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Mechanism-Based Predictions of Dose-Response Relationships: Why Low Doses of CCl_4 are Non-ToxicAdvisory Committee

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SUMMARY

Activation of hepatocytes arrested in G_2 by low doses of CCl_4 may represent a novel cellular triage function in response to chemical insult. Activation of arrested G_2 hepatocytes by low doses of CCl_4 limits injury by stimulating tissue repair. High doses of CCl_4 ablate the G_2 activation response, thereby eliminating an adaptive response normally inducible by a low dose. Elimination of this response prevents early and immediate gene expression known to increase competency of other cells. This results in delayed and attenuated tissue repair response. Much too late and much too little response may account for the rapid rise in CCl_4 toxicity at high doses and thus its characteristic hockey-stick-like dose-response curve. Inactivation of this function is also believed to be the principal mechanism accounting for the proposed enhancement of CCl_4 - induced hepatotoxicity by chlordecone (Kepone®). Prevention of prompt tissue repair response and attenuation of that response lead to the rapid progression of tissue injury upon exposure to toxic chemicals at high doses.

INTRODUCTION

Governmental regulation of manufacturing and products consumers use is governed by the scientific input concerning the potential for adverse effects on public health, social and economic impact of such regulation as well as political considerations. Increasingly, we as members of the public demand greater objectivity in making such regulatory decisions. While the prediction of what may happen to human health after exposure to low levels of

pollutants over a lifetime is difficult to make with a respectable degree of confidence, scientists are nevertheless asked to provide exactly that kind of information so that the legislative and regulatory branches of our government may make black and white, yes and no type of decisions that enable them to carry out their societal obligations. Assessment of risk associated with the use of a product and/or exposure to chemicals, radiation and other types of pollutants over a lifetime, requires highly reliable scientific basis to enable us to make such objective decisions which have to be balanced by social, economic, political and other societal considerations.

We are exposed to combinations of chemicals. However, our regulatory decisions are based on exposure to singular chemicals

With this background, let me now turn to a problem faced by scientists and regulators worldwide. Testing of chemicals is conducted with one chemical at a time, and the risk associated with exposure to that chemical is evaluated. However, it is common knowledge that we are never exposed to one chemical at a time. Exposure to combinations or mixtures of chemicals is the rule. So, what is the risk from exposure to combinations of chemicals even though they may be present in our food, water and air at levels that are considered harmless? Unfortunately, our present knowledge and know-how does not permit us to predict the outcome of lifetime exposure to combinations of chemicals at very low or legal levels. What is frightening is that we are not able to accurately predict the outcome of exposure to combinations of chemicals, even for a short duration of time. With this question in mind, approximately two decades ago we set out to investigate this problem in a laboratory setting. We determined that a systematic approach to that problem should involve development of a laboratory animal model in which such combinations of chemicals may be tested. With this objective in mind, we set out to first demonstrate if exposure to combinations of chemicals at individually nontoxic levels, can result in highly exaggerated toxicity. For our experiments, we assumed two ground rules. Because the problem is so complex, we began testing only two chemicals in combination. If we understood the basis of interaction of two chemicals which results in highly exaggerated toxicity, then we could begin to investigate the impact of exposure to three, four, and higher numbers of chemicals and combinations. The second assumption was that both of these chemicals would be used at levels which are individually nontoxic. In other words, we would expose animals to two chemicals, each at harmless levels. After many experiments, we discovered one model where exposure to two chemicals resulted in an unprecedented level of highly exaggerated toxicity. Exposure of laboratory rats to their diet containing 10 parts per million (ppm) of chlordecone (Figure 1) for 15 days, resulted in no detectable adverse or harmful effects in the animals. Likewise, administration of a single dose of a common chlorinated solvent, carbon tetrachloride (CCl_4) at 100 µl/kg body weight of the animal results in no adverse effect of

any consequence to the animal. When this amount of carbon tetrachloride is administered to rats maintained for 15 days on a diet contaminated with chlordecone, all of the rats died from massive liver failure. Upon closer examination, we discovered that the toxicity of chlordecone was not at all expressed in these animals. Instead, it was the toxicity of carbon tetrachloride that was amplified. Furthermore, it was discovered that this interaction resulted in a 67-fold increase in the killing effect of carbon tetrachloride. The mechanism by which this kind of unprecedented level of interactive toxicity could be expressed became an intensive area of our investigations. The toxicity of chloroform and other related compounds is also amplified by chlordecone (Figure 2).

