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PUTATIVE MECHANISMS OF TOXICITY OF 3-METHYLINDOLE: From Free Radical to Pneumotoxicosis

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INTRODUCTION

Damage to lung tissue can result not only from exposure to chemicals by inhalation, but also from internal exposure to a variety of drugs or chemicals by ingestion. For example, the use of antineoplastic agents in the treatment of cancer (i.e. bleomycin, cyclophosphamide, carmustine, methotrexate), or the inadvertent ingestion of pesticide residues (i.e. paraquat, trialkylphosphorothioates), or natural plant compounds (4-ipomeanol, plant pyrrolizidine alkaloids) are known to cause lung injury (1). In contrast to these ingested drugs or xenobiotics that produce critical lung disease, 3-methylindole (3MI) is produced by resident microorganisms in the digestive system from tryptophan (TRP), an essential amino acid in the diet of humans and animals (2–4). Production of 3MI in the rumen in response to sudden changes in the feeding regimen can become detrimental to cattle by causing fatal pneumotoxicosis (5). 3MI is also produced in the colon of humans and animals, and the quantity varies with dietary composition, nutritional status, or disease (6). In addition to internal exposure to 3MI, humans can inhale 3MI from cigarette smoke because it is a pyrolysis product of TRP in tobacco leaves (7).

Investigation of the etiology of a naturally occurring lung disease in cattle,

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acute bovine pulmonary edema and emphysema (ABPE), led to the discovery of 3MI as the causative agent (8). As a consequence, prevention of ABPE can be achieved by practical means of livestock management to reduce the production of 3MI (9, 10). Studies of 3MI toxicity, however, continue to provide a fascinating research area for many scientists. 3MI has several unique characteristics as a pneumotoxin. 3MI toxicity is specific or selective for particular species, organ, and cell types. The specific lung lesions resulting from 3MI toxicity are similar to many other chemically induced lung diseases (1, 11). Thus 3MI can serve as a prototype pneumotoxin to examine the pathogenesis of lung damage induced by toxins and as an excellent model to study the effect of diet on disease. This review focuses on the mechanisms of 3MI toxicity relative to its species and organ specificity with emphasis on the interaction of the reactive intermediate of 3MI with cellular macromolecules and its biological consequences. Other aspects of 3MI toxicity have been reviewed by researchers with different perspectives and areas of expertise (11–16).

METAMORPHOSIS OF A NUTRIENT TO TOXIN: Conversion of TRP to 3MI

TRP is the indole derivative most widely distributed in nature. In addition to being an essential building block in proteins, TRP serves as a precursor for the synthesis of biologically important small molecules that include serotonin, a neural transmitter involved in the control mechanisms of the central nervous system, and niacin, a vitamin that influences vital metabolic pathways. However, a small percentage of dietary TRP that escapes absorption is metabolized via a deamination and decarboxylation pathway to indole-3-acetic acid (IAA), 3MI, and other indolic compounds by microorganisms in the colon or in the rumen (17). Appreciable levels of 3MI can be produced from this pathway. Concentrations of approximately 5 μg 3MI/g feces in healthy individuals ranging to 80–100 μg/g feces in individuals with digestive disturbances have been reported (18), and concentrations of up to 9.0 μg 3MI/ml in the rumen have been measured within 24 hr of intraruminal administration of TRP (4).

The conversion of TRP to 3MI is affected by diet, e.g. there is a 100-fold increase in rats fed a meat diet compared to a chow diet (19). Incubation of ^{14}C-TRP with ruminal fluid confirms that the formation of 3MI is the major fermentation product, with smaller amounts of IAA and indole also being produced (2, 20). The microorganisms involved in the conversion of TRP to 3MI seem to be resilient to the deprivation of energy in the nutritive medium. It has been shown that decreasing concentrations of glucose (110–0 mM) in the incubations results in a progressive increase in the conversion

of ^{14}C-TRP to ^{14}C-3MI (20). Short term starvation may alter the microbial population in the rumen by inhibiting the growth of one type of microorganism, but not of the others. Refeeding of TRP-rich feeds after starvation may enhance or amplify the production of 3MI from the resilient microorganisms that are provided with increased concentrations of substrate (TRP) and decreased populations of competing microorganisms. This is demonstrated by the onset of ABPE when cattle are moved from poor quality forage to lush green pastures in late summer (21), or possibly even when refeeding a high energy diet after shipping great distances to feed lots. The quantity and quality of dietary protein consumed is often altered in conjunction with elevation of the availability of TRP.

The role of the conversion of TRP to 3MI by the ruminal microflora in the development of ABPE has been thoroughly examined. Only oral administration of TRP to cattle can mimic the naturally occurring disease, while intravenous or intraperitoneal infusion is ineffective, which suggests that TRP itself is not the causative agent (22). Although indole and 3MI are prevalent end products of TRP metabolism in the rumen (4, 5, 20), pulmonary lesions developed only upon infusion of 3MI, but not indole (23). Both intraruminal and intravenous administration of 3MI experimentally induce ABPE in cattle, sheep, and goats (2, 3, 24, 25). The clinical signs and pathological lesions are identical to those of naturally occurring ABPE. The severity of pulmonary lesions is proportional to the dose given and turnover of 3MI in plasma (4). Non-ruminants have different susceptibility and distinct pathologies to 3MI toxicity than ruminants, and the cause of death is unknown, but is not necessarily attributed only to lung damage. For example, the LD_{50} of 3MI in goats, mice, and rabbits is 30–40, 57 and in excess of 900 mg/kg, respectively (12). The risk of exposure to 3MI in humans is still unknown. In attempting to elucidate the mechanism of 3MI pneumotoxicity, ruminants appear to be the best animal model because severe 3MI toxicity is targeted to the lung exclusively and occurs rapidly. Non-ruminant species with inherent metabolic differences, however, can also be used as models, especially for comparison in sorting out the species specificity for toxicity.

CHEMICAL AND METABOLIC PROPERTIES OF 3MI RELATED TO TOXICITY

The changes of biochemical parameters, the manifestation of clinical signs, and the presence of pathological lesions induced by a toxin only provide clues to the pathogenesis of diseases. It is important to differentiate between the initial toxic event and its subsequent consequence and to recognize the differences between the causative and the contributing factors. This distinc-

tion is important with 3MI because its chemical and metabolic properties contribute to the clinical pathology of 3MI-induced pneumotoxicity; however, it is the metabolic activation of 3MI that initiates the specific toxic event and may be responsible for the organ-specific toxicity.

