should be versatile enough to handle most other candidate P/T compounds. The Plan is designed to aid the drug discovery process to identify potential antivesicant compounds with four basic guidelines used in the design of the Plan: (1) that compounds will be evaluated *in vitro* prior to evaluation in animal models, (2) that both animal and test drug requirements be the minimum needed to provide valid comparisons of proposed therapies, (3) that proposed compounds showing no promise be eliminated as rapidly as possible, and (4) that testing strategies allow for possible quantitative structure activity relationship studies.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
[X] UNCLASSIFIED/UNLIMITED [ ] SAME AS RPT. [ ] DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
UNCLASSIFIED

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MCMR-UV-D

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

Preface

In August 1985, the Commander, U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) appointed an *ad hoc* tech base drug discovery task area working group composed of members from USAMRICD and Walter Reed Army Institute of Research (WRAIR). The overall scope of the working group was to recommend improvements in the medical chemical defense tech base drug discovery program. At the 15 January 1986 meeting, the working group recommended the establishment of Technical Evaluation Committees (TECs) to evaluate and make recommendations relating to drug screening. Since at that time formal decision networks for tech base drug discovery did not exist, the working group recommended that the TECs develop these networks as an item of high priority. The concept of the TEC was implemented at USAMRICD in June 1986 by the formation of the Drug Assessment Technical Evaluation Committee (DATEC). DATEC recognized that separate plans were needed for screening different classes of drugs for each kind of chemical threat agent being countered. Soon after the first DATEC meeting, subcommittees were appointed to develop specific drug decision networks. Because the networks had a tree-like branching structure, they soon became known as Decision Tree Networks (DTNs). Over the years additional subcommittees were appointed to develop DTNs that were not originally conceived. Other drug discovery approaches have been designated as "Plans" to avoid the impression of a more rigid structure to the assessment process. Where the knowledge base is incomplete, model development (in particular for primary screens) is ongoing, and compound accession is intermittent and varied, use of the term "Plan" is preferred. The subcommittee's work addressed the following DTNs:

NERVE AGENT DTNs

Pretreatment - to identify compounds which, when used on a short-term basis before expected nerve agent exposure, are effective (in combination with therapy) in the amelioration of organophosphorus agent injury.

Reactivator - to identify compounds that have efficacy based upon the ability to reactivate organophosphorus-inhibited acetylcholinesterase (AChE).

Cholinolytic - to identify compounds clearly superior to atropine with respect to efficacy against organophosphorus agents.

Anticonvulsant - to identify compounds having anticonvulsant properties which are effective in either the pretreatment or treatment modes against organophosphorus agents.

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BLOOD AGENT DTN

Anticyanide - to identify compounds having efficacy (by any mechanism) in the prevention or therapy of cyanide poisoning.

VESICANT PLAN

Pretreatment/Treatment - to identify compounds for oral or parenteral administration that have efficacy in the prevention or therapy of the vesicant injury.

Topical Treatment - to identify topical formulations that effectively ameliorate or prevent development of cutaneous lesions after vesicant exposure.

MISCELLANEOUS PLANS OR DECISION NETWORKS

Topical Protectant - to identify topical formulations that, when applied to skin before exposure, limit or prevent vesicant or nerve agent contact with skin.

The objective of the tiered approach to the screening and development project begun in 1986 was to provide a management concept and program strategy that would facilitate the rapid identification of the best candidate compounds. Proponents believed that DTNs or Drug Assessment Plans would provide the following advantages:

- Rapid selection of the most promising candidates,
- Quick elimination of poor candidates and conservation of resources,
- Assurance of comparability of data,
- Provision of quantitative data for new drug design, and
- Facilitation of prioritization of resources.

Contributors

The testing strategy reflected in the Antivesicant Pretreatment/ Treatment (P/T) Drug Assessment Plan represents the scientific consensus of the Antivesicant P/T Plan Subcommittee. The Subcommittee met on a frequent basis, in 2- to 4-hour sessions, over the course of a 6-month period. The Subcommittee presented the Antivesicant P&T Plan to DATEC who recommended its implementation to the Commander, USAMRICD in June 1987. The Plan recommended to the Commander represents the best effort of the scientists who staffed the committee. The Subcommittee members were as follows:

Walter E. Sultan, Chairman
Principal Investigator
Drug Assessment Division

Philip Chan
Principal Investigator
Pharmacology Division

Clark Gross
Principal Investigator
Pharmacology Division

Philip Hammond
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Since the development of the original Plan, ongoing vesicant research has greatly enhanced our knowledge. The Plan presented here is modified from the original Plan approved in 1987. The various modifications were recommended by DATEC at several meetings since 1987.

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Introduction

The Antivesicant P/T Plan is designed to rapidly identify compounds which, when given orally or parenterally before or shortly after vesicant exposure, will be effective in the prevention or reduction of vesicant-induced cutaneous injury. Antivesicant compounds are being considered for both pretreatment and treatment modalities. Pretreatment refers to the administration of compound before exposure to vesicants, whereas treatment refers to the administration of compound after exposure but before the onset of incapacitating signs. In both cases, the aim is to prevent or reduce the lesions associated with vesicating agents. This Plan is not designed to identify compounds which, when given after the development of lesions, will speed healing of those lesions. Ideally, both pretreatments and treatments should be void of undesirable side effects since administration of the drugs would occur before the onset of incapacitating lesions.

