Protein Targets of Monocrotaline Pyrrole in Pulmonary Artery Endothelial Cells*

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A single administration of monocrotaline to rats results in pathologic alterations in the lung and heart similar to human pulmonary hypertension. In order to produce these lesions, monocrotaline is oxidized to monocrotaline pyrrole in the liver followed by hematogenous transport to the lung where it injures pulmonary endothelium. In this study, we determined specific endothelial targets for 14C-monocrotaline pyrrole using two-dimensional gel electrophoresis and autoradiographic detection of protein metabolite adducts. Selective labeling of specific proteins was observed. Labeled proteins were digested with trypsin, and the resulting peptides were analyzed using matrix-assisted laser desorption ionization mass spectrometry. The results were searched against sequence databases to identify the adducted proteins. Five abundant adducted proteins were identified as galectin-1, protein-disulfide isomerase, probable protein-disulfide isomerase (ER60), b- or c- cytoplasmic actin, and cytoskeletal tropomyosin (TM30-NM). With the exception of actin, the proteins identified in this study have never been identified as potential targets for pyrroles, and the majority of these proteins have either received no or minimal attention as targets for other electrophilic compounds. The known functions of these proteins are discussed in terms of their potential for explaining the pulmonary toxicity of monocrotaline.

The pyrrolizidine alkaloid monocrotaline (MCT)1 is a phytotoxin used experimentally to cause a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vascu- litis, pulmonary hypertension (PH), and cor pulmonale (1–3). Although MCT intoxication is used as a model for studying human PH, the initiating mechanism(s) by which this agent produces PH have remained elusive. To produce pulmonary insult, MCT must first be activated by the liver to the putative electrophile monocrotaline pyrrole (MCTP) (4, 5) which has characteristics of a bifunctional cross-linking agent and has a half-life of ~3 s in aqueous environments near neutral pH (6). Stabilization of MCTP by red blood cells facilitates subsequent transport to the lung (7). The evidence for the involvement of the pulmonary endothelium as the target for MCT intoxication is supported by the circulatory proximity of the lung to the liver endothelium, evidence of increased thymidine uptake and decreased 5-hydroxytryptamine clearance by endothelial cells, and extravasculature leakage of large macromolecules (2, 8, 9). Human primary PH is hypothesized to be an inherited dysfunction of the pulmonary vascular endothelial cells (10). In primary PH, disturbance of the endothelial cell surface is suspected to be the initiating factor in the formation of platelet aggregates (11) and cause the presence of in situ thrombosis (12, 13). In vitro experiments with bovine pulmonary artery endothelial cells have shown that MCTP can cause a moderate decrease in their ability to act as a permeability barrier (14), cell proliferation is inhibited (15), prolonged cell cycle arrest in G2-M occurs (16, 17), and cells unable to correct or compensate for electrophilic insult often undergo apoptosis (18). Apoptosis has recently been shown to occur in rat pulmonary artery endothelial cells following the in vivo administration of MCT (19).

Previous work has supported the involvement of endothelial cells as the target for MCT-induced pulmonary hypertension; however, the mechanism(s) by which these cells lose their ability to function correctly is unknown. With respect to pyrrole adduct formation this has been restricted to the measurement of covalent binding to endothelial cell DNA (16, 20). MCTP has been shown to react in a facile manner with the thiol groups of cysteine and glutathione (21–25). A carbonium ion can be generated at both the C7 and the C9 positions on the pyrrole ring with the pyrrole structure being stabilized by resonance structures that share a charge with the bridge head nitrogen (26). This delocalization of charge for MCTP confers soft electrophile characteristics (27, 28) in line more with reactivity toward soft nucleophile protein side chains than with nucleic acids, which are harder nucleophiles. It has previously been shown that MCTP reacts with thiol groups on proteins such as hemoglobin (22, 29, 30). Of the limited number of proteins identified as specific targets for MCTP, cytochrome P450 3A, which is responsible for the dehydrogenation of MCT, has also been shown to form adducts with pyrroles (31). In this study we have coupled the use of two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization (MALDI) to identify five major MCTP target proteins in human

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‡The abbreviations used are: MCT, monocrotaline; PH, pulmonary hypertension; MCTP, monocrotaline pyrrole; MALDI, matrix-assisted laser desorption ionization; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate; PVDF, polyvinylidene difluoride; HPLC, high pressure liquid chromatography; ACTH, adrenocorticotropin hormone; PSD, post-source decay; TBS, Tris-buffered saline; PDI, protein-disulfide isomerase; ECM, extracellular matrix.

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lungs endothelial cells and discuss their potential relevance to the enigmatic process of pulmonary hypertension.