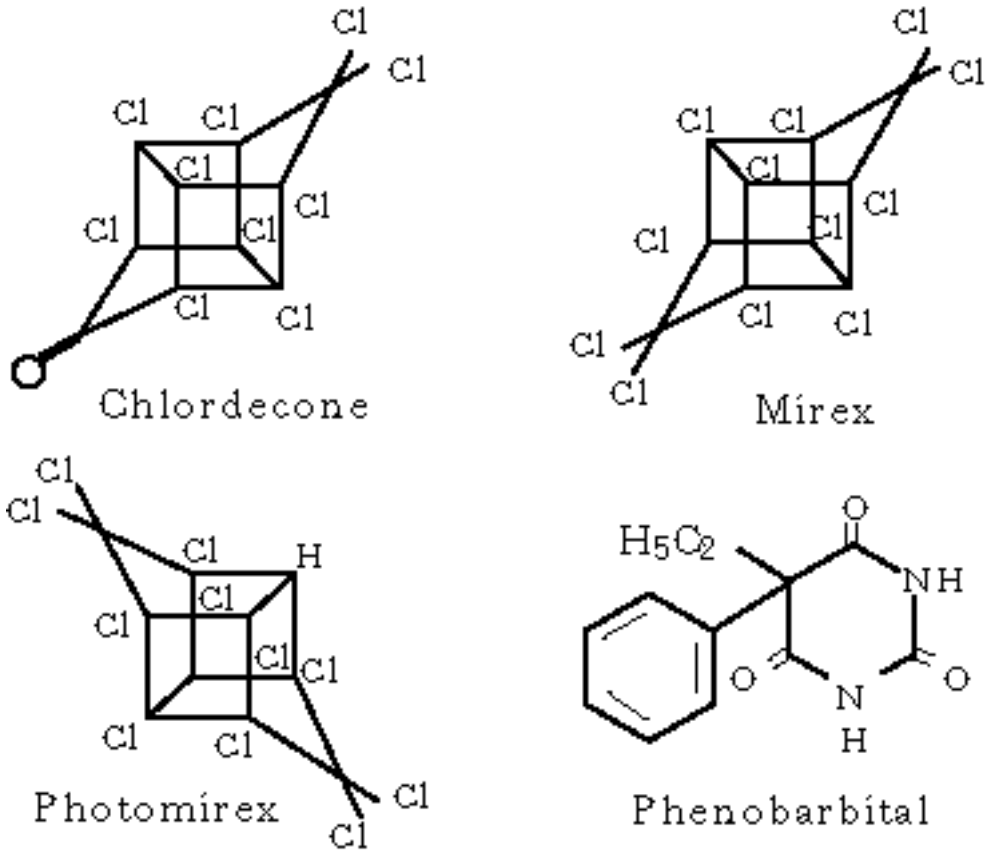
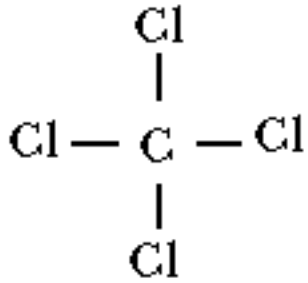
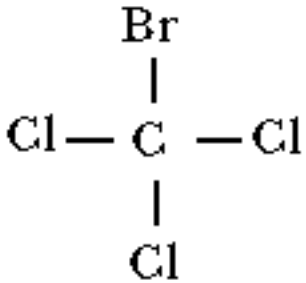


Figure 1. Structures of chlordecone, mirex, photomirex and phenobarbital. At subtoxic doses, chlordecone amplifies the hepatotoxic and lethal

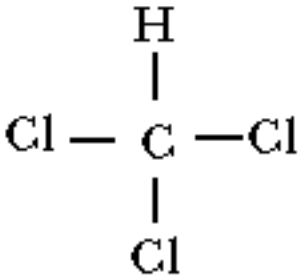
effects of halomethanes such as CCl_4 and related solvents. Neither the closely related structural analogs of chlordecone nor phenobarbital share chlordecone's propensity to amplify the toxicity of these solvents. The specificity of chlordecone in combination with CCl_4 is closely related to the depletion of liver glycogen and cellular ATP, which leads to incapacitation of the liver to repair the injured tissue. Such dramatic depletion of cellular ATP does not occur when same dose of CCl_4 is administered in combination with phenobarbital or mirex.



Carbon tetrachloride



Bromotrichloromethane



Chloroform

Figure 2. Structures of carbon tetrachloride, chloroform and bromotrichloromethane. Hepatotoxicity as well lethal effects of these solvents is amplified by prior exposure to nontoxic levels of chlordecone.