The DATEC has expressed an interest in screening compounds with the following activities for potential use as antivesicants: compounds that (a) scavenge vesicants, (b) inhibit poly (ADP-ribose) polymerase, (c) maintain NAD and/or ATP levels, (d) prevent loss in membrane function/integrity, (e) inhibit release of relevant mediators and/or proteases, (f) inhibit relevant mediator function, (g) inhibit relevant protease activity, (h) stimulate repair of alkylated DNA, and (i) slow or arrest cell cycling. While the Plan is designed with these classes of compounds in mind, it should be versatile enough to handle most other candidate P/T compounds. If other classes of compounds become of interest that are not adequately addressed here, then additional *in vitro* assays may be added.

This Plan is designed to aid the drug discovery process to identify potential antivesicant compounds, and does not describe the predevelopment or development tests necessary for fielding a drug. The subcommittee fully expects that these preliminary tests be followed by more complete safety and efficacy evaluations as part of the predevelopment and development testing stages.

Four basic guidelines were used in the design of the Plan:

- That compounds will be evaluated *in vitro* and demonstrate potential for efficacy before being evaluated in animal models,
- That both animal and test drug requirements be the minimum needed to provide valid comparisons of proposed therapies,
- That proposed compounds showing no promise be eliminated from consideration as rapidly as possible, and
- That the testing strategy provide the maximum amount of useful information possible for quantitative structure activity relationship (QSAR) studies.

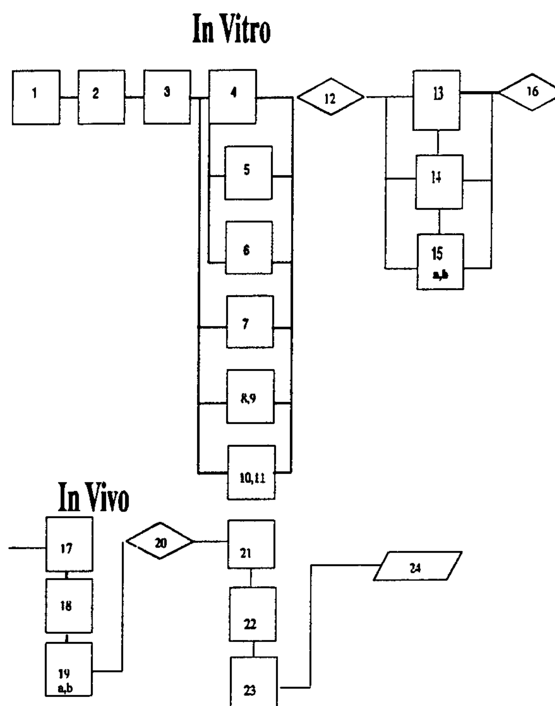

FIGURE 1

Plan for the Identification of Antivesicant Pretreatments/Treatments

Although highly structured to provide maximum definition to the drug discovery program, all Plans are flexible working documents which may be altered to reflect changes in program objectives or availability of resources. Since the mechanism by which vesicating agents cause blisters is unknown, developing a network for the assessment of candidate compounds is difficult at best. While tests included in this Plan have been developed previously and used by a number of different investigators and organizations, they have not been evaluated in the sequence or for the purpose that is proposed. The subcommittee, therefore, expects that a validation study using several members of each proposed antivesicant class will be conducted to ensure the validity of the screen up to the final selection stage. In fact, this process has indeed occurred for many of the proposed test modules.

The subcommittee recognizes that, while there are many possible alternatives to the sequence of the testing modules, the one defined in this document is a reasonable approach that should meet the objectives of the Medical Chemical Defense Program. Elements of this Plan have been presented for review and comment at DATEC meetings in 1988, 1991, 1992 and 1993. However, this document represents the first publication

of the entire structure of the Drug Assessment Plan for Evaluation of Antivesicant Compounds.

Overview of the Antivesicant Pretreatment/Treatment Decision Tree Network

The major segments of the antivesicant P/T Plan are composed of a series of testing modules arranged in a logical sequence (Figure 1). The Plan has three decision points designed to ensure that only the most promising compounds undergo further testing. At each decision point, the results for test compounds with the same expected mode of action are compared with each other and only the best compounds are selected to proceed.

During the first two phases of the Plan, a series of *in vitro* tests will be conducted to determine the activity, lack of cytotoxicity, and possible mechanism of action of a test compound before safety and efficacy tests are performed in animal models. During the first phase only those compounds that are miscible or can be uniformly suspended in aqueous buffers will be examined. A compound will have an individualized pattern of passage through the first phase of the Plan depending on its suspected mechanism of action. The *in vitro* tests are designed to identify compounds which prevent vesicant-induced cell death, limit cellular secretion of proteases or mediators, and/or stimulate the cell's ability to repair DNA and reproduce after vesicating agent exposure. Even though a correlation between *in vitro* activity and *in vivo* efficacy has not been established, the *in vitro* tests are useful to assess compound potency for well-characterized, desirable effects and to provide information on mechanism of action; therefore, results from the *in vitro* evaluations will be used to prioritize the available compounds for *in vivo* screening. Compound prioritization will be a dynamic process in which only the best candidate(s) available at any given time will be advanced to *in vivo* testing.