**MATERIALS AND METHODS**

Production of 14C-Monocrotaline Pyrrole—All reagents unless otherwise indicated were obtained from Fisher. *Crotalaria spectabilis* was grown under a confined atmosphere of 14CO2, and the 14C-monocrotaline (14C-MCT, purity >98%) was extracted and purified as described before (32). 14C-MCT was converted to 14C-monocrotaline pyrrole (14C-mCTP, 1.95 mCi/mmol) by the method of Mattocks et al. (35) using tetrabromo-1,2-benzoquinone (Aldrich); the product was recrystallized in hexane/diethyl ether. Using fast atom bombardment mass spectrometry the conversion of MCT to MCTP was found to be complete; daughter spectra contained ions characteristic of the pyrrole (34). 14C-MCTP was stored in N,N-dimethylformamide at −80 °C until just prior to use.

**Tissue Culture and Sample Preparation—**Normal human pulmonary artery endothelial cells (passage 7–8) (Clonetics, San Diego, CA), from a 34-year-old female were grown to 80–90% confluence prior to treatment. Cells in 175-cm² flasks (Falcon polystyrene) were incubated at 37 °C, 5% CO2 with humidity in EGM-2 medium (Clonetics). The medium was replaced with EGM-2 without 10% fetal bovine serum immediately before exposing cells to 105 μCi/mL-14C-MCTP delivered in 12.9 μl of N,N-dimethylformamide (DMF) containing 25 μl of water. Cells were removed from flasks using 39-cm cell scrapers (Sarstedt, Newton, NC). After pelleting, the cells were washed three times with isotonnic phosphate-buffered saline (136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na2HPO4, and 1.76 mM KH2PO4, pH 7.4). The supernatant was removed, and the cells from five flasks were combined, lysed in a 9 μl urea (pure urea) solution containing 4% CHAPS (99%), all obtained from Amersham Pharmacia Biotech plus 40 mM Tris and 0.1% SDS at 55 °C. Triplicate samples were extracted from the PVDF membrane by a 1:500 (wt/vol) ethanol (Aldrich, spectrophotometric grade): 50% formic acid (purity 99%) to the digestion medium and sonicating vials in a Branson ultrasonic cleaner (Shelton, CT) for 30 min. The extract was concentrated to dryness in 100-μl glass AccuMicro Vials (Kimble-Kontes, Vineland, NJ) using a Savant Speed Vac concentrator (Laboratory Equipment Company, Hayward, CA); samples were stored at −80 °C until they were analyzed by MALDI.

**Mass Spectrometry**—Molecular masses of tryptic peptides were determined using a Voyager-DE STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA), using a nitrogen laser (337 nm) for ionization. A 10 mg/ml solution of recrystallized c-4-aminocaproic acid (Aldrich) matrix was prepared in 50% aqueous acetone containing 0.5% trifluoroacetic acid. Peptide digests were dissolved in 10 μl of 50% aqueous acetonecontaining 0.1% formic acid, and a 0.5-μl aliquot was mixed on the sample target with 0.5 μl of matrix solution and allowed to dry under ambient conditions. Multi-point mass axis calibration was performed using external standards (angiotensin I, ACTH(1–17), ACTH(18–39), ACTH(7–38), and bovine insulin. Initial screening was performed in linear mode, and more complex molecular isomorphs were observed in reflector mode. To determine the identity of selected spots, MS-Tag and in some cases MS-Tag were used to search data bases for peptide mass fingerprints and to match fragment ions observed in post-source decay (PSD) spectra, respectively (37, 38). In addition to the above data bases we have used the ExPaSy Proteomics tool ProtScale to accomplish hydropathicity calculations (39). These calculations were used to determine the potential accessibility of the tryptic peptides for MALDI detection.

**Western Analysis of PDI and Galectin-1**—An additional blot and the corresponding autoradiographic analysis were performed for antibody detection of PDI and galectin-1. PVDF membranes were stained with sulforhodamine B, and the proteins were matched with autoradiographic spots. The location of spots previously identified by peptide mass fingerprinting correspond to galectin-1 and PDI were marked by recording their horizontal and vertical position prior to destaining with 100% methanol and a final rinse with 70% acetonitrile. Blots were sectioned in half, and one portion developed for galectin-1 and the other PDI. Membranes were first blocked with 3% nonfat milk (Bio-Rad) in 150 mM NaCl, 50 mM Tris buffer (TBS), pH 7.4, for 30 min at ambient conditions. This was followed by an overnight incubation with primary antibodies at 4 °C. The primary antibodies were polyclonal rabbit raised against rat galectin-1 (generously provided by D. N. W. Cooper from UCSF), used at a concentration of 100 μl of serum/20 ml of 3% milk TBS and a mouse monoclonal raised against rat protein-disulfide isomerase synthetic peptide (amino acids 499–509) with a working concentration of 2 μg/ml. The latter was obtained from StressGen Biotechnologies Corp., Victoria, British Columbia, and is known to react with both PDI and calsequestrin. After the overnight incubation the blots were washed four times with TBS, and the secondary antibody either goat anti-rabbit or anti-mouse conjugated with alkaline phosphatase (Bio-Rad) was applied at a 1:2000 dilution in 3% milk TBS. Incubations were carried out for 1.5 h at ambient conditions, and the blots were subsequently washed with 0.05% Tween-20 in TBS (two times) followed by four washes with TBS. Blots were developed...
enhance spot visualization.