Widely accepted mechanisms did not explain why the combination of chlordecone and carbon tetrachloride was deadly

It was also discovered that exposure to Mirex, a closely related chlorinated pesticide to chlordecone, and carbon tetrachloride combination, did not result in such an explosive amplification of toxicity. In the scientific literature, models where exposure to high levels of phenobarbital, a barbiturate drug used in epilepsy, and carbon tetrachloride were described. The mechanism by which phenobarbital potentiates the toxicity of carbon tetrachloride in the liver

was widely believed to be understood. In this context the mechanism by which carbon tetrachloride causes toxicity is of interest since it is the toxicity of this compound that is increased in these models. It should be noted that carbon tetrachloride is metabolized in the liver to a trichloryl carbon (€CCl3) free radical (a harmful form of the compound) which reacts with the cellular components resulting in further damage, cell death and liver injury. If a sufficiently large dose of carbon tetrachloride is administered, then a larger number of liver cells would die causing liver failure and eventually, animal death. Exposure to phenobarbital results in increased bioactivation of CCl_4 and lipid peroxidation leading to increased liver injury. The mechanisms of bioactivation and cellular disintegration brought about by a process known as lipid peroxidation might have been increased as a result of the animal's exposure to food contaminated with chlordecone. In our experiments, we employed phenobarbital as a positive control so that we could have a reference point as to whether the chlordecone potentiation of carbon tetrachloride toxicity was mediated by mechanisms similar to those understood to be operating for phenobarbital + carbon tetrachloride model. Some very important differences began to surface between phenobarbital and chlordecone potentiation of carbon tetrachloride toxicity. One key difference was that in spite of the increased liver injury, phenobarbital treated animals survive while chlordecone treated animals die.

Neither increased bioactivation of CCl_4, nor increased lipid peroxidation would explain the enormously increased toxicity of CCl_4 by dietary exposure to chlordecone

After a series of intensive research studies, it became apparent that neither increased bioactivation nor enhanced lipid peroxidation were the mechanisms underlying chlordecone + carbon tetrachloride interactive toxicity. These and other studies lead our research group to propose that none of the mechanisms that were available in the literature were adequate to explain the enormously increased toxicity resulting from the combination of chlordecone and carbon tetrachloride. Since the existing information was not helpful in resolving this problem, we then undertook a series of investigations which resulted in the final unfolding of the mystery behind this enormously amplified carbon tetrachloride toxicity.

Why is a low dose of CCl_4 ordinarily not toxic?

What we had learned about this model allowed us to frame a different question than we were thus far asking. We asked the question, "Why is ordinarily a low dose of carbon tetrachloride not toxic?" Asking such a question causes a significant stir among the scientific community since we had published many papers indicating that the low dose of

carbon tetrachloride being employed in our study was nontoxic. Furthermore, asking as to why something is not toxic causes problems since it is difficult to look for something that is not. So the question attracted significant stir in that we were setting out to look for something that wasn't. Nonetheless, we designed an experiment by making another assumption. The assumption was that we did not know anything about carbon tetrachloride and how it causes toxicity. This assumption is difficult to make when the literature abounds reports on various aspects of carbon tetrachloride and how it causes toxicity. Nevertheless, in order that we make progress with the puzzling question that we had posed, we made this second assumption. Consequently, since we knew nothing about this compound, we could now go into a laboratory and inject a tiny amount of this compound (the same amount we have been using in our experiments) and examine the animals at various times in a variety of different ways. This approach lead us to find out some intriguing information about the biology of the liver.

Low dose of CCl_4 causes limited injury, but this is completely overcome by stimulated tissue repair

What we discovered was that the tiny dose of carbon tetrachloride administered to the laboratory rats caused no detectable injury up to 4 hours after its administration. At 6 hours detectable injury was evident. At 12 hours there was greater injury. By 24 hours, most of this injury had disappeared. The liver looked normal. By 36 hours and later, the animal completely recovered. This study indicated to us that the tiny dose of carbon tetrachloride did cause a small amount of injury, and that the reason it was not ultimately toxic to the animal was the ability of the liver to overcome that injury. We refer to this phenomenon to overcome tissue injury as tissue repair.