A word on Multiple Endpoint Assays: Multiple endpoint assays using fluorescent probes of specific cellular structures/enzymes have been proposed as effective means to examine *in vitro* toxicity. Multiple assays have several advantages: (1) they may avoid false negatives or positives associated with using only one endpoint, (2) use of batteries of assays may reveal mechanisms or determine the sequence of events of cellular description following HD exposure, (3) fluorescence-based assays can allow for simultaneous evaluation of cellular viability, plasma membrane integrity, lysosomal integrity, GSH of levels, intracellular calcium, mitochondrial activity, etc., (4) use of such fluorochromes allows for rapid throughput of samples using certain instrumentation, and (5) results can be compared with results obtained through ultrastructural or fluorescence microscopy. The feasibility of such approaches is being evaluated in the Drug Assessment Division under protocol no. 1-05-94-000-A-680.

Prior to *in vivo* testing chemical stability and ease of laboratory scale-up of compound synthesis will be determined to ensure that detailed studies are not begun with unstable materials or compounds too costly for consideration in large-scale use.

Alternatively, ethical considerations do not allow for experimentation in man. The initial *in vivo* screens evaluate compound toxicity to determine a safe dose of compound to use in efficacy studies. Since soldiers must perform their missions while being protected by pretreatment compounds, behavioral testing is evaluated, and efficacy testing is performed at doses no higher than the no observable effect level (NOEL). During the efficacy evaluation, several endpoints will be examined which include, but are not limited to, the separation of the epidermis from the dermis, edema formation, and development of erythema. It is recognized that a compound is likely to reduce or prevent one of these endpoints without affecting the other endpoints.

It is important to note that much of the data generated during the initial and advanced screening phases is reported back to the synthesis contractors in order to facilitate quanitative structure-activity relationships (QSAR). In addition, summary information for each test module is entered into the Drug Assessment Compound Tracking System (DACTS), a computerized program information system. This system tracks each compound through the screening process.

Finally, a thorough review of all data derived from the testing modules will be conducted. A clear recommendation for either selection or rejection of the candidate compound will emerge from this formal review. All data generated from these studies will be made accessible to all interested parties in the U.S. Army Medical Research and Materiel Command. This review and data access will provide guidance for both program management and QSAR, and is critical to the success of the testing program.

Detailed Description

IN VITRO SCREENING

In vitro screening is used to provide information about the potential cytotoxicity, possible mechanism of action, and potency, of a test compound before safety and efficacy tests are performed in animal models. The potency of the compound for some desirable effect will be determined and compounds will be prioritized for *in vivo* testing based on their potency. This segment consists of compound accession, solubility determination, cytotoxicity and several other *in vitro* tests.

Designing appropriate *in vitro* assays to identify compounds with potential efficacy against vesicating agents is difficult at best since the mechanisms of action of vesicating agents are unknown. The endpoints for the *in vitro* tests are chosen with the current knowledge and/or belief of the mechanisms of vesicant-induced injury in mind. The *in vitro* tests are designed to identify compounds which prevent vesicant-induced cell death, maintain cellular energy levels, limit cellular secretion and/or activity of proteases or inflammatory mediators, stimulate the cells' ability to repair DNA, and/or stimulate the cells' ability to replicate after vesicating agent exposure. When further knowledge is obtained concerning the mechanism of injury induced by vesicating agents, other *in vitro* assays may need to be added to this Plan.

The title, purpose/rationale, and description of each testing module of the Antivesicant P/T Plan are described below. Numbers correspond to the numbering of the network modules illustrated in Figures 1 through 3.

1. Compound Accession

Purpose/Rationale: Provide storage, audit, and tracking procedures which document disposition and testing of candidate compounds in contractor and government laboratories. Identify which *in vitro* screens a potential compound will be tested in based on knowledge concerning the possible mechanism of therapeutic action.

Description: Compounds entering this Plan will be limited to those compounds that have a reported biochemical/pharmaceutical mode of action that DATEC believes may be of benefit in preventing or reducing the vesicant injury. Compounds submitted for testing are processed through WRAIR, which serves as the chief repository for the chemicals. WRAIR assures that the materials are properly identified, stored, and shipped and maintains or obtains sufficient quantities for testing. All compounds received at USAMRICD are logged into the inventory tracking system of the DACTS. Entries into the

data base are keyed to the Division of Experimental Therapeutics by Bottle #, ICD #, WRAIR #, quantity received, and quantity used during testing. Each compound will be assigned an individualized pattern of passage through the *in vitro* phase of the Plan depending on its suspected mechanism of action. A compound may undergo testing in more than one *in vitro* module if deemed appropriate, but care should be taken to prevent a compound from being screened in inappropriate modules (e.g., a protease inhibitor screened in the DNA repair module).

Reference: USAMRICD SOP # SGRD-UV-DB-6-87.