The membrane was then incubated with alkaline phosphatase conjugation substrate kit (Bio-Rad), incubated for 10 min with alkaline phosphatase conjugation substrate kit (Bio-Rad), and subsequently stained with sulforhodamine B. Proteins were identified by MALDI, excised portions taken on a squared appearance. The following numbers correspond with the following proteins: 1, PDI; 2, ER60; 4, β- or γ-cytoplasmic actin; 6, cytoskeletal tropomyosin; 7, galectin-1. Library searches performed for the peptide mass fingerprints of spots 3 and 5 resulted in the absence of definitive matches.

**Antibody Recognition of Galectin-1 and PDI**—Because these two proteins can be more simply identified through the use of antibody techniques, results are recorded in Fig. 4. The commercially available antibody to PDI was found to react with the protein previously identified using MALDI. The antibody also reacted with another protein, which had an estimated pI and molecular weight that corresponded to calreticulin, a protein specified by the peptide mass fingerprint. There are also a number of discrete radiolabeled spots that travel with the dye front. Because the number of known metabolites and breakdown products of MCTP (24, 41, 42) cannot account for this heavily labeled area, we suspect that these are proteolysis products derived from adducted proteins.

Antibody Recognition of Galectin-1 and PDI—Because these two of the more interesting proteins found to be adducted by pyrroles, we determined if for future experiments they could be more simply identified through the use of antibody techniques. Results are recorded in Fig. 4. The commercially available antibody to PDI was found to react with the protein previously identified using MALDI. The antibody also reacted with another protein, which had an estimated pI and molecular weight that corresponded to calreticulin, a protein specified by the peptide mass fingerprint. There are also a number of discrete radiolabeled spots that travel with the dye front. Because the number of known metabolites and breakdown products of MCTP (24, 41, 42) cannot account for this heavily labeled area, we suspect that these are proteolysis products derived from adducted proteins.

**RESULTS**

**Two-dimensional Gel Electrophoresis, Autoradiography, and Peptide Mass Fingerprinting**—Results recorded in Fig. 1 show that a selective number of proteins form covalent adducts with MCTP. This pattern was consistent from separation to separation, with six separate autoradiographic profiles showing the identical pattern of labeling. Of the 13 labeled spots, seven were chosen for analysis based on the amount of radioactivity associated with them and the intensity of the corresponding sulforhodamine B stain. The latter was used to pick protein spots that were in sufficient quantity and purity to have a reasonable chance of producing an unambiguous match with protein data bases. Of these seven spots, five were identified (Table I) as probable protein-disulfide isomerase precursor ER-60 (EC 5.3.4.1, Swiss-Prot P30101), protein-disulfide isomerase precursor (PDI, EC 5.3.4.1, Swiss-Prot P07237), β- or γ-cytoplasmic actin (Swiss-Prot P02570 and P02571, respectively), cytoskeletal tropomyosin (TM30-NM, Swiss-Prot P12324), and galectin-1 (Swiss-Prot P09382). As recorded in Table I, both the apparent molecular weights and estimated pIs from the two-dimensional separations were in close agreement to data base values with the actual tryptic peptide masses showing excellent coverage and agreement with the expected theoretical values. Typical MALDI mass spectra for tryptic digests of galectin-1 (linear mode) and PDI (reflector mode) are shown in Figs. 2 and 3, respectively. PSD spectra were generated to confirm assignments of tryptic fragments from each of these two proteins. The PSD ions described here follow the nomenclature of Biemann (40); fragmentation designation is followed in parenthesis by the m/z value. For galectin-1 the peptide sequence DSNNLCLHFNPR was chosen. The PSD ions presented the MH+ -45 (loss of the threonine side chain), y3 (417), NQ-NH3 (b ion, 226), FIFIDSDHT-H2O (b ion, 1059), LFIFIDSHT (b ion, 1190), and FIFIDSHTD (a ion, 1164). Spots three and five produced no meaningful matches considering apparent molecular weight, estimated pI, or the peptide mass fingerprint. There are also a number of discrete radiolabeled spots that travel with the dye front. Because the number of known metabolites and breakdown products of MCTP (24, 41, 42) cannot account for this heavily labeled area, we suspect that these are proteolysis products derived from adducted proteins.