Indolic compounds, including 3MI, are disruptive to biological membrane structure and function. These compounds are a class of nonpolar, lipophilic compounds capable of intercalating between fatty acid chains and artificial lecithin micelles (26). The lysis of mammalian erythrocytes (27), the release of intracellular markers in human lung fibroblasts in culture (28), and the bacteriostatic action seen in many enteric bacilli (29) in response to treatment with indolic compounds provide evidence for the direct effect of 3MI due to its chemical properties. 3MI-induced pneumotoxicity, however, cannot be attributed to its direct effects on membranes since other indoles behave similarly, but are unable to induce lung damage (Table 1) (14, 23). Disruption of membranes by 3MI does not account for its organ and cell type specificity either. However, membrane disruption does appear to contribute to early clinical signs and reversible ultrastructural damage in capillary endothelial cells during 3MI circulation or infusion (25, 30).

The characteristic of lipid solubility is common in xenobiotics. Metabolism of such nonpolar xenobiotics to more polar forms is essential for their excretion and ultimate detoxification. Ironically, the common means of disposing of toxic compounds through detoxification can often inadvertently involve metabolic activation of the compound to detrimental reactive intermediates. The extensive study of metabolism of 3MI is essential in uncovering the mechanism of toxicity. 3MI can be metabolized to inert compounds

Table 1 Qualitative comparison of some effects of indole and 3MI

	3MI	Indole
Lung lesions		
Membrane effects	+	—
Hemolysis	+	+
Protozoa lysis	+	+
Lysosome lysis	+	+
Arthritic damage	+	+
Lecithin micelles	+	+
Metabolism		
Oxindole derivatives	+	—
Indoxyl derivatives	—	+

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that are excreted as detoxification products, but 3MI can also become a reactive intermediate producing pneumotoxicity (31). Jugular infusion of [methyl-^{14}C]3MI in goats resulted in a minute accumulation of ^{14}C-3MI in plasma with a half-life of 20–25 min (32, 33). The pulmonary concentration of the parent compound of 3MI was low, yet the presence of 3MI metabolites was high. Most of the infused 3MI was metabolized, then excreted in the urine within 72 hr (8). Ten distinct urinary metabolites were identified by ion exchange chromatography, but no unmetabolized 3MI was detected (34). A majority of the urinary metabolites from goat were identified as 3-methyloxindole (3MOI) or related derivatives, and indole-3-carboxylic acid and its conjugates. Administration of 3MOI or indole-3-carbinol (precursor of indole-3-carboxylic acid) did not result in pneumotoxicosis (31). Even though none of the urinary metabolites of 3MI were toxic, it was found that species with susceptibility to 3MI have distinct urinary metabolites from those species that are resistant. The major urinary metabolite of 3MI in goats, 3MOI, is not even detected in mice (35). The major murine urinary metabolite of 3MI is 3-hydroxy-3-methylindole and is also present in human urine (35, 36). In vivo experimental evidence precludes the involvement of these end-products as the toxic agents in 3MI-induced pulmonary toxicity. Furthermore, it implicates the metabolism of 3MI to a reactive intermediate, which may vary with susceptibility of species, as the main event responsible for the induction of lung toxicity.

MECHANISM OF 3MI PNEUMOTOXICITY

The mechanism of toxicity for most toxins usually involves the interaction of many factors and, therefore, an all encompassing mechanism of toxicity is usually difficult to discern. Determination of the various parameters contributing to overall toxicity is further complicated by the cell's ability to metabolically compensate for damage to optimize its survival. However, some initial toxicological event is usually responsible for launching the sequence of cascading events that follows. The alteration of biochemical and histological parameters often provide indicators of the potential mechanism of toxicity. The interpretation of these indicators must distinguish between the actual cause and consequence of the initial toxicological event. A broad perspective of the potential factors contributing to the species, organ, and cell specificity of 3MI toxicity is presented here.

Enzymatic Requirement for Metabolic Activation and Toxicity of 3MI

Lipophilic xenobiotics undergoing metabolism often induce proliferation of the smooth endoplasmic reticulum (SER), which has been associated with

induction of activity of cytochrome P450 (37), a family of mixed function oxidases (M
sionally activates toxins as well. Electron microscopic studies of 3MI-induced pneumotoxicity reveal an unusual proliferation of SER in alveolar type I cells and Clara cells, the type of lung cells most susceptible to damage by 3MI (38, 39). In addition, ^{14}C-3MI metabolites selectively accumulate in the subcellular microsomal fraction of these cells where MFO is known to be located (40, 41). Thus this family of enzymes is primarily considered to be involved in 3MI toxicity. The involvement of MFO in 3MI-induced pneumotoxicity was first demonstrated in vivo by use of compounds known to alter MFO activity (3
MFO inhibitor, piperonyl butoxide, had a prolonged plasma half-life of 3MI and a reduced pneumotoxicity (30, 32). This indicated that the 3MI parent compound was not the cause of pneumotoxicity itself since its plasma half-life was prolonged and 3M
versely, phenobarbital, a MFO inducer, shortened the plasma half-life of 3MI and enhanced the pneumotoxicity (3
concerning the involvement of MFO in 3MI-induced pneumotoxicity contributed significantly to the generation of extensive in vitro studies including examination of the formation of the activated 3MI, which initiates toxicity, the form(s) of isoenzymes that are responsible for the activation of 3MI, the interaction of activated 3MI with cellular macromolecules and subsequent disruption of cellular functions. These studies provide valuable insight into the understanding of the mechanism of 3MI toxicity.

In vitro studies using the incubation of 3MI with isolated microsomes confirmed the in vivo evidence that the metabolic activation of 3MI was a NADPH-, cytochrome P45
reactive intermediates was first indicated by the covalent binding of radioactive 3MI to microsomal proteins (41). No binding was observed when 3MOI was used as the substrate (42). Microsomes isolated from goat lung had a greater capacity to metabolically activate 3MI than liver or kidney (42). Incubation with MFO inhibitors significantly decreased covalent binding of [^{14}C]-3
was later demonstrated when 3MI was incubated in the microsomal system (43). The in vitro evidence also supports the hypothesis that MFO is required for metabolic activation of 3MI.