Mitigated tissue repair leads to permissive progression of injury

The animal which had received both the compounds (chlordecone + CCl_4), also experienced limited liver injury about 6 hours after carbon tetrachloride was administered. However, two events in this animal are different in comparison to the other animal which received only carbon tetrachloride: first, there was no recovery from this injury; second, there was an acceleration of injury. These experiments indicated to us that the animals receiving the combination of the chemicals were unable to overcome tissue injury due to their inability to respond by stimulation of tissue repair.

The basic unit of tissue repair is cell division, meaning when cells die either due to natural causes or due to injury, other surrounding cells must divide and replace the dead or dying cells. This process results in restoration of tissue architecture and thereby tissue function.

It is instructive to understand that cells divide through a process known as cell cycling. (Figure 3) When the cells progress through various phases until they divide, they use energy for a very complex series of biochemical changes in preparation to remodel the entire cellular architecture such that one cell now may divide into two new daughter cells. Normally, cells exist in the tissue at various phases of the cell cycle. Most of the cells exist in resting phase (G0). A very small number of cells exist in G1, S, and G_2 phase. Normally, very few cells need to divide in a resting liver. It should be noted that normally 3 to 6% of the cells in the entire liver exist in G_2 phase. Upon administration of a low dose of carbon tetrachloride, we discovered that within 6 hours the small population of cells arrested at the G_2 phase are immediately mobilized to proceed forward so that they may divide into two new daughter cells in order that the injured tissue may be repaired with the new stronger and better cells. Furthermore, additional cells in G0 phase are stimulated to undergo cell cycle progression so that these cells may now divide and provide additional new healthy cells to replace the dead or dying cells. What we observed was that these two events, one occurring at 6 hours and the other occurring approximately 36 to 48 hours, provide sufficient number of cells to replace the dead or dying cells resulting in complete restoration of tissue architecture and function. Upon administration of the same dose of carbon tetrachloride to rats which had been eating food contaminated with chlordecone, we found that the first process whereby the small population of G_2 cells divide was completely ablated. Furthermore, the additional stimulation of the resting G0 cells to go through the cell cycle progression was significantly attenuated. This impairment of stimulation of tissue repair that normally occurs upon exposure to a toxic chemical facilitates the permissive progression of injury without any mechanism to arrest such acceleration of tissue injury. This mechanism whereby impairment of tissue repair results in acceleration of progressive injury can be likened to the mechanism by which forest fires occur. Note that a small brush fire can be started with simply one lighted match stick. Our mechanism to prevent a forest fire from occurring is to send a fire fighter who would promptly pour water and prevent a forest fire. If the fire fighter is prevented from arriving at the scene promptly, the same brush fire progresses to a forest fire. In this analogy, we do not need any additional lighted match sticks to get from a brush fire to a forest fire. Likewise we do not need any additional reactive metabolites, or any additional dangerous forms of chemicals to cause much greater and accelerated liver injury. This concept has been tested in a variety of experiments, and has been confirmed and experimentally verified.