**DISCUSSION**

Auroradiographic analysis showed that MCTP forms covalent adducts with specific proteins. Of these, we identified the following abundant endothelial proteins as targets for pyrrole adduct formation: galectin-1 (Swiss-Prot P09382), PDI precursor (EC 5.3.4.1, Swiss-Prot P07237), probable protein-disulfide isomerase ER-60 (EC 5.3.4.1, Swiss-Prot P30101), β- or γ-cytoplasmic actin (Swiss-Prot P02570 and P02571, respectively), and cytoskeletal tropomyosin (TM30-NM, Swiss-Prot P12324).
Table I
Summary of MALDI masses obtained from tryptic digests of pyrrole adducted proteins

| Spot no. | Experimental m/z | Experimental Δm | Proposed peptide sequence<sup>a</sup> | Peptide sequence numbers | Protein match (Swiss-Prot accession no.) | Percentage of sequence coverage observed in MALDI spectrum | Calculated molecular mass<sup>c</sup> | Molecular mass from gel Da | pI from gel | Calculated pI | pI from gel |
|----------|------------------|-----------------|----------------------------------------|--------------------------|------------------------------------------|-----------------------------------------------------------|----------------------------------|--------------------------|-------------|--------------|-------------|
| 1        | 608.299          | −41.4           | (K) LSNFK (T)                          | 272–276                  | Protein-disulfide isomerase (precursor), human (P07297) | 43%                                                       | 57,116                           | 58,900                   | 4.76        | 4.8         |             |
|          | 761.341          | −38.6           | (K) AEGSEIR (L)                        | 72–78                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 763.385          | −50.8           | (K) IFGGEIK (T)                        | 248–254                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 777.420          | −31.1           | (K) DGGVLFK (K)                        | 201–207                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 870.346          | −35.9           | (K) FFPASADR (T)                       | 445–452                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 928.484          | −41.7           | (K) VHSFTPLK (F)                       | 437–444                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 962.411          | −41.2           | (R) ITEFCimHR (F)                      | 339–345                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 966.528          | −38.2           | (R) ILEFFGLK (K)                       | 301–308                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 988.491          | 2.6             | (K) KEECimFAVR (L)                     | 309–316                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 991.524          | −22.6           | (K) ENLDPFK (H)                        | 222–230                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1002.471         | −87.4           | (K) LKAEGSEIR (L)                      | 70–78                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 1037.424         | −66.4           | (R) NNFEGVTK (E)                       | 214–222                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1066.470         | −46.9           | (R) TVIDYNGER (T)                      | 453–461                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1081.635         | −42.4           | (K) THILLFLP (K)                       | 255–263                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1158.539         | −51.7           | (K) SNFAEALAAHK (Y)                    | 32–42                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 1213.501         | −36.7           | (K) NFEDVAFDEK (K)                     | 376–385                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1451.680         | −21.8           | (K) YKPESEELTAER (I)                   | 327–338                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1780.794         | −41.3           | (K) VDATEESDLAQYQG−VR (G)             | 82–97                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 1833.884         | −29.5           | (K) IILFIFSDHDTDQ−R (I)                | 286–300                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1965.005         | −39.6           | (K) HNQLPLVIEFTEQTA−FK (I)            | 231–247                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 2935.451         | −43.1           | (R) TGAATTLPDAAA−ESLVESSEAVGFFK−D     | 133–162                  | Protein-disulfide isomerase, human (P30101), ER60 |                                                            |                                  |                          |             |             |             |
| 2        | 877.499          | 9.7             | (K) LNFAVASR (K)                       | 297–304                  |                                      |                                                            |                                  |                          |             |             |             |
|          | 1172.568         | 27              | (K) FVMQEEFSR (D)                      | 336–344                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1188.555         | 19.1            | (K) FVNe−oxQEEFSR (D)                  | 336–344                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1191.582         | −19             | (R) LAPEYAAAAT (L)                     | 63–73                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 1236.539         | 25.7            | (R) DGEAAAGYDGPR (T)                   | 108–119                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1341.704         | 19.7            | (R) GFPTIYFSPANK (K)                   | 449–460                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1359.674         | 15.5            | (R) FLQDYFDGNLK (R)                    | 352–362                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1368.721         | 56.3            | (K) SEPIPESNDGPVK (V)                  | 367–379                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1370.726         | 30.4            | (R) ELSDFSYIQLR (E)                    | 472–482                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1619.736         | −48.5           | (K) DLLIAYDYYDYEK− (H)                 | 259–271                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1664.750         | −9              | (K) MDATANDVPSPYEV−R (G)               | 434–448                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1680.810         | 56.1            | (K) Met−oxDATA−NDVPSPYEV (G)          | 434–448                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 2348.263         | 190.7           | (K) DASIVGGFDFDS−EHSEFLK (A)          | 153–173                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 2575.338         | 33.6            | (K) TSFHELSDFGLES−TAGEIPVVAIR (T)      | 306–329                  |                                          |                                                            |                                  |                          |             |             |             |
| 4        | 644.352          | −21             | (R) LDLAGR (D)                         | 178–183                  | β-actin (P02570) or γ-actin (P02571) or cytoplasmic, human | 29%                                                       | 41,737                           | 47,700                   | 5.29        | 5.4         |             |
|          | 795.459          | −13.9           | (K) IIAJP (K)                          | 329–335                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 976.449          | 1.1             | (K) AGFAGDDAPR (A)                     | 19–28                    |                                          |                                                            |                                  |                          |             |             |             |
|          | 1132.532         | 4.5             | (R) GYSFTTIAER (E)                     | 197–206                  |                                          |                                                            |                                  |                          |             |             |             |
|          | 1499.691         | 14.3            | (K) pyr−GluEYESGSPSIVHR (K)            | 360–372                  |                                          |                                                            |                                  |                          |             |             |             |