The selectivity of 3MI for tissue damage cannot be explained entirely by the action of the MFO system. In addition to MFO, prostaglandin H synthetase (P
MFO in the development of cytotoxicity by toxins. In fact, the activity of PHS is considerably higher in lung than liver, while activity of MFO is relatively low in lung and high in liver. Type II and Clara cells in the lung

contain particularly high concentrations of PHS (44). In addition to its ability to activate toxins, PHS can produce prostanoids, potent substances that exert their physiological effect locally and therefore may potentiate organ-specific toxicity. Thus the possible involvement of PHS in the mechanism of 3MI toxicity must also be considered.

Ram seminal vesicles (RSV) are enriched in PHS while deficient in MFO, thus they are often used to study the effect of PHS on metabolic activation (45). Treatment of RSV microsomes with 3MI enhanced PHS activity, which resulted in an increase in PHS-catalyzed prostaglandin synthesis. These effects could be inhibited by indomethacin, an inhibitor of PHS (46). Similar results were obtained with goat lung microsomes where PHS activated 3MI to an electrophilic species that could bind microsomal protein (47). These responses were dependent on arachidonic acid and were inhibited by indomethacin. The existence of both MFO and PHS systems in the activation of 3MI was demonstrated by the absence of cross inhibitions by their specific inhibitors, piperonyl butoxide and indomethacin.

Inhibitors of PHS suppress activation of 3MI in vitro and in vivo. Pretreatment with aspirin and indomethacin prior to administration of 3MI to goats attenuated the pneumotoxicity of 3MI in a manner similar to the inhibition of MFO by piperonyl butoxide (48). PHS inhibitors are only effective when administered before 3MI dosing, which indicates that the role of PHS in the development of pneumotoxicity occurs during the metabolism of 3MI, and not afterwards (49) when the biosynthesis of the prostanoids has been modulated. This study confirms the role of the PHS system in 3MI toxicity in vivo.

The protection of goats from 3MI toxicity by MFO and PHS inhibitors is an interesting observation. The comparison of these two enzymes using in vitro systems, however, may misrepresent the relative importance of the role of PHS in 3MI toxicity since PHS is a self-destructive enzyme (50), while MFO is an NADPH-regenerating system. The relevance of MFO as well as PHS should be considered as part of the comprehensive scheme of 3MI toxicity.

3MI Reactive Intermediate

Many xenobiotics must be metabolized to an electrophilic intermediate in order to become toxic. A vast number of nucleophilic compounds are present in the cell, so the events contributing to cell and tissue damage by a xenobiotic substance may be complex and difficult to decipher. Disruption of an early event of a significant pathway, however, usually initiates toxicity. Once a xenobiotic is enzymatically activated by MFO or PHS to a reactive intermediate, it poses a potential threat to the integrity and functioning of the cell. Covalent binding of proteins by xenobiotics or toxins appears to

implicate the formation of an electrophilic intermediate that is vital for toxicity. 3MI is known to participate in covalent binding of proteins in vitro and in vivo. Thus the activation of 3MI to an electrophilic intermediate by MFO or PHS, which can interact with cellular macromolecules such as proteins, lipids, and DNA, has been proposed.

The removal of hydrogen from the 1-position of indoles produces an N-centered free radical and is easily achieved by irradiation, or treatment with KO_2 (43). Of the indoles tested, only 3MI produced an N-centered free radical from enzymatic activation in the microsomal system (43). The significance of the N-centered free radical of 3MI to its toxicity has been the focus of much research. It is possible that the N-centered free radical of 3MI is the initial reactive intermediate that binds directly to cellular macromolecules and causes cytotoxicity. Alternatively, internal molecular rearrangement of N-centered free radical of 3MI could lead to the formation of a C-centered free radical at the 3-position or a methylene imide. Either of these intermediates could bind directly to cellular macromolecules and cause cytotoxicity. Of course, metabolism of 3MI may also produce other unknown reactive intermediates that might mediate toxicity. Two different approaches for determining the structure and identity of the reactive intermediate of 3MI have been pursued. Both consider the initial production of a 3MI free radical to be crucial.

Kubow et al discovered that treatment of microsomes with 3MI led to the generation of free radicals that could be trapped by a spin trapping agent, a-phenyl-tert-butyl-nitrone (PBN) (43, 51). In the presence of NADPH and O_2, optimal for MFO activity, an N-centered free radical was generated early in the incubation, followed by development of a C-centered free radical over a 1 hr time period (51). The C-centered free radical was found to be analogous to that produced by lipid peroxidation induced by $FeSO_4$ (51). A free radical was also detected when 3MI was metabolized by horseradish peroxidase, a model system for the hydroperoxidase of the PHS (46). Since free radicals of 3MI can be generated by both metabolic-activating systems, i.e. MFO or PHS, and inhibition of these enzymatic systems can eliminate the onset of disease, the theory that the formation of the N-centered free radical of 3MI is the initial toxicological event was proposed.

A more refined detection system was recently implemented by Chen et al, which enables the free radicals generated from microsomal activation of 3MI to be trapped by PBN, isolated and purified by HPLC, and identified by EPR (52). The persistence of both N-centered and C-centered 3MI free radicals after a 3 hr incubation period was demonstrated. Furthermore, in addition to a lipid peroxidation product, a signal produced by a C-centered free radical generated from 3MI itself was detected. In this study, the N-centered 3MI free radical isolated from the HPLC eluate was found to

be long-lived, a finding that differs from the first study. This difference can be attributed to the immensity of the C-centered radical, which overwhelmed the N-centered signal in the detection system used in the first study. The role of the N-centered or C-centered free radicals in the development of toxicity was not determined in this study (52). However, it is possible that the persistence of both N-centered and C-centered free radicals could contribute to the mechanism of 3MI toxicity.

The effect of antioxidants on free radical generation from 3MI was examined to test the theory that free radicals are involved in 3MI toxicity. Levels of tissue vitamin E and glutathione (GSH), cellular free radical scavengers, were manipulated by supplementation of vitamin E, a GSH precursor (cysteine, CYS), and a GSH-depleting agent (diethyl maleate) to goats in vivo (53). Tissue levels of GSH were the major determinant of the concentration of free radicals measured by EPR spin trapping with PBN, regardless of vitamin E level. The elevation of GSH resulted in a lower incidence of free radical production in the lung and reduced symptoms of pneumotoxicity, while depletion of GSH led to pronounced generation of free radicals in the lung and more severe pneumotoxicity. The level of lung GSH was inversely related to the pneumotoxicity of 3MI. It was proposed that the primary cause of toxicity is initiated by the production of a 3MI free radical that binds to cellular macromolecules.