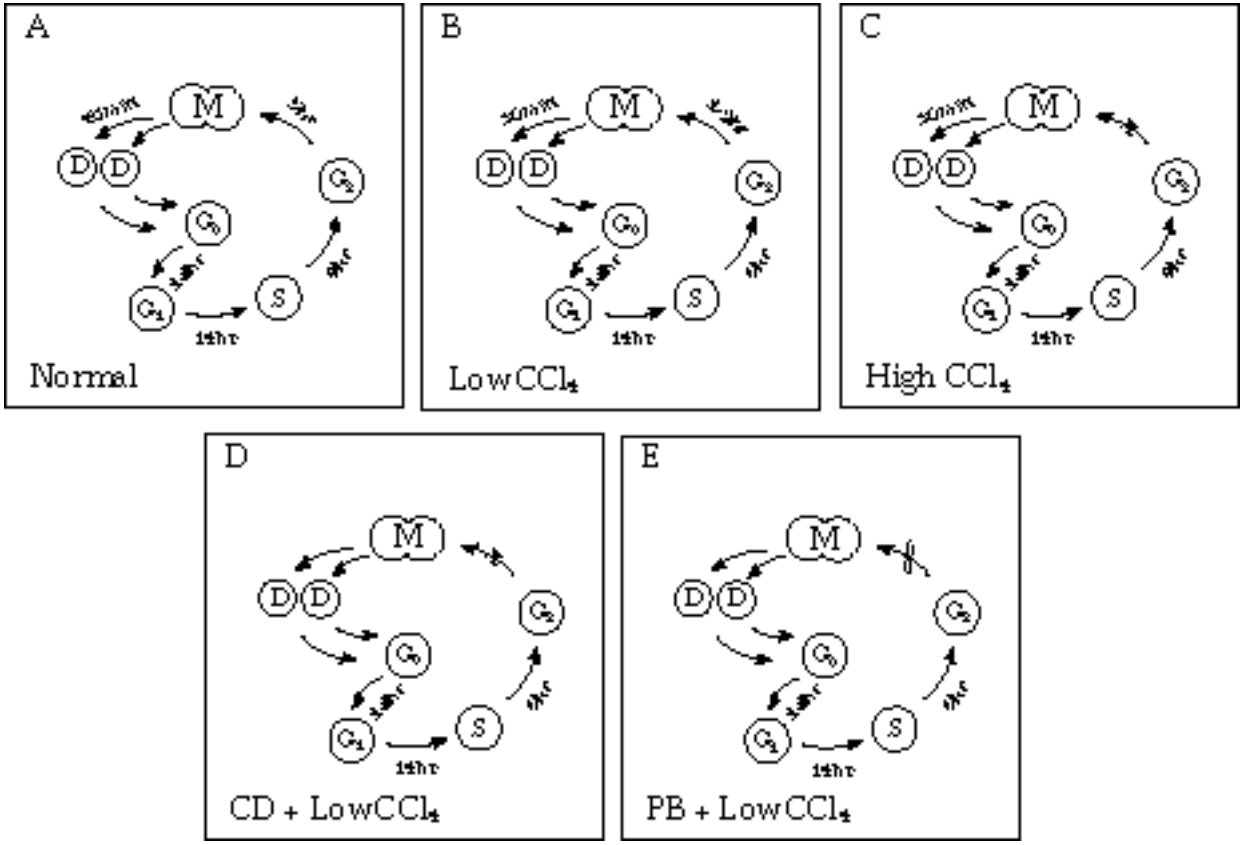


Figure 3. Illustration of hepatic cell cycle and the involvement of G_2 cells in the toxicology of low dose of carbon tetrachloride, chlordecone + carbon tetrachloride, phenobarbital + low dose of carbon tetrachloride, and high dose of carbon tetrachloride.

A. Normal cell cycle is illustrated. Normally cell division is very quiescent in adult livers, is sufficient to replace apoptotic loss of cells and restoration of lobular structure and function. At any given time, a normal liver

cell population might be ranked approximately as follows, Go ~ > > G1 > > S > G_2 > M. Prereplication changes may take approximately 14 hours and then another 14 hours for S phase DNA synthesis, followed by approximately 8 hours for G_2 phase. Cells in G_2 phase will reach M phase relatively quickly.

B. After the administration of a low dose of carbon tetrachloride, cells (thick arrow) in G_2 phase are stimulated to divide giving rise to the early-phase response (6 hours). S Phase is also known to be stimulated by a low dose of carbon tetrachloride. Furthermore, the entire cell cycle is enhanced resulting in liver cell division peaking between 36 - 48 hours.

C. After high dose of carbon tetrachloride, the early-phase forward progression of G_2 to M phase is inhibited. The second wave of cell division, resulting from forward progression G1 cells is stimulated, but this response is delayed because of the lack of early gene expression, and progressive necrotic and degenerative events consequent to the inhibited release of G_2 cells. Hepatic injury becomes progressive and a dose-dependent expression of

ultimate toxicity is observed.

D. Upon exposure to chlordecone the early-phase cell division normally seen after administration of a low dose of carbon tetrachloride does not occur due to insufficient energy to drive the G_2 cells into mitosis. Forward progression of G1 ~ S G_2 ~ M is stimulated, but it is delayed and attenuated.

E. Upon exposure to phenobarbital the early-phase stimulation of liver cell division by a low dose of carbon tetrachloride is decreased. This decrease is related to a transient and marginal decrease in cellular ATP. However, the overall cell cycle time is significantly decreased as evidenced by a very substantial increase in cell division starting at 24 hours and peaking at 48 hours. These events enable the rats to overcome liver injury and survive the combination treatment. This mechanism explains why the rats survive even though liver injury in these rats is approximately twice as severe as seen in rats treated with chlordecone + carbon tetrachloride combination.

The following mechanism of this highly amplified toxicity exemplified in chlordecone + carbon tetrachloride

interaction is illustrated below in (Figure 4). Ordinarily, limited injury caused by a low dose of carbon tetrachloride results in recruitment of a biological response that starts even before the injury becomes evident and results in stimulated tissue repair and complete recovery. In contrast to this, the prior exposure to chlordecone results in ablation of the early tissue repair response and considerable attenuation of the second phase of tissue repair response such that the tissue injury permissively progresses until the liver fails leading to animal death.