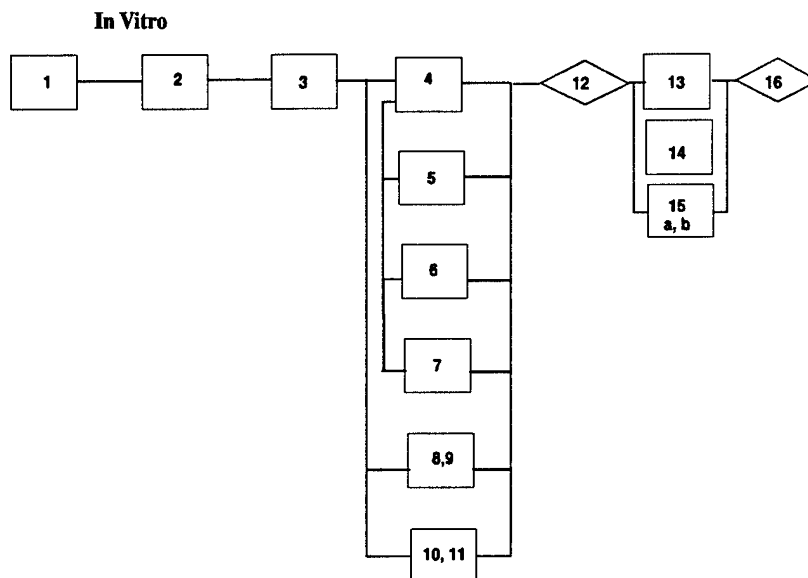

FIGURE 2
In Vitro Segment

2. Solubility Determination

Purpose/Rationale: To identify the limit of solubility of the candidate compound in an aqueous medium (saline) and to identify the solvent in which the compound is most soluble. The limit of solubility in aqueous media is needed for *in vitro* tests and the ideal solvent is needed for *in vivo* tests. Only physiologically compatible solvents will be used.

Description: The solubility of each candidate compound will be determined in a number of solvents suitable for human use. Maximum solubility is determined in mg/ml.

Reference: USAMRICD SOP # SGRD-UV-DB-1-87.

3. Cytotoxicity

Purpose/Rationale: To determine the highest concentration of candidate compound that is not toxic to cells.

Description: Cells will be exposed to at least five concentrations of candidate compound and cell viability will be determined at 24 hours post exposure to the compound using a dye exclusion assay. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being either the concentration at maximum solubility or 1 mM, whichever is lower. A concentration of 1 mM was chosen as the highest concentration of compound to be examined to prevent problems with solubility of other components of the cell growth medium. If the candidate compound is non-cytotoxic at 1 mM, then the highest non-cytotoxic concentration will be reported as >1 mM. Other cytotoxicity endpoints may be examined concurrently with dye exclusion; however, more weight will be given to the dye exclusion results in determining the highest non-cytotoxic concentration.

Reference: USAMRICD Protocol No. 1-11-90-000-A-584

4. Reduction of Vesicant-induced Cell Death

Purpose/Rationale: To identify candidate compounds that are capable of preventing or reducing vesicant-induced cell death. This module will be used to test all candidate compounds except for the following classes: anti-inflammatory agents and protease inhibitors. These compounds will be tested in other *in vitro* modules. Human lymphocytes were chosen as the cell type to be used in this assay because they are a sensitive site for systemic HD poisoning, and because they are non dividing cells. Using these cells, biochemical cell death induced by vesicating agents may be examined in the absence of replicative cell death that results when a cell tries to replicate damaged DNA.

Description: Lymphocytes will be isolated from blood drawn from normal human volunteers under an approved human use protocol. The lymphocytes will be pretreated with at least five concentrations of a candidate compound and exposed to an approximate EC87 concentration of HD (170 μM). Cell viability will be determined at 24-26 hours post-exposure using a dye exclusion assay and a flow cytometer. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an EC50 concentration of compound for the reduction of vesicant-induced cell death will be determined. The EC50 concentration will be used as an indicator of potency. If the compound is not effective or potentiates vesicant cytotoxicity, or if an EC50 cannot be determined, then the compound will be reported as not effective. If a compound

has an EC50 greater than niacinamide or equal to or greater than the lead compound (whichever is lower), then it will proceed to the second phase. All other compounds will proceed to the other appropriate *in vitro* tests as assigned in paragraph 1, Compound Accession.

References: USAMRDC SOP Log No. A-3575, and USAMRICD Protocol No. 1-11-90-000-A-584

5. Biochemical Marker Assay

Purpose/Rationale: To identify compounds that prevent vesicant-induced loss of cellular energy (ATP). This assay will be used to test compounds that are known or suspected to intervene in the vesicant's action prior to or at its ability to inhibit glycolysis.

Description: An assay will be developed to quantitate the level of ATP in human lymphocytes exposed to vesicants. After incubation in the presence or absence of vesicant (HD or L), the lymphocytes will be acid lysed. The supernatants will be neutralized and their ATP content measured. ATP will be measured by the luciferase assay on a luminometer. Concentrations will cover a two log rymthic unit range. If the candidate compound is effective in a concentration dependent manner, then an EC50 will be calculated. The EC50 concentration will be used to rank order the compounds.

Reference: USAMRICD Protocol No. 1-11-90-000-A-582

6. DNA Repair Assay

Purpose/Rationale: To identify compounds that stimulate the repair of HD damaged DNA. This assay will be used to test compounds that are known or suspected to stimulate DNA repair.