Another approach used to identify the 3MI reactive intermediate was to examine the bond structure of 3MI adducts in order to deduce the reactive species of 3MI. The determination that the methyl group of 3MI was conjugated to the sulfhydryl of GSH was the impetus for the second hypothesis concerning the structure of the 3MI reactive intermediate. Nocerini et al proposed that a methylene imine electrophile (3-methyleneindolenine) is the ultimate reactive intermediate that initiates the toxicity of 3MI. The structure of the 3MI-GSH adduct was shown to be 3-[(glutathion-S-yl)-methyl]indole, which was formed in a microsomal system by covalent binding of the sulfhydryl group of GSH to the exocyclic methyl group of 3MI (54). The importance of the C-H bond of the methyl group of 3MI was also examined in vivo by replacing the three hydrogens of the methyl group with three deuteriums (55). It was found that administration of 3MI containing CD_3 as the methyl group resulted in a reduced pneumotoxicity compared to the parent 3MI compound (56). Incubation of lung cell isolates, including Clara and type II cells, with CD_3-3MI in vitro also led to a reduction in cytotoxicity compared to the parent 3MI compound. These findings led to the proposal that the oxidation of a C-H bond on the methyl group of 3MI is a significant event in the mechanism of metabolic activation of 3MI to a toxic intermediate. The first oxidative step in the activation of 3MI, however, is thought to involve an electron abstraction

from the N of 3MI, which forms an N-centered radical cation, while internal molecular rearrangement of the N-centered radical cation of 3MI to form a methylene imine electrophilic species is the next step (12). The in vivo formation of 3MI-GSH adduct has also been demonstrated. A urinary metabolite of 3MI-GSH, 3-[(N-acetylcysteine-S-yl)-methyl]indole (3MI-NAC), was recently isolated from goats, mice, and rats, which also implicates methylene imine as the reactive intermediate involved in its formation (57). The collective experimental evidence strongly supports the theory that methylene imine is the ultimate reactive intermediate of 3MI, which binds the sulfhydryl group of GSH and provides protection against cytotoxicity.

Both approaches used to identify the structure of the ultimate reactive intermediate of 3MI involve the production of free radicals of 3MI. Generation of N-centered free radical of 3MI by the MFO or PHS activating systems is thought to be the precursor for the formation of the methylene imine. Whether the molecular rearrangement of the N-centered free radical of 3MI to a C-centered free radical is the only pathway required to initiate toxicity, or whether the N-centered free radical can act as reactive intermediate itself has yet to be revealed, but its identification will be essential for discovering the mechanism of 3MI toxicity.

Macromolecular Targets that Cause Tissue Damage

Regardless of the ultimate structure of the reactive 3MI intermediate, the next important question to be addressed is how does the reactive intermediate mediate toxicity? Many toxins appear to be enzymatically activated to reactive species that can chemically interact with cellular macromolecules. Several hypotheses to describe a common mechanism for toxicity have been proposed. At the time when disruption of cell membranes was thought to be the primary cause of cell death, lipid peroxidation of cell membranes by reactive intermediate of toxins was regarded as the initial toxic event. Covalent binding of activated toxins to protein was also found to be a measurable parameter and considered a prerequisite for toxicity. Of course, binding of toxins to DNA often results in mutagenicity, so DNA could also be a target for covalent binding of activated 3MI. Interaction of 3MI with any or all of these macromolecules could be considered as a possible mechanism for toxicity.

INVOLVEMENT OF LIPID PEROXIDATION IN 3MI-INDUCED TISSUE DAMAGE

Lipid peroxidation is of basic importance in damage to cells by metabolically derived electrophilic species. Lung tissue contains an abundance of polyunsaturated fatty acids, a critical component of surfactant, which are susceptible to peroxidative damage. It seemed instinctive to examine the role

of lipid peroxidation in 3MI toxicity. This concept was supported by the experimental generation of a C-centered free radical species upon treatment of lung microsomes with 3MI, which was analogous to that produced during lipid peroxidation induced by $FeSO_4$ (51). However attractive this hypothesis may have been, it was disproved by subsequent experiments that directly measured lipid peroxidation. For example, treatment of goat lung microsomes with 3MI led to a dose-dependent inhibition instead of propagation of NADPH-supported lipid peroxidation, as indicated by the cessation of malondialdehyde (MDA) production (58). Furthermore, respiratory ethane production, an index for lipid peroxidation in vivo, was not affected in 3MI-treated rats; in fact, 3MI was found to act as an antioxidant in rats fed a diet deficient in vitamin E and selenium (59). In addition, in vivo pretreatment with vitamin E was not sufficient protection against 3MI toxicity (60). These experiments strongly argue against the involvement of lipid peroxidation as the mechanism of 3MI toxicity. If lipid peroxidation plays any role in 3MI-induced toxicity, it may be a consequence, rather than the cause of 3MI-induced tissue damage.

COVALENT BINDING OF 3MI REACTIVE INTERMEDIATES TO PROTEINS The covalent binding of xenobiotics to cellular proteins has been used as an index of toxicity for many drugs and toxins since Brodie et al proposed such a mechanism for bromobenzene-induced toxicity more than 30 years ago (61). Similarly, covalent binding of 3MI to proteins has been proposed to be the mechanism of 3MI pneumotoxicity. Covalent binding of 3MI to protein is dependent on the metabolic activation of 3MI. The reactive intermediate of 3MI produced by activation with microsomal MFO or PHS systems can covalently bind microsomal proteins (41, 42, 47). Inhibitors of MFO and PHS circumvent covalent binding of 3MI in lung and alter the severity of 3MI toxicity in vivo (30, 32, 56). The extent and distribution of protein binding by 3MI varies between organs and species. The species that are most susceptible to 3MI toxicity preferentially bind 3MI in lung. Infusion of radioactive 3MI in goats and cattle results in accumulation of greater amounts of radioactive metabolites, but not 3MI itself, in the lung than in the liver (33, 41). Those species that are less prone to 3MI toxicity, such as rabbit, may have comparable binding in liver in the absence of hepatotoxicity (40). Consequently, greater concentrations of 3MI are required to elicit pneumotoxicity in rodents than ruminants (59, 62), and spleen has been reported to be a target of 3MI toxicity in rodents (63). The hypothesis that covalent binding to protein is the mechanism for 3MI toxicity is further supported by experiments in which tissue GSH concentration was manipulated. As mentioned previously, GSH is protective against 3MI toxicity in ruminants (64, 65). Elevation of GSH levels in vitro or in vivo can protect