<sup>a</sup>The difference between the measured monoisotopic mass and calculated mass.

<sup>b</sup>Parentheses denote residue before and after the peptide. Cim, denotes iodoacetamide-derivatized Cys; Ac-, acetylated N terminus; Met-ox, methionine sulfoxide; pyro-Glu, N-terminal Gln converted to pyroglutamine.

<sup>c</sup>Masses according to MS-FIT.

<sup>d</sup>Acetylated alanine labeled as first residue.
the environment of a thiol group can increase its reactivity to
was more pronounced than with cysteine alone, indicating that
tryptophan, and histidine. Adduct formation with glutathione
MCTP, could alkylate, in decreasing order of reactivity, cysteine,
drotronecine, a pyrrole formed from the reaction of water with
activity observed is not surprising considering earlier work. Dehy-
have identified or attempted to characterize. The degree of selec-
tively observed is not surprising considering earlier work. Dehy-
that are potentially important in the maintenance of the endo-
mechanistic link between MCTP treatment and the progres-
that are potentially important in the maintenance of the endo-
imine (43). The proteins identified in this study serve functions

| Spot no. | Experimental | $\Delta \text{a}$ | Proposed peptide sequence$^b$ | Peptide sequence numbers | Protein match (Swiss-Prot accession no.) | Percentage of sequence coverage observed in MALDI spectrum | Calculated molecular mass$^c$ | Molecular mass from gel | Calculated pl | pl from gel |
|----------|--------------|----------------|--------------------------------|--------------------------|------------------------------------------|----------------------------------------------------------|-----------------------------|---------------------------|-------------|-------------|
| 1515.769 | 19.3         | (K) IWHHTFYNELR(V) | 85–95 | Tropomyosin, cytoskeletal, human (P12324) | 26% | 29,032 | 35,600 | 4.75 | 4.9 |
| 1516.730 | 26.8         | (K) EYDESGPSIVH-R(K) | 360–372 | | | | | | |
| 1790.926 | 33.5         | (K) SYELPDQVQIVIT-L GNER(F) | 239–254 | | | | | | |
| 1954.097 | 32           | (R) VAPECHPVLTE-APLMPK(A) | 96–113 | | | | | | |
| 2231.057 | −8.4         | (K) DLYANTVLSGGT-Met-oxYPGIADR-(H) | 292–312 | | | | | | |
| 6        | 630.381      | 23.7 | (K) VIENR(A) | 93–97 | Galectin-1, human (P08382) | 74% | 14,584 | 14,100 | 5.34 | 5.1 |
| 7        | 786.399      | −0.7 | (R) GEVAPDAK(S) | 21–28 | | | | | |
| 968.491 | 18.1         | (K) LPDGYEFK(F) | 100–107 | | | | | | |
| 1041.605 | 35.7         | (R) GVGEVAPDAK(S) | 19–28 | | | | | | |
| 1076.501 | 24.9         | (K) DGGWGETQER(E) | 64–73 | | | | | | |
| 1486.734 | 47.9         | (K) DSNLCLmHF-PR(F) | 37–48 | | | | | | |
| 1800.897 | 49.2         | (R) LNEAINyMet-oxAXAGSFXK(T) | 112–127 | | | | | | |
| 2014.069 | 47.6         | (−)Ac-ACimGLV-ASNLKPGECim-LR(V)$^d$ | 1–18 | | | | | | |
| 2867.490 | 61           | (R) EAVFPFPGSSVAE-VCimITFPQANLIVK(L) | 74–99 | | | | | | |