Description: An assay will be developed to quantitate DNA repair in HD exposed cells. Incorporation of ^{3}H-Thymidine may be a useful endpoint for this assay. The assay may be conducted with or without standard compounds that reduce cell death or that reduce cell cycling. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependent manner, then an EC50 concentration of compound for stimulation of DNA repair will be determined. The EC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an EC50 cannot be determined, then the compound will be reported as not effective.

Reference: USAMRICD Protocol No. 1-11-92-000-A-639

7. Cell Cycling Assay

Purpose/Rationale: To identify compounds that arrest cell reproduction long enough so that the cell can repair of vesicant damaged DNA. This assay will be used to examine compounds that are known or suspected to reduce the rate of DNA replication.

Description: An assay will be developed to quantitate the rate of cell cycling in HD exposed cells. Incorporation of ^{3}H-Thymidine may be a useful endpoint for this assay in the presence or absence of hydroxyurea. The assay may be conducted with or without standard compounds that reduce cell death. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an EC50 concentration of compound for the reduction of cell cycling will be determined. The EC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an EC50 cannot be determined, then the compound will be reported as not effective.

Reference: SOP/Protocol in preparation

8. Mediator Release Assay

Purpose/Rationale: To determine the ability of compounds to inhibit the vesicant-induced secretion of inflammatory mediators from isolated cells. Inflammatory mediators are thought to be involved in the formation of the vesicant lesion, but the mediator(s) involved has not been identified. Compounds that are known or suspected to prevent cellular secretory mechanisms will be evaluated to determine their ability to inhibit the release of the mediator(s) of interest.

Description: An assay will be developed to quantitate the release of the mediator(s) involved in blister formation. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an EC50 concentration of compound for the reduction of mediator release will be determined. The EC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an EC50 cannot be determined, then the compound will be reported as not effective.

Reference: USAMRICD Protocol No. 1-11-92-000-A-639

9. Mediator Inhibition Assay

Purpose/Rationale: To identify compounds that inhibit the activity of the mediator(s) involved in blister formation. Inflammatory mediators are thought to be involved in the formation of the vesicant lesion, but the mediator(s) involved has not been identified. Compounds that are known or suspected to inhibit mediator activity or to compete with the mediator for binding to its receptor will be evaluated to determine their ability to inhibit vesicant-induced mediator activity.

Description: An assay will be developed to quantitate the inhibition of mediator activity involved in blister formation. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an IC50 concentration of compound for the inhibition of mediator activity will be determined. The IC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an IC50 cannot be determined, then the compound will be reported as not effective.

Reference: SOP/Protocol in preparation

10. Protease Release Assay

Purpose/Rationale: To determine the ability of compounds to inhibit the vesicant-induced secretion of proteases from isolated cells. Proteases are thought to be involved in the formation of the vesicant lesion, but the protease(s) involved has not been identified. Compounds that are known or suspected to prevent cellular secretory mechanisms, will be evaluated to determine their ability to inhibit the release of the protease(s) of interest.

Description: An assay will be developed to quantitate the release of the protease(s) involved in blister formation from cells. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an EC50 concentration of compound for the reduction of protease release will be determined. The EC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an EC50 cannot be determined, then the compound will be reported as not effective.

Reference: To be developed

11. Protease Inhibition Assay

Purpose/Rationale: To identify compounds that inhibit the activity of the protease(s) involved in blister formation. Proteases are thought to be involved in the formation of the vesicant lesion, but the protease(s) involved has not been identified. Compounds that are known or suspected to inhibit protease activity will be evaluated to determine their ability to inhibit vesicant-induced protease activity.

Description: An assay will be developed to quantitate the inhibition of protease activity involved in blister formation. The assay should be developed with isolated protease(s) and a synthetic substrate if possible. Several concentrations of the candidate compound will be studied to determine its ability to decrease the cleavage of the substrate by the protease. Only the specific protease(s) responsible for the cleavage of the epidermis from the dermis will be used in this assay. The concentration range of the candidate compound will cover at least 2 log orders of magnitude with the highest concentration examined being the highest non-cytotoxic concentration determined in module 3 above. If the candidate compound is effective in a concentration dependant manner, then an IC50 concentration of compound for the inhibition of protease activity will be determined. The IC50 concentration will be used as an indicator of potency. If the compound is not effective, or if an IC50 cannot be determined, then the compound will be reported as not effective.

Reference: To be developed

12. Decision Point 1

At this point, the effectiveness and potency of many compounds should be known. Compounds will be prioritized within a class based on potency; the higher the potency of the compound, the higher the priority for further screening. As a guide, compounds with EC50 or IC50 values in any of the *in vitro* assays equal to or less than 10 μM or less than 1% of its LD50, should be considered for high priority testing. Compounds that were not effective in any of the *in vitro* assays will be given the lowest priority for *in vivo* screening. Priority between compound classes will be determined by DATEC. At this point all data obtained from the *in vitro* screening will be sent back to synthesis contractors for QSAR analysis. All compounds sent on in the screen will also require DATEC asking WRAIR to perform preliminary stability studies to be done at WRAIR under SOP # SGRD-UWM-MC-1-87. WRAIR will also be asked to supply a minimum of 10-100 grams of the compound depending on the potency of the compound. This larger amount is required for animal testing in Phase III and IV (*in vivo* testing).