cellular proteins from 3MI binding and toxicity (66–68). There is an inverse correlation between conjugation of 3MI to GSH and covalent binding to protein. Furthermore, activation of 3MI by microsomes from various species and tissues to bind proteins in vitro demonstrates distinct specificities (69). Since conjugation of 3MI to GSH is important in the detoxification of 3MI, it would follow that covalent binding of 3MI to cellular proteins in the same manner could be the mechanism of toxicity. Inhibition of GSH synthesis with L-buthionine-(S,R)-sulfoximine (BSO) causes a selective depletion of hepatic GSH in mice that results in a switch in the order of covalent binding of 3MI to kidney proteins in preference to lung; i.e. there is a switch from pneumotoxicity to nephrotoxicity (70). Therefore, organ toxicity is associated with the relative amount of protein binding.

It has also been proposed that the amount of covalent binding of 3MI to protein depends on the tissue- and species-specific isozymes of the superfamily of P450, which activate 3MI. Incubation of goat lung microsomes with 1-aminobenzotriazole (ABT), a suicide substrate with broad specificity for several P450 isozymes, inhibits activation of 3MI. However, α-methylbenzylaminobenzotriazole (α-MB), with specificity for a different (or distinct) P450 isozyme, is a better inhibitor of 3MI activation by goat lung microsomes than ABT (71). Similar results were obtained using an in vitro viral expression system where it was shown that only certain P450 isozymes could metabolize 3MI to an intermediate that would covalently bind to proteins (72). In addition, within one isozyme (1A2) there was variation between species. The mouse isozyme 1A2 activated 3MI to bind protein, while its human counterpart could not. Since in vitro experimental evidence suggests that several P450 isozymes can activate 3MI to varying degrees, with some being more effective than others, it is quite possible that the organ and species specificity of 3MI toxicity could be related to the P450 isozyme present. Further experiments concerning selective inhibition of these P450 isoenzymes that demonstrate a direct relationship to their in vivo toxicity would strengthen the argument for a isozyme-specific activation of 3MI that is related to pneumotoxicity.

If covalent binding to a protein is involved in the mechanism of toxicity, identification of the target protein is crucial to support this hypothesis. The target protein should be vital for specific cellular survival and organ functioning. It is conceivable that the toxin could selectively bind to a regulatory or structural protein and disrupt the catalysis of a reaction that is vital to the functioning of the organ. In addition, oxidative damage of a protein could activate cellular protein repair mechanisms and thus accelerate the degradation of the target protein and alter cellular function. Attempts have been made to identify the protein target of 3MI. When 3MI was incubated with goat lung microsomes in the presence of NADPH, several

proteins were alkylated by 3MI, but one of 57 kd was found to be the predominantly alkylated protein (73). The alkylated proteins were present to a greater extent in microsomes from goat lung than goat liver. In addition, alkylated proteins were detected in human liver microsomes, but not human lung microsomes (73). Recently these major alkylated proteins from goat lung microsomes were hydrolyzed, and [3-(cystein-S-yl)methyl]indole was identified as the major amino acid adduct. The structure of this adduct was identical to the GSH-3MI conjugate (74). Although this indicates that 3MI is bound to CYS of microsomal proteins, the precise protein that disrupts cellular function is still unidentified.

Another approach for identifying the specific proteins involved in 3MI toxicity involves the development of an antibody that recognizes proteins bound with 3MI. We have recently produced polyclonal antibodies to a 3MI protein conjugate that works effectively in competition assays with bovine blood, but relatively high nonspecific binding occurred. Since monoclonal antibodies often provide improved specificity, we are attempting to develop a monoclonal antibody to protein-bound 3MI. Then the identity of a protein(s) bound to 3MI after administration in vivo can be determined.

Alternatively, some researchers suggest that the binding of toxins to proteins may be more accurately considered as a consequence of the activation of a xenobiotic by certain isozymes that can be tissue- and species-specific and one that does not contribute directly to tissue pathology. Instead, random binding of toxins to non-vital proteins in the cell may simply offer an additional means of detoxification. 3MI conjugation to the sulfhydryl group of CYS residues in cellular protein is similar to that of GSH. The formation of adducts of 3MI with protein probably depends on the number of available reactive sulfhydryls, and the target is probably randomly chosen. There are other examples of cellular proteins binding to toxins that serve a protective function. CYS-containing proteins such as metallothionein appear to act as common regulators for metal toxicity (75). It is known that species that are not susceptible to 3MI pneumotoxicity have appreciable binding of 3MI to hepatic proteins, but do not experience hepatotoxicity (62, 69). In species that are susceptible to 3MI pneumotoxicity, 3MI also binds to hepatic proteins, yet only the lung is susceptible to 3MI toxicity (35, 41, 66). Covalent binding of 3MI to cellular proteins cannot entirely explain the mechanism of toxicity of 3MI, but may be a reflection of its metabolic activation by specific enzymes. In addition, the consequence of 3MI binding to intracellular proteins is subject to the natural occurrence of protein turnover, which enables the damaged proteins to be replenished. However, the interaction of activated 3MI, which persists after detoxification, to another cellular macromolecule, DNA, may have a greater impact on cellular function and survival.

INTERACTION OF 3MI WITH NUCLEIC ACIDS It is often believed that protein binding to an activated toxin is associated with short-term toxicity such as necrosis, while nucleic acid binding is usually associated with chronic disease such as carcinogenesis. Thus previous research focused mainly on the binding of 3MI to cellular proteins, rather than DNA, as the potential mechanism of 3MI toxicity. Although the direct interaction of activated 3MI with nucleic acids has not been investigated, researchers have proposed that covalent binding of reactive metabolites of 3MI to nucleic acids could contribute to 3MI toxicity (38). In addition, it was suggested that 3MI-induced damage to DNA could inhibit the differentiation of type II to type I cells and subsequent regeneration of appropriate lung structure during the course of 3MI toxicity (15). This hypothesis is indirectly supported by the high nuclear and microsomal concentrations of radioactivity localized in the lung after intratracheal administration of radioactive 3MI in rabbits (40). In comparison, the majority of radioactive 3MI was located in the soluble cell fraction of the liver, which is not a target organ for 3MI toxicity (40).