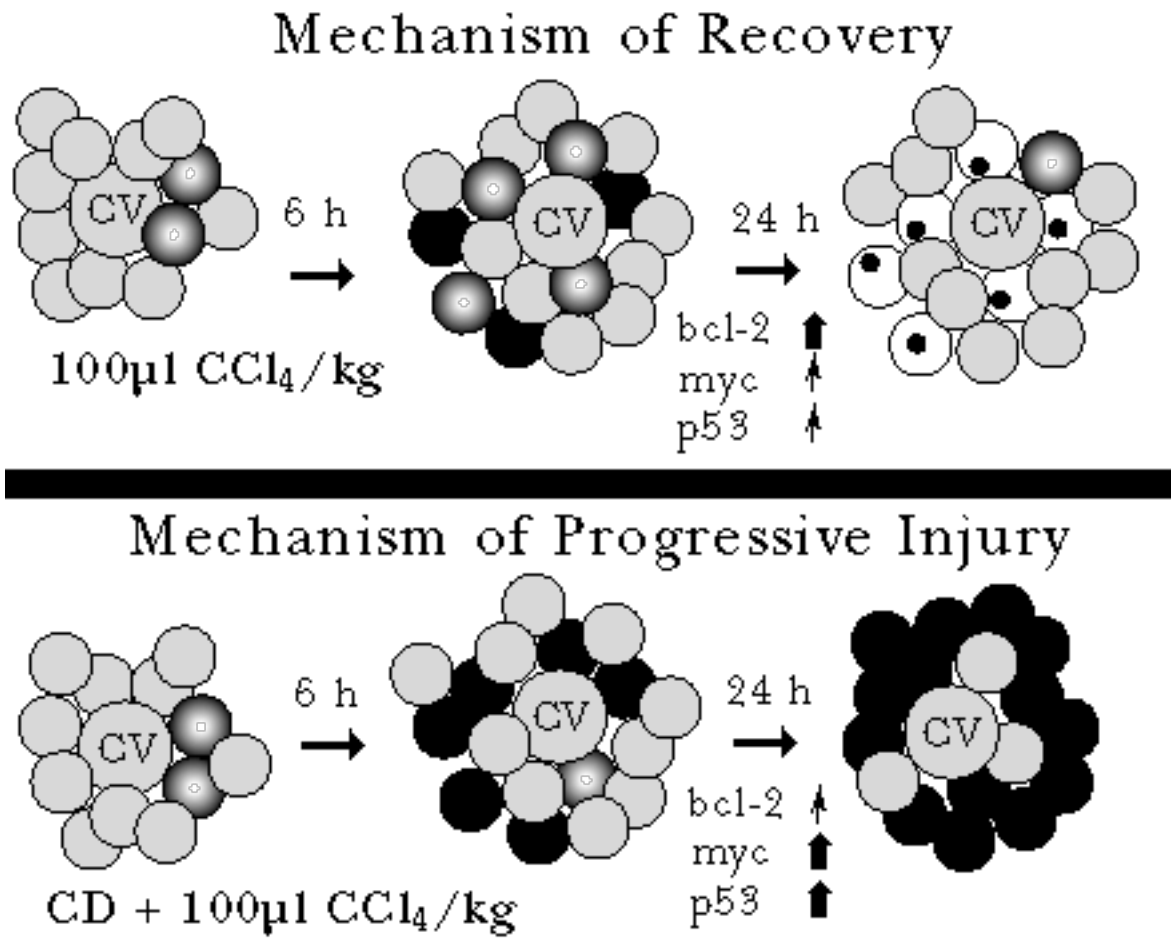


Figure 4. Mechanisms of rapid recovery from limited injury from CCl_4 or accelerated progression of injury in rats treated with a combination of

chlordecone and CCl_4. Proposed recovery mechanisms involve expression of protooncogene bcl-2 to inhibit injury-stimulated apoptosis. Inhibition of apoptosis and availability of new daughter cells resulting from released G_2 cells to divide leads to quick recovery from limited injury. Conversely, inadequate expression of bcl-2 may lead to continued apoptosis and further deterioration of liver tissue.