In Vitro Organ Culture Screening

In vitro organ culture screening consists of modules designed to study the ability of candidate compounds to be effective in tissue preparations. These tissue preparations of human skin, lung, and/or eye will study the effectiveness of the candidate compounds to perform in a complicated mix cell matrix. The assay in this phase of the Plan will measure the ability of the compound to preserve the integrity of the intercellular matrix and to prevent the release of inflammatory mediators and proteases. These assays will indicate whether a candidate compound will exert the same effect on a cell in the mixed cell tissue environment as it did on single cell suspensions.

13. Morphological Effects on Skin Organ Cultures

Purpose/Rationale: To identify compounds that reduce the pathological effects of vesicants on human skin sections maintained in culture. This module is designed to test all compounds of interest except anti-inflammatory agents.

Description: Human skin obtained from surgical specimens will be dissected free of subcutaneous fat and the lower portion of the dermis. Skin sections will be floated on the top tissue media containing the compound of interest. The sections are exposed to vesicant and skin organization and cellular changes will be assessed at 24 hours post-exposure by histopathology.

Reference: To be developed

14. Inhibition of Secretion in Chopped Skin

Purpose/Rationale: To identify compounds that reduce vesicant-initiated secretion of cellular inflammatory mediators and proteases from skin sections maintained in culture.

Description: Human skin obtained from surgical specimens will be dissected free of subcutaneous fat and the lower portion of the dermis. Skin cut into 1 mm sections will be immersed in tissue media containing the compound of interest. The sections are exposed to vesicant, and at various times, the supernatant will be assayed for related inflammatory mediators and proteases.

Reference: To be developed

15. Feasibility Studies

15.a. Preliminary Stability Study

Purpose/Rationale: To provide an early assessment of potential stability problems.

Description: Stability of the test compound is evaluated by measuring rate of degradation in environmentally stressed samples.

Reference: WRAIR SOP# SGRD-UWM-MC-1-87

15.b. Feasibility of 100-kg Lot Synthesis

Purpose/Rationale: To assess any problems involved in producing sufficient compound needed for *in vivo* testing.

Description: An investigation will be conducted and a report made to determine the availability of starting chemicals and the economic feasibility of producing a 100 kg lot of test compound.

Reference: WRAIR SOP# SGRD-UWM-MC-2-87.

16. Decision Point 2

Candidate compounds will be prioritized for *in vivo* testing based on their previous potency scores and on their ability to reduce pathology/secretion in skin sections. All data from the second stage screening will be sent back to DATEC and the synthesis contractor for QSAR determinations.

***IN VIVO* SCREENING**

In vivo screening consists of modules to study the safety and efficacy in animal models when given either orally or parenterally to animals. The third and fourth phases of the Plan consist of *in vivo* screening in two animal models. It is in these areas that the greatest scientific risk occurs. Seventy-five years after the first application of mustard (HD) in warfare, we still lack an unequivocable animal model of vesicant effects. The next few assays evaluate the compounds toxicity, pharmacokinetics, and behavioral side effects and help determine a safe dose of compound to use in efficacy studies. Since soldiers must perform their missions while being protected by pretreatment compounds, behavioral testing is evaluated, and efficacy testing is performed at doses no higher than the no

observable effect level (NOEL). During the efficacy evaluation, several endpoints will be examined which include, but are not limited to, the separation of the epidermis from the dermis, edema formation, and development of erythema. It is recognized that a compound is likely to reduce or prevent one of these endpoints with out affecting the other endpoints.

17. Acute Toxicity in the Hairless Guinea Pig

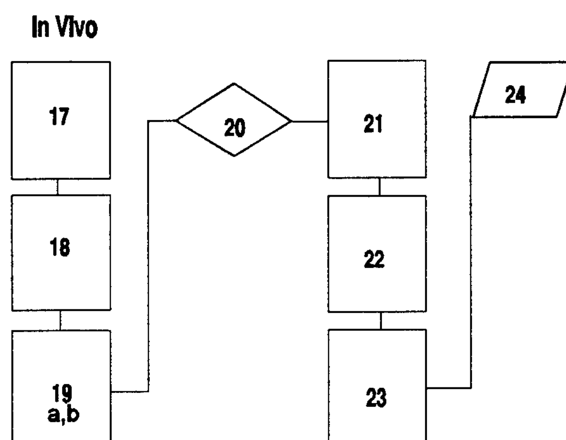

FIGURE 3
In Vivo Segment

Purpose/Rationale: To obtain an initial toxicity assessment of each compound, and to provide a rational basis for selecting doses for additional testing.

Description: If a good estimate of the LD50 cannot be found in the literature search, then an intramuscular and/or oral LD50 will be determined using a 24-hour end point. A dose of 400 mg/kg will be chosen as the highest dose to be tested, based both on practical use considerations and a desire to conserve sample. An LD_{50} value will be computed by the Moving Average Method of Thompson and Weil [Thompson, W.R., and Weil, C.S., "On the Construction of Table for Moving Average Interpolation," Biometrics 8, 51-54 (1952)]. Twenty-four-hour survivors will be evaluated for physical performance decrements. In animals used to evaluate potential protease inhibitors, blood will be drawn and a PPT clotting test performed to be sure that no prolonged bleeding times results in treated animals.