We recently examined the capacity of activated 3MI to bind DNA using calf thymus DNA (CT DNA) and a microsomal-activating system of MFO or PHS (76). ^{14}C-3MI activated by microsomal MFO was found to covalently bind to CT DNA, and this binding was dependent on 3MI dosage, incubation time, and microsomal protein concentration. Not only was activated 3MI capable of binding DNA, but it also occurred in a species- and tissue-specific manner. Figure 1 depicts the species specificity in which activation of 3MI by sheep lung microsomes results in higher binding to CT DNA than in the comparable rat lung microsomal system. Figure 2 demonstrates the tissue specificity in which 3MI activated by lung microsomes had greater binding to CT DNA than 3MI activated by liver microsomes in sheep, which are susceptible to 3MI pneumotoxicity. There was not a detectable difference in the CT DNA binding activated by lung or liver microsomes from rats, which are less susceptible to 3MI toxicity than ruminants. Similar to the binding of 3MI to protein, binding to CT DNA requires activation by MFO or PHS. Preincubation of sheep and rat microsomes with piperonyl butoxide or indomethacin, inhibitors of MFO and PHS, respectively, suppressed the formation of 3MI-CT DNA adducts. Furthermore, preferential binding of MFO-activated 3MI to specific nucleic acid bases was demonstrated using homopolyribonucleotides instead of CT DNA. The purines (Poly[A] and Poly[G]) were bound over twice as much as the pyrimidine, Poly [C]. Such preferential binding is found with many aromatic amines and nitroarenes.

Although binding of 3MI to DNA in vitro is fascinating, conclusions can only be speculative since there is a lack of intracellular compartmentation and DNA repair mechanisms that exist in vivo. Also, the tissue- and species-specific binding of 3MI to DNA in vitro could merely be a function

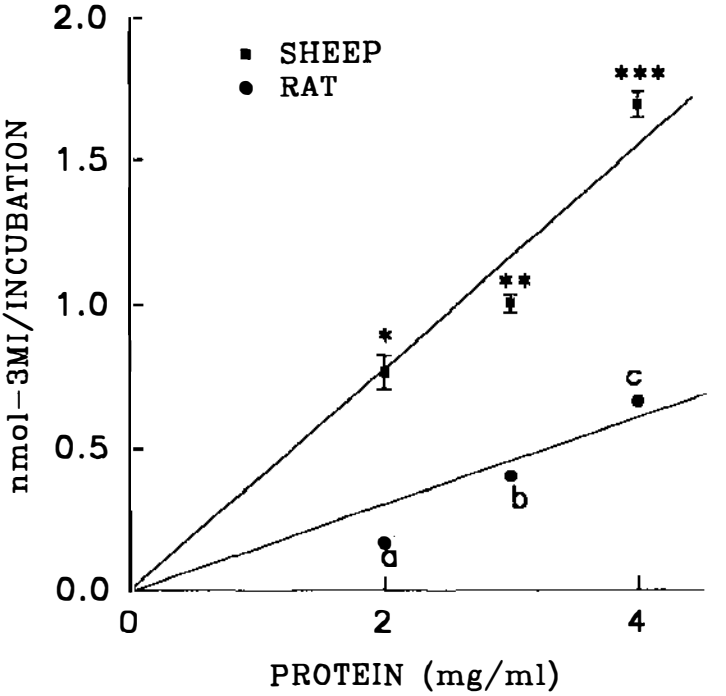

Figure 1 Effect of microsomal protein concentration on binding of [^{14}C]3MI activated by sheep or rat lung microsomes to calf thymus DNA. Values for boiled controls were subtracted from all activities. Incubations were carried out for 60 min. Points represent mean ± SE of duplications from three groups of rats or three sheep. Means with different letters and number of asterisks are significantly different ($p>0.05$), by one-way ANOVA by Tukey's test (from 76).

of the enzyme-activating system, rather than being indicative of a unique event. Therefore, demonstration of binding of 3MI to DNA in vivo in relation to species- and organ-specific toxicity becomes essential for establishing its relevance to the mechanism of 3MI toxicity. It is known that SER contains the highest concentration of drug-metabolizing enzymes; however, nuclear and mitochondrial membranes have also been shown to contain P450 monooxygenase activities, which are inducible in a similar manner to microsomal P450 (77). It is conceivable that 3MI could be activated by nuclear drug-metabolizing enzymes and subsequently bind to DNA. Preliminary evidence showing that 3MI is capable of binding DNA in vivo and is responsive to different doses is depicted in Figure 3. 3MI-DNA adducts were detected in DNA isolated from liver and lung of female mice (C57BL/6J) 24 hr following a challenge with two doses of 3MI. The higher

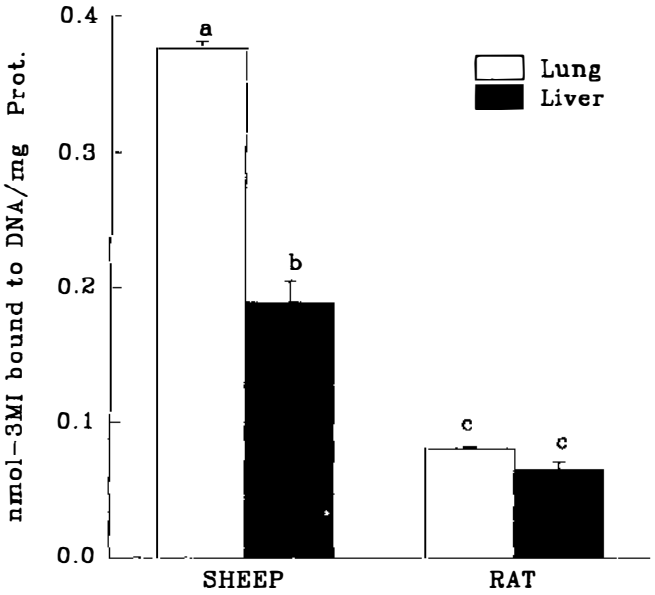

Figure 2 Binding of [^{14}C]3MI activated by sheep lung or liver microsomes vs rat lung or liver microsomes to calf thymus DNA. Values for boiled controls were subtracted from all activities. Results represent mean ± SE of duplicates from three groups of rats or three sheep. Means with different letters are significantly different ($p>0.05$) by one-way ANOVA by Tukey's test (adapted from 76).