Lack of cellular energy leads to incapacitation of cells to divide

These studies indicate to us that exposure to combinations of chemicals even at very low doses, can result in highly exaggerated toxicity and in this particular instance, the mechanism seems to interfere with the endogenous tissue repair response. We have investigated the mechanism resulting in failure of cells to divide. Interestingly, the reason for failure of tissue repair stimulation is the lack of adequate cellular energy. Cellular energy in the form of adenosine triphosphate (ATP) is depleted as a result of a number of cellular events that result in rapid depletion of glycogen (the liver's principal form of stored energy), and this coupled with a large demand on cellular energy under toxic stress results in 60 to 80% depletion of cellular energy. Therefore, at 6 hours when the small population of G_2 cells should have divided, because cellular energy is not available, these cells fail to divide. This results in the unavailability of a small population of cells which would have formed the seed for additional cell multiplication, and therefore tissue is deprived of repair.

High doses are lethal because tissue repair is inhibited

In addition to learning that exposure to combination of chemicals can result in highly exaggerated toxic injury, we have benefitted a great deal from this scientific inquiry. For example, we have now begun to appreciate the importance of this biological response that accompanies toxic injury. A number of chemicals are capable of stimulating tissue repair. However, at high doses, the tissue repair is inhibited. A fundamental principle of toxicology is "dose-response" relationship. When the dose administered is increased, increased injury results. This

relationship is referred to as dose-response. Our new findings suggest that because the biological response includes stimulation of tissue repair, measuring tissue repair in addition to injury, should be more useful in predicting toxic effects upon exposure to chemicals, with greater precision.

Infliction of tissue injury and stimulation of tissue repair are opposing and dynamic biological responses

Let me cite one example of work we have adopted recently which indicates that such an approach might be extremely beneficial. We took a model compound known as thioacetamide which causes liver injury and if a larger dose is administered, the liver fails and the animal dies. We tested a 12 fold range of doses of thioacetamide in animals. We measured injury in the liver simultaneously by measuring the rate at which tissue repair was stimulated. There was no dose-related increase in thioacetamide injury up to a 6-fold range of thioacetamide doses. The next higher dose caused a significantly greater injury followed by animal death. When the rate at which stimulated tissue repair was measured, we found an excellent dose-related increase in stimulation of tissue repair for the first three doses. Note that even though as we increased the dose, there was greater stimulation of tissue repair, with each incremental dose, the stimulation of tissue repair was delayed by an increment of time. Note that the highest dose resulted in significantly decreased stimulation of tissue repair on the one hand, and considerable delay in the onset of tissue repair on the other. Therefore, too little and too late tissue repair was seen after the administration of a dose above the threshold for stimulation of tissue repair. These findings suggest that the rate at which tissue repair is stimulated is an important determinant of the ultimate outcome of toxic injury. In this case, we could predict animal death by the imprompt and insufficient tissue repair stimulated by the highest dose of thioacetamide.

The so-called 'mechanisms of toxicity' offer adequate explanation of how toxicity begins, but do not help in predictive toxicology

There are additional biochemical and public health implications of the foregoing discoveries and resulting concepts. For instance, studies have enabled us to propose that the toxicology of chemicals may be viewed as an event consisting of two stages. This "two-stage model" of toxicity incorporates a number of the foregoing novel findings into this concept. The so-called "mechanisms of toxicity" enable us to adequately explain how toxicity begins upon exposure to a given chemical. In other words, mechanisms involving bioactivation of chemicals, formation of free radicals or other forms of harmful moieties, generation of harmful forms of oxygen, etc., are able to inflict injury in a given tissue. These mechanisms help us to understand whether and how much injury may be initiated. However, in

contrast to the widely held expectation, these mechanisms cannot help us in predicting the ultimate outcome of that toxic injury. Prediction of the ultimate outcome of that injury with any reliable accuracy requires an understanding of the endogenous mechanisms of tissue repair or other similar compensatory mechanisms that may or may not be sustained in a given circumstance. In other words, if the tissue repair and similar compensatory mechanisms are not permitted to occur, regardless of how it is initiated, injury progresses causing organ failure and leading to more severe toxicities culminating in mortality. On the other hand, if tissue repair is permitted to occur, regardless of how injury is initiated, and even regardless of the extent of injury initiated, that injury might be inconsequential to the ultimate outcome of that toxicity. Since from the public health perspective, we are interested in the ultimate outcome of toxic injuries (particularly viewed in terms of long term effects on public health measured as mortality and morbidity), our ability to accurately predict the ultimate outcome is really the critical issue in protecting public health. The direct biomedical application of this concept becomes evident if one considers that we might be able to therapeutically manipulate and enhance the endogenous compensatory mechanisms thereby enabling us to overcome even the most severe instances of toxic injury and avert the ultimate disastrous outcome of that injury.