Gel images in Fig. 1, A and B, confirm this selectivity; numerous abundant proteins, in and around the 66-kDa zone, are
devoid or show nondiscrete traces of associated radiolabel. This is readily observable in the abundant protein just above spot 3.
The lower left quadrant, a portion of the blot containing proteins closer to an overall neutral pl, contains numerous protein spots; however, they are not associated with detectable levels of $^{14}$C. Other prominent examples of selective adduct formation can be seen in the area of spot 6. This area contains many sulforhodamine B-stained spots, yet only spot 6 shows distinct and reproducible evidence of radiolabel incorporation. Among the seven spots that we selected for identification, it is also apparent that the degree of labeling does not correspond with the amount of protein present. Gels stained with silver (not shown) or blots developed with sulforhodamine B show that spot 5 is an abundant protein, which is qualitatively equal in content to spots 1–4, 6, and 7. However, the degree of label associated with spot 5 appears to be less when compared with these spots. Of the protein spots that consistently contain $^{14}$C, the two unmarked spots (not identified) near spot 7 also show intense radiolabeling and are not as abundant as the proteins we have identified or attempted to characterize. The degree of selectivity observed is not surprising considering earlier work. Dihydroetroncine, a pyrrole formed from the reaction of water with MCTP, could alkylate, in decreasing order of reactivity, cysteine, tryptophan, and histidine. Adduct formation with glutathione was more pronounced than with cysteine alone, indicating that the environment of a thiol group can increase its reactivity to pyrrole (21). Adduct formation would thus be more favorable for proteins containing a higher percentage of accessible cysteine residues and a microenvironment that aids in thiol activation. Such selectivity has been observed for a number of electrophiles, one of the best studied being the arylation of proteins by acetaminophen-derived electrophile, N-acetyl-p-benzoquinone imine (43). The proteins identified in this study serve functions that are potentially important in the maintenance of the endothelial cell barrier. Alterations in these functions could be a mechanistic link between MCTP treatment and the progression of vascular remodeling evident in the lung. However this statement is tempered by the fact that for most compounds attempts have failed to definitely link the event of covalent ad-
duct formation with cellular proteins to a toxic event observed in a particular organ. Even for well studied compounds such as acetaminophen this link is casual in that covalent binding is merely seen to parallel the degree of hepatotoxicity (44).

Additional peaks that could be identified as added tryptic peptides were not observed in the MALDI mass spectra. Re-
covers of pyrrole adducts were expected to be low owing to the liability of the pyrrole adducts during work up in 50% formic acid solutions or because of incomplete digestion of protein-
containing intramolecular pyrrole cross-links. We have previously shown that pyrrole-glutathione conjugates and pyrrole adduct peptides of glutathione transferases are not stable to

$^a$ M. W. Lamé, A. D. Jones, D. W. Wilson, S. K. Dunston, and H. J. Segall, unpublished results.
separations on silica-based reverse phase HPLC supports using trifluoroacetic acid in the mobile phase.

Previous examination of adducts formed from the reaction of MCTP with synthetic peptides have revealed that the amino acids cysteine, tryptophan, and histidine are the most likely targets for pyrrole adduct formation (21, 30). Because these groups are present in many proteins, accessibility of side chain nucleophiles and local microenvironments that enhance side chain nucleophilicity are expected to form the basis of selectivity in adduct formation. Galectin-1 contains six cysteine residues (4.5% of total amino acids), all of which are in the reduced state (45). Hydropathicity calculations have indicated that at least 50% of these cysteines are potentially exposed to the aqueous environment. Both PDI and ER60 have two thioredoxin domains/protein molecule that are redox active (46) and would provide reduced cysteines to bind with pyroroles. Another interesting facet of both PDI and ER60 is the presence of an acidic group that is positioned close to the cysteines involved in the catalytic center of these proteins. Chivers and Raines (47) have proposed that aspartate or glutamate participate in a general acid/base catalysis scheme at the active site (Cys-Xaa-Xaa-Cys) involved in the thiol/disulfide oxidoreduction carried out by *Escherichia coli* thioredoxin. These authors pointed out that this feature is also present in human PDI. Examination of ER60 also shows a similar sequence of amino acids and the presence of glutamate. For example *E. coli* thioredoxin (Swiss-Prot no. P00274) has the sequence DFWEAEWCPCM (residues 26–37), human PDI precursor (Swiss-Prot no. P07237) is EFYAPWGHCKRA, (residues 47–58), and ER60 (Swiss-Prot # P30101) is EFAPWGHCKR (residues 51–62). The importance of the proximity of the glutamate residue to the thiol group stems from the potential for the carboxylate to enhance thiol reactivity via general base catalysis. Because the reactivity of MCTP or dehydroretronecine toward protein nucleophiles involves first the release of the alkaloid ester or hydroxyl groups (respectively) producing a carbonium ion the presence of acidic groups could increase the alkylation potential of pyrroles (48). Protons donated by glutamic or aspartic acid drive this release under the general mechanism of acid catalysis. Therefore, proton donors near cysteine would be expected to

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FIG. 2. MALDI spectrum (linear mode) of peptides derived from the digestion of galectin-1 with trypsin. Numbers positioned above ions represent the peptide sequence in the parent protein. Ion masses corresponding to the tryptic fragments are listed in Table I, except for span 29–36 (m/z 878) and the peptide generated by a single missed cleavage by trypsin 74–111 (m/z 4334).