dose of 3MI resulted in significantly elevated binding of 3MI to DNA isolated from both liver and lung. The binding of 3MI to DNA is on the order of magnitudes lower than binding to protein, which could be related to the higher protein concentration in the cell (78). Considering the small dose of 3MI administered, the presumably even smaller presence of relative reactive intermediate, and the considerably minute quantity of DNA compared to protein in the cell, it is remarkable that binding of 3MI to DNA in vivo occurs. However, as seen in rodent studies where covalent binding of 3MI to proteins is measured, there is no correlation between tissue selectivity of 3MI-induced toxicity and levels of 3MI-DNA binding in C57BL/6J female mice. Since mice are not as susceptible to 3MI pneumotoxicity as ruminants, the in vivo comparison of 3MI binding to DNA from lung vs liver in ruminants is necessary before the impact of DNA binding by 3MI to pneumotoxicity can be evaluated.

The macromolecular target of activated 3MI is probably randomly chosen, but the formation of 3MI-DNA adducts may have a greater impact on

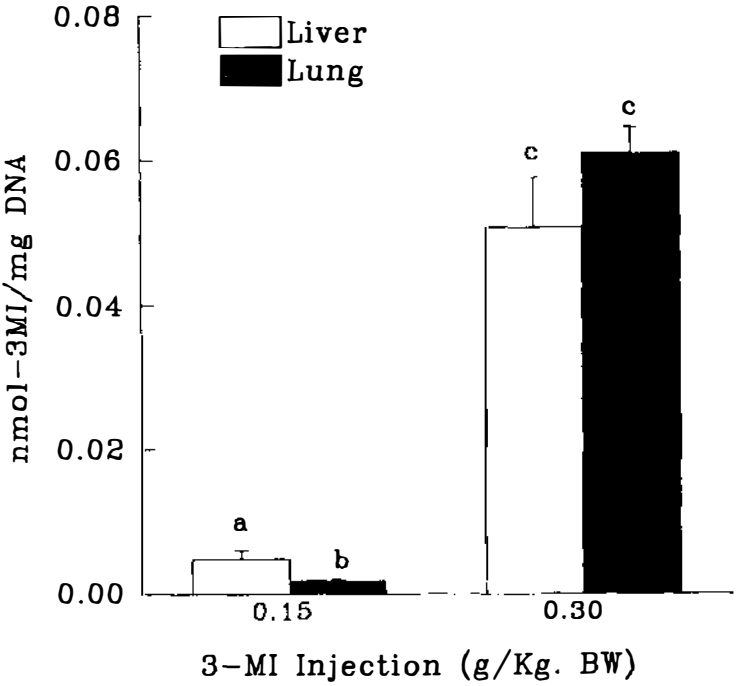

Figure 3 In vivo 3MI-DNA adduct formation in liver and lung from 3 week old C57BL/6J female mice. The animals received IP injections of either 0.15 or 0.30 g/kg body weight [^{14}C]3MI in corn oil. Results represent mean ± SE from three groups of mice. Means with different letters are significantly different ($p>0.05$) by paired comparison (from 76).

cellular function and survival than protein adducts. It is speculated that the interaction of a toxin with a protein could be repleted by the normal process of protein turnover, but alteration of DNA could result in a change in the cell's ability to express protein. Binding of 3MI to DNA could inhibit DNA replication or synthesis of critical proteins. In addition, only cell-type-specific characteristic genes are actively being expressed, which might contribute to the cell specificity of 3MI toxicity. The fraction of activated 3MI that binds to DNA and persists may prevent the cells from responding normally to a stimulus for cell division and lead to enlargement of the cell, as is seen with abnormal proliferation of type II cells (with high nuclear to cytoplasmic ratio) in 3MI-treated goat lungs, and ultimately lead to cell death. Inhibition of DNA replication could affect the proliferation of type II cells and conversion to type I cells. Interference in protein expression could participate in the initial cytotoxic event or could, instead, affect

progression of 3MI cytotoxicity. Since the DNA was isolated from whole organ, the levels of 3MI bound to DNA might be underestimated in particular regions of the lung (i.e. highly susceptible cells such as Clara cells).

The prospect of a possible contribution of 3MI interaction with DNA to pneumotoxicity is exciting. 3MI-induced cytotoxicity does not involve the onset of tumorigenesis, but is a much more rapid and acute pulmonary insult, which is ultimately fatal. There are reports of several drugs that bind DNA without any known mutagenicity. For example, the commonly used analgesic, acetaminophen/paracetamol, is known to cause acute hepatotoxicity, but is not mutagenic (79, 80). Both compounds are capable of binding DNA and causing considerable DNA single-strand breakage (80). Pentamidine and related diamines used in the treatment of African trypanosomiasis undergo tight DNA binding and inhibit cell growth and division, but are not mutagenic in experimental test systems (81). Similar to the previous examples, 3MI causes acute pneumotoxicity, but gives negative results for mutagenicity in the Ames test. Further studies determining the biological consequences of the 3MI binding to DNA in a specific cell type, which is related to acute lung injury, could contribute to the understanding of the mechanism of 3MI toxicity.

METABOLIC CONSEQUENCES OF BINDING OF 3MI TO MACROMOLECULES The experimental evidence that the initial toxicological event involves the formation of a reactive intermediate of 3MI that binds cellular macromolecules is convincing. However, the sequence of events between 3MI binding of cellular macromolecules and the onset of pulmonary pathological lesions has not been clearly described. The description of this linkage has been attempted by numerous experimental approaches including microscopic, histopathological, biochemical, and physiological time-course evaluation and assessment in response to 3MI administration (38, 82–92). There are several candidates for the subsequent toxicological events in specific cell types that could lead to organ-specific disfunction. From histological studies it is known that alveolar type I cells and Clara cells are initial targets of 3MI. The SER undergoes considerable proliferation in these cell types, which are most susceptible to, yet capable of surviving, 3MI toxicity (38, 39). The SER, where cytochrome P450 and PHS are located, is responsible for detoxification and activation of 3MI. Other enzymes inherent to the SER may be primary targets for disruption by activated 3MI because of their proximity. The observation by electron microscopy that surfactant phospholipids (PLs) were replaced by neutral lipids in lamellar bodies in type II cells and that intracellular glycogen was abnormally accumulated in type I cells in the lung of goats treated with 3MI provided early clues in the search for enzymes in the SER that could be responsible for such histopa-

thology (93). Examination of the effect of 3MI on the enzymes involved in the metabolism of phospholipid and glycogen was of primary interest. The organ-specific metabolic changes induced by 3MI have been extensively reviewed (15), but a brief synopsis is given below.