Lethal outcome of a toxic dose of acetaminophen can be averted by preplacing stimulated tissue repair

Our recent experiments have included a test of this possibility. If the extent and the promptness of the stimulation of tissue repair is really the critical determinant of the ultimate outcome of toxic injury, experimentally one should be able to preplace and enhance tissue repair and thereby deny the ominous outcome of lethal injury from a lethal dose of a toxic chemical. We employed a lethal dose of acetaminophen (Tylenol) which at high doses causes massive liver injury leading to liver failure and death. By preplacing stimulation of tissue repair, one should be able to deny lethal action of a lethal dose of acetaminophen. At very low doses thioacetamide (just as many other chemicals) is known to stimulate tissue repair. We employed a low dose of thioacetamide to stimulate tissue repair, and 36 hours after its administration (when maximal stimulation of cell division has taken place), a lethal dose of acetaminophen was administered. The lethal action of a massive dose of acetaminophen is completely mitigated by the preplacement of tissue repair by thioacetamide administration. Such experiments conducted with other toxic models give us additional confidence that the concept being developed is very sound and valid. Biomedical applications of this concept must await the unfolding of the understanding of molecular events that lead to stimulated tissue repair. For instance, even though in an experimental setting we employ thioacetamide to initiate a series of biochemical events in the cells and ultimately in the tissue, an understanding of what biochemical molecular events mediate this

response would ultimately lead us to use that information in medical treatment of massive poisoning. Ultimately, an understanding of the role of growth factors, cytokines and other biologically active cellular molecules will enable us to develop appropriate therapeutic agents which could be administered even after exposure to toxic chemicals to bring about greater stimulation of tissue repair and thereby overcoming toxic injury.

Implications to public health

There are additional implications to public health. Clearly, this example of interactive toxicity employing only two chemicals amply illustrates the potential for adverse health effects from combinations of chemicals even when they are present at individually nontoxic levels. This serves as an example of the potential threat from combinations of chemicals (which is a rule for human exposure rather than an exception), thereby alerting us to modify chemical regulation as presently conducted in order to protect public health. New ways of estimating risk from exposure to combinations of chemicals must be developed. In doing so, the scientists as well as the regulators will be challenged by public demands for greater precision in the assessment of risk. The science of risk assessment must reach a higher level of sophistication and fine-tuning.

Animals to man data extrapolation

In risk assessment, we rely on the information obtained from laboratory experimental animals challenged with various toxic chemicals. Humane ethics do not permit us to experiment in humans. Therefore, we must rely on the experimental animal data to extrapolate the humans. This leads to another problem. Animals vary in their sensitivity to toxic chemicals. Species and strain variations are well known. An experimenter may use one or more species or strains of laboratory animals to conduct tests. Often, it is customary to choose the most sensitive animals so that any assessment of risk through extrapolation for humans may be made on a conservative basis. However, because of the demand for greater accuracy in risk assessment, our ability to more accurately extrapolate information from animals to man, is hampered by a lack of understanding of the mechanisms by which the animal species and strains differ in their sensitivity to toxic chemicals. Because of uncertainties involved in this area, currently we assume that humans are 10-fold more sensitive than the most sensitive animal being used for these tests. The concept that the rate of tissue repair determines the ultimate outcome of toxicity, has opened up the possibility of a greater understanding of the mechanisms underlying species and strain differences. For instance, we found that gerbils are 35-fold more sensitive to a very low dose to carbon tetrachloride in comparison to rats. We hypothesized that extremely high sensitivity of gerbils might be explained on the basis of a very sluggish rate of tissue repair in this

species of animals. When we investigated this possibility, we found that the gerbil is indeed a species in which carbon tetrachloride results in only a very small increase in tissue repair and this tissue repair occurs quite late (42 hours after exposure to a tiny dose of carbon tetrachloride). Recall that in the rat stimulation of tissue repair occurs as early as 6 hours after exposure to carbon tetrachloride. These experiments have indicated that the species differences and sensitivity might be accounted for by the differences in rates of tissue repair stimulated upon exposure to toxic chemicals. These findings have provided additional new possibilities for us to fine-tune the prediction of species differences, in determining risk assessment while we extrapolate animal data to humans. By understanding the precise mechanism behind species and strain differences, the uncertainty inherent in routine risk assessment procedures especially those concerned with interspecies extrapolations may be markedly reduced.

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