FIG. 3. MALDI spectrum (reflector mode) of peptides derived from the digestion of PDI with trypsin. Numbers positioned above ions represent the peptide sequence in the parent protein. Ion masses corresponding to the respective trypsin-generated peptides are listed in Table I.
increase their susceptibility to alkylation by pyrroles through an SN1 mechanism. Based upon nucleic acid sequencing, PDI has six cysteine residues in its mature form. The two cysteine residues that are not in thioredoxin-like sites are reactive to iodoacetamide (49) but only under denaturing conditions; this would leave only the hyperreactive Cys-Xaa-Xaa-Cys motifs for MCTP to target. Both β-actin cytoplasmic 1 and cytoskeletal tropomyosin contain 1.6% cysteine, and both also have acidic pls, 5.29 and 4.75, respectively. Hydropathicity calculations indicate that the cysteine residues of cytoskeletal tropomyosin at positions 154, 226, and 233 are more negative than the β-actin cytoplasmic 1 residues at 257 and 272, which are suspected to be in contact with the aqueous environment. From the autoradiograph it also appears that tropomyosin is more extensively labeled, which would correlate with its more accessible thiol groups.

The potential biological relevance of these proteins in an emerging model of MCTP-induced pulmonary hypertension will be discussed with an emphasis being placed on exploring the known functions of these proteins and the observed pulmonary physiological alterations, which typify the disease.

Galectin-1 is a lectin or carbohydrate-binding protein, which in its dimeric state possesses two galactoside binding sites (50). The dimeric state can thus participate in both intramolecular and intermolecular cross-linking through the interaction of more than one sugar residue (50). Intermolecular cross-linking capabilities have been used as an assay for galectin-1 activity by exploiting its agglutination of erythrocytes (45). The proposed biological functions of galectin-1 include adhesion in cell-cell and cell-extracellular matrix interactions, concentration-dependent induction and inhibition of cell proliferation, and the ability to induce apoptosis (50). In rat lung, the β-galactoside-binding proteins are expressed in smooth muscle cells, type I alveolar epithelial cells, and are concentrated extracellularly in elastic fibers of the pulmonary parenchyma and blood vessels (51, 52). Human aortic and umbilical vein endothelial cells have also been shown to express galectin-1 in vitro (53). In human aortic endothelial cells, the bulk of galectin is located within the interior of the cell with ~5% on the surface. Stimulation of human aortic endothelial cells with minimally oxidized low density lipoprotein resulted in a 47–79% increase in galectin on the cellular surface (53). Similar findings were reported for human umbilical vein endothelial cells. Both smooth muscle cells and pulmonary artery endothelial cells have been observed to undergo cellular proliferation when exposed to various lectins including galectin (54). It has been suggested that galectin-1 could play a significant role in stimulating smooth muscle growth in developing alveolar wall vessels and the development of pulmonary capillaries (54). Mitogenic versus sugar binding activity may be related to the state of galectin sulfhydryl groups, because formation of two intramolecular disulfide bonds caused the protein to act as a mitogen but lacks sugar binding properties (55). The protein may exist in two different states; as a monomer it has transforming growth factor qualities, and as a dimer it acts as a lectin (55). MCTP binding could either result in intramolecular or intermolecular cross-linking of sulfhydryls with functional consequences for the sugar binding or growth factor activities.

The conversion of galectin-1 to a mitogen could explain some of the observed pulmonary changes following the in vivo administration of MCT. Feeding of C. spectabilis seeds to rats results in a significant proliferative response in endothelial cells, fibroblasts, and smooth muscle cells as measured by the uptake of [3H]thymidine (8). Galectin-1 has also been shown to form associations with extracellular matrix (ECM) proteins such as laminin and fibronectin (50) and promote cell adhesion to the ECM. Its interaction with ECM proteins has been implicated in the modulation of the spread and migration of vascular smooth muscle cells (56). Alteration in the ECM because of changes in galectin-1 may result in a matrix unable to act as a sufficient anchor for endothelial cells resulting in either loss of cells or the microvascular leakage that is observed in the MCT model.

Galectin-1 has also been shown to effect the release of cytokinin from murine macrophage and human monocytes (57). One of the earliest morphological changes in the pulmonary vasculature of MCT-exposed rats is the accumulation of mononuclear inflammatory cells in the small intraocular vessels and this is associated with or precedes the accumulation of edema fluid and inflammation (58).