Enzymes located on the SER catalyze the final steps in the synthesis of major PLs (94); therefore, it was proposed that 3MI could interfere with the synthesis of PLs from neutral lipids. Incubation of 3MI with goat lung tissue slices inhibited the incorporation of acetate into all phospholipids [sphingomyelin, phosphatidylinositol (PI) + phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC)] examined, but the PC fraction was most affected (95). PC is the active component of surfactant and the predominant PL in membranes. Subsequent experiments were designed to study the effect of 3MI on PC synthesis. Incorporation of choline into PC declined with 3MI treatment, but was unexpectedly accumulated in CDP-choline, which indicated that P-choline transferase could be rate limiting (96). Under normal conditions, P-choline cytidyltransferase is considered the regulatory enzyme for PC synthesis. However, P-choline transferase is present only in the SER, so it is possible that it could become rate limiting if disrupted by 3MI activation. These experiments did not differentiate between decreased synthesis of PC for membrane turnover and surfactant production. Thus the effect of 3MI on PC synthesis is not limited to cells that specialize in the synthesis of surfactant. Cultures of fibroblasts, unable to synthesize surfactant, require PC for membrane biogenesis during proliferation. Addition of 3MI to fibroblasts also results in a alteration in synthesis of PLs with a decrease in PC synthesis relative to neutral lipids (97).

An organ such as lung, which relies on surfactant for function, is especially sensitive to a decline in phospholipid synthesis. The effect of 3MI on surfactant function in vivo was examined in goats infused with 3MI. In contrast to the in vitro experiments, the profile of PLs in air space was not significantly altered, but the functional characteristics of surfactant in isolated lavage was altered (98, 99). These results are not surprising since it is prudent for lung cells to maintain an adequate supply of surfactant, which is crucial for the organ function. Thus it is apparent that an uncomplicated deficiency of surfactant is not responsible for the initial injury to type I epithelial cells following 3MI administration. Proliferation and differentiation of type II cells to type I cells is critical in the process of lung repair (100). However, regeneration of type I cells damaged by 3MI toxicity is absent. Proliferation of type II cells in response to injury of type I cells is stimulated; however, the differentiation of type II cells to type I cells is inhibited in 3MI toxicity. The defect in PL synthesis probably affects its availability for membrane synthesis required for differentiation of type II cells to type

I cells, which typically contain an abundance of epithelial membranes. In addition, the possible damage to DNA by 3MI could also lead to inhibition of differentiation of type II cells. The abnormal accumulation of the type II cells contributes to the clinical symptoms of pulmonary disfunction in 3MI toxicity.

Glycogen rarely accumulates in adult lungs; however it does occur in 3MI-induced lung toxicity. Glycogen is stored in the fetal lung as a source of energy for active surfactant synthesis prior to parturition. Initially, it was hypothesized that the loss in the control of glycogen metabolism was linked with PL metabolism in 3MI toxicity. A deficiency of energy and provision of precursor from glycogen metabolism to be used for surfactant synthesis could lead to a pathology similar to the hyaline membrane disease seen in premature infants. However, the glycogen accumulation observed in 3MI toxicity is apparently unrelated to deficiency of energy required for PL synthesis as previously hypothesized because a deficiency in surfactant synthesis does not occur (101). Instead, 3MI toxicity is possibly related to the balance of the activities of glycogen synthase and glycogen phosphorylase. Both enzymes exist in phosphorylated and dephosphorylated forms, with glycogen synthase being inactivated by phosphorylation, and glycogen phosphorylase activated upon phosphorylation. The SER contains the phosphatase enzyme responsible for the dephosphorylation of the glycogen enzymes, which is increased by 3MI treatment of goat lung homogenates. Upregulation of the phosphatase activity would activate glycogen synthase while inactivating glycogen phosphorylase, thus resulting in accumulation of glycogen. However, the direct contribution of glycogen accumulation in the lung to development of 3MI toxicity is unknown.

None of the examples of cell-specific responses to 3MI investigated provides a complete description of the mechanism of 3MI toxicity. Examination of most of the cellular responses has focused on specific cellular protein targets of 3MI and has not explored the potential relationship to DNA. Yet, all of these responses could be involved in the progression of 3MI toxicity. The link between the initial toxicological event and onset of the pathological lesion, which would include multiple stages in the progression of the disease, is biologically important in determining the mechanism of toxicity.

CONCLUSIONS

The toxicity of 3MI is cell-, organ-, and species-specific; however, the mechanism by which 3MI causes this specific tissue damage is quite complex. Extensive work has been done to determine the formation of the ultimate reactive intermediate of 3MI that initiates toxicity. The formation

of a free radical is essential in the activation of 3MI; however, following molecular rearrangement, the chemical species of 3MI that binds GSH and the CYS of cellular proteins has been identified as methylene imine. There is a definite relationship between the generation of 3MI free radicals, the cellular macromolecules that are targeted, and the impact on specific cell functions. The pathogenesis of 3MI related to its organ and species specificity is still not entirely explained by the mechanisms proposed so far. The initial insult may be mediated by one common mechanism followed by disruption of cellular events that will affect the organ-specific functions. However, the progression of pneumotoxicity induced by 3MI probably involves multiple targets and a cascade of events. Determination of the cellular processes that are disrupted by 3MI may lead to the elucidation of the sequence of toxicological events and eventually contribute to the understanding of the tissue-specific damage. Identification of the specific proteins bound by the reactive intermediate of 3MI and their role in 3MI toxicity will be enlightening. There are several interesting aspects to be pursued in the area of 3MI binding to DNA, such as examining the effect on expression of certain proteins, comparisons of DNA binding between species, and determining whether DNA binding is the ultimate cellular target that initiates toxicity. Understanding the etiology of 3MI-induced pneumotoxicity in ABPE allowed for an effective means with a sound scientific base to solve an economic problem in the livestock industry. In addition, studying the mechanism of 3MI toxicity provides an ideal model for understanding the cell-, tissue-, and species-specific pathogenesis of diseases induced by toxins in animals and humans.

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