Reference: To be developed

18. Pharmacokinetic Studies

Purpose/Rationale: To assess the half-life and bioavailability of candidate compounds to the skin when given either orally or parenterally and to provide information needed to determine the dosing scheme for efficacy studies.

Description: A bioanalytical method to measure the compound(s) of interest in the skin will be developed. Pharmacokinetic studies are then conducted in guinea pigs to determine bioavailability and half-life of the candidate compound in skin. Any compound generating greater than a +2 Draize swelling at the sight of application or any over pathology will be returned to DATEC for re-evaluation.

Reference: USAMRICD Protocol No. 1-11-92-000-A-648 and follow-on protocols

19. Initial Efficacy Determinations

19a. Initial Efficacy Determination in the Hairless Guinea Pig

Purpose/Rationale: To identify compounds that reduce the separation of the epidermis from the dermis, reduce erythema, and reduce edema initiated by vesicant exposure. The hairless guinea pig-vapor cup model was selected for this module based on the uniform response to HD, ease of HD application, and similarity of skin structure and pathology to humans. Unlike the initial efficacy modules in other Plans where a single dose of candidate compound is given, the candidate compound is given in such a manner to approach steady state conditions before, during, and after exposure to vesicating agent to give the compound the best possible chance to demonstrate efficacy. This dosing scheme is necessary due to the long period between exposure and onset of clinical signs. Since a compound has the potential to interfere anywhere in the cascade of events that lead to blister formation, the compound must be present at high enough levels before exposure and remain throughout the entire cascade of events.

Description: Hairless guinea pigs will be pretreated with four doses of candidate compound (either orally or parenterally) given at the pharmacologic half-life of the compound in skin (as determined in module 18). Using this dosing scheme the levels of compound in the skin should be at 90% of steady state levels. The dose will be determined based on the combined results from modules 17 and 18. The animals will then be exposed to a 7-minute vesicant vapor challenge using the vapor cup method. After HD exposure the animals will be treated with the candidate compound at each pharmacologic half-life. At 24 hours post-exposure to vesicant the animals are euthanized and skin punch biopsies are taken and prepared for histopathology. If the candidate compound is an anti-

inflammatory agent then erythema measurements will be taken at 4-6 hours post exposure and edema measurements will be taken at 12-16 hours post-exposure.

Reference: USAMRICD Protocol No. 1-11-91-000-A-626

19b. Mouse Ear Inflammation Model

Purpose/Rationale: At the time of final development of this plan, the hairless guinea pig had been, and would remain, unavailable. Efforts to develop a suitable replacement efficacy screen have moved in several directions. The mouse ear inflammation model was one of these. The croton oil vesicant mouse ear model is a well developed assay for the evaluation of the cutaneous effects of inflammation. It has been suggested as a method available for determining topical activity of anti-inflammatory responses. Several investigators have further quantitated the chemically induced inflammatory responses in the mouse ear by measuring radioactivity as counts per minute (CPM) of ^{125}I-albumin. Analogues of these methods have been developed in-house. For very specific types of inflammation which may be produced by mustard, the mouse ear assay may prove fruitful.

Reference: USAMRICD Protocol No. 1-05-93-000-A-677 and follow-on protocols

20. Decision Point 3

Candidate compounds will be prioritized for the next level of *in vivo* testing based on the data from Module 19, efficacy in the hairless guinea pigs. The results from stability testing and ability of large volume manufacturing will be used to help rank order which compounds go into the next phase of animal testing. This next phase of screening should be performed in either a pig model or a non-human primate model.

21. Behavioral Effects in Second Animal Model

Purpose/Rationale: To determine behavioral side effects of a candidate compound. Also, to determine the no observable effect level (NOEL) for both orally and intramuscular injections of the candidate compounds.

Description: The test would be dependent on the animal model of choice. Serial probe recognition could be used in the non-human primate.

22. Further Pharmacokinetic Studies

Purpose/Rationale: To determine the half-life and bioavailability of candidate compounds to the skin and possibly the lungs of the second animal mode. This would provide data for the dosing regimens in both the behavioral toxicity module and the efficacy module.

Description: The analytical methods used to measure the compound(s) of interest in the blood and tissue (skin and/or lung) of hairless guinea pigs should be usable in the second animal model, but this would have to be verified. The pharmacokinetics of the compound(s) will have to be determined for both the oral and intramuscular routes of administration. These pharmacokinetic studies will have to determine the bioavailability and half-life of the candidate compound in blood and skin.

Description: Note on Isolated, Perfused Skin Models: Perfused skin preparations such as the isolated perfused porcine skin flap (IPPSF) may offer new insights into the mechanisms of cutaneous absorption of toxicants, as well as their pharmacokinetics and metabolism. IPPSF fit somewhere between *in vitro* diffusion models and whole animals (*in vivo* studies.) IPPSFs may allow us to (1) study mechanisms of absorption, (2) quantify cutaneous metabolism of toxicant or potation therapy, (3) study the effects of altered skin physiology on absorption and metabolism, and (4) design transdermal drug delivery systems with requisite pharmacokinetics for therapeutic effects on HD poisoning.

Reference: MREF Task 92-31

23. Initial Efficacy Determination in a Second Animal Model

Purpose/Rationale: To identify compounds that reduce the separation of the epidermis from the dermis, reduce erythema, and reduce edema. A second efficacy module will be developed when a suitable animal model is discovered. The dosing scheme will be similar to that described for the hairless guinea pig. See also comments regarding the mouse ear inflammation model (Section 19.a).

Description: It has been reported that the weanling pig is susceptible to vesicant exposure, especially in the abdominal and inguinal areas. Other attributes of weanling pig skin that make it a potentially useful model are the facts that (1) the thickness of the epidermis is similar to man, (2) pig skin has density, like man, is sparse, and (3) lipid composition and carbohydrate biochemistry is similar to man, (4) dermal microcirculation is similar to man, and (5) epidermal hormone kinetics are similar to man. Thus, we have

been evaluating morphological and histopathological endpoints, erythematous reactions, and epidermal biochemistry in the weanling pig to determine the usefulness of this model.

Reference: MREF Pretasks 93-19 and 93-20

24. Formal DATEC Review

At this point the data from modules 15-21 will be assembled for review by DATEC. The DATEC will conduct a formal review and make written recommendations to the Commander, USAMRICD, as to which compound(s) should be advanced to the predevelopment stage for the anti-vesicant pretreatment and treatment compounds.

SUMMARY

This Plan will provide data on the utility of candidates as either pretreatment and/or treatment compounds in two species. In all cases emphasis has been placed on pretreatment, but the possibility of finding a useful treatment compound has not been ignored. Data to allow for a correlation of *in vitro* tests with *in vivo* results will be available. Preliminary data on efficacy against vesicant-induced cutaneous lesions will also be available. Some *in vivo* pharmacokinetic data will have been determined and the extent of protection at a NOEL dose level in two animal models will be known. There will be data on the stability and feasibility of synthesizing compounds. *In vitro* and *in vivo* data will be available to send back to synthesis contractors for QSAR analysis. The Antivesicant Drug Assessment Plan will provide sufficient data on which to base a recommendation for advancing a compound to predevelopment. The rank ordering of all compounds tested provides for continuous selection of the "best of the best" compounds for evaluation in advanced predevelopment testing.

Rationale for Animal Model Selection

The hairless guinea pig serves as the initial screening *in vivo* model. A second animal model will have to be developed for module 21. Hairless guinea pigs were selected for use in this Plan for the following reasons:

a. Genetically pure strains are available in the large numbers required.

b. Hairless guinea pigs are easily housed and handled.

c. Their small size enables testing of sufficient animals to allow statistical validity with a minimum quantity of test compound.

d. Hairless guinea pig skin is thicker and more morphologically similar to human skin than normal guinea pig skin.

e. The lack of hair on the hairless guinea pig facilitates the application of vesicant to the skin.

f. A database of hairless guinea pig responses to various HD vapor exposure times exists and can be used as a historical control group.

The weanling pig and mouse ear inflammation models, at the time of writing, appear to be suitable alternative models. Reasons for selection of the weanling pig are below:

a. The density, epidermal turnover kinetics, and other morphology of porcine skin is similar to human skin.

b. Dermal microcirculation, enzyme histochemistry, and arrangement of dermal collagen and elastic fibers, are also reported to be similar to human skin.

c. The skin of the weanling pig shows similarities to the human subcutaneous response to HD, including formation of small gross blisters.

Reasons for use of the mouse ear inflammation test (MEIT) include the following:

a. The MEIT has been validated as an accurate, sensitive and efficient predictor of human contact hypersensitivity.

b. The MEIT is economical in terms of labor, resources, animals, and costs.

Definitions

Antivesicant: A compound that prevents or reduces the cutaneous response to vesicating agents.

Candidate Compound: A drug or formulation selected for drug assessment.

Decision Tree Networks (Plans): A defined sequence of testing modules and selection criteria which identifies active compounds and, through prioritization, advances the best candidates.

Drug Assessment: The quantitative evaluation of drugs and formulated products for efficacy and safety (in *in vitro* and animal models) through the application of screening protocols.

Drug Assessment Technical Evaluation Committee (DATEC): A select panel of U.S. Army Medical Research and Development Command scientists who provide to the Commander, USAMRICD technical evaluation and recommendations regarding the Medical Chemical Defense Tech Base Drug development Program.

Lewisite (L): Chlorovinyldichloroarsine, an arsenical vesicant.

Mustard Gas (HD): Sulfur mustard, β,β'-dichloroethyl sulfide, a chemical warfare vesicating agent.

Pretreatment Agent: A drug that is given before exposure to a chemical warfare agent exposure but which is intended to mitigate the effects of the chemical warfare agent.

QSAR: Quantitative structure activity relationship

Screening: The systematic testing of candidate compounds for efficacy and safety in the prevention and treatment of chemical agent exposure.

Testing Module: An experimental procedure, defined in a written protocol or Standing Operating Procedure, which is designed to evaluate specific characteristics of the compound being screened.

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