Galectins have been implicated as participants in cell adhesion, cell growth, immunomodulation, inflammation, embryogenesis, apoptosis, pre-mRNA splicing, and metastasis. Although there are numerous biochemical and molecular studies relative to galectins, their in vivo role is basically unknown (59); however, the properties of galectin-1 make it an attractive and never before considered participant in the pathology induced by MCT treatment.

PDI is located in the lumen of the endoplasmic reticulum at concentrations approaching mM levels (49) and is responsible for the insertion of disulfides into folding proteins as well as correcting errors in disulfide formation (60). This process is achieved by a mechanism of thiol/disulfide exchange (60). PDI has two active sites each containing two cysteines separated by glycine and histidine (61–63). PDI also has been shown to act as a chaperone that binds to unfolded proteins and prevents their aggregation with other proteins (64–67). It acts as a subunit for prolyl 4-hydroxylase as well as microsomal triglyceride transfer protein (68). Because of the numerous functions...
of PDI, alteration or loss of activity because of alkylation or indirect perturbation in the redox environment by pyroles could rapidly endanger the homeostasis of the endothelial cell.

Peptide mapping of spot 4 indicated the presence of either the β- or γ-form of cytoskeletal actin but could not distinguish between the two. Previously we have shown, using SDS-polyacrylamide gel electrophoresis, Western blotting, and antibodies, that β-actin was a potential target for pyrrole adduct formation (69). Actin is important in the maintenance of the endothelial permeability barrier. Its contractile interactions with myosin regulates the endothelial permeability barrier and serves as an effector for inflammatory and procoagulant-induced vascular leak. At cell-cell adherens junctions, actin is linked to the plasma membrane through interactions with α-actinin, which associates with vinculin; vinculin is linked to catenins and plakoglobin, which both interact with membrane-associated cadherins. Cadherins in turn associate with cadherins from other cells through their extracellular domains. In the subendothelial matrix, fibronectin or vitronectin are bound to integrins, which bridge the plasma membrane and in turn bind through talin to vinculin, which is linked to α-actinin thus to actin filaments (70, 71). Alterations in actin or other proteins involved in the normal functioning of the endothelial barrier could result in extravascular leakage of plasma proteins and fibrin, which could stimulate vascular wall remodeling.

Studies with nonmuscle isoforms of cytoskeletal tropomyosin have shown it to be important in the process of actin filament formation and structure and in the defining of domains along the actin filament (72). Tropomyosin can block the bundling of actin by villin (73), the interaction of actin with filamin and α-actinin (74, 75), and inhibits fragmentation by gelsolin (76). In budding yeast, the loss of the single copy gene for tropomyosin results in the disappearance of cytoplasmic actin cables and reduced cell growth (77). These functions make tropomyosin essential for normal cell division, locomotion, and shape changes (72). This protein, like the actin it influences, could be essential for preservation of the osmotic barrier provided by functional endothelial cells and, along with DNA adducts, could potentially explain some of the observed perturbations observed in the cell cycle following MCTP treatment. Using methodologies similar to those employed in this study, acetalaminophen has recently been shown to form adducts with the hepatic derived cytoskeletal tropomyosin (37).

The limited number of proteins that are bound by MCTP suggests particular motifs that may be important for the binding of pyroles and thus narrows the search for other proteins that may be important to the toxicology of MCT. Only a limited number of proteins possess thioredoxin sites Cys-Xaa-Xaa-Cys (78). A few of these that were not observed in this study, which are present in endothelial cells, are fibronectin, thioredoxin, von Willebrand factor, and human RNA polymerase. Fibronectin, which has PDI activity, is an important component of the ECM and is necessary for endothelial integrity (79). The von Willebrand factor is produced by endothelial cells and plays an important role in platelet adherence to the interstitial matrix following endothelial damage (80). One of the manifestations of primary PH is the evidence of circulating platelet aggregations (11) and the presence of in situ thrombosis in pulmonary lesions (12, 13). Human RNA polymerase contains three Cys-Xaa-Xaa-Cys regions with one of these groups falling within a zinc finger. Perturbation in the transcription of DNA into RNA would have obvious consequences. Some of the 14C spots that remain to be identified could contain proteins with the Cys-Xaa-Xaa-Cys motif or accessible reactive cysteine(s). Additional proteins may be identified using different pH gradients and blotting conditions. The technique employed in this paper probably missed many of the higher molecular weight proteins because of the soaking technique employed and the gel composition of commercially available IGP strips (81, 82).

In the search for important molecular target(s) for MCTP, it should be considered that only small quantities of electrophile (MCTP) are essential to elicit pulmonary insult. Doses as low as 1 mg/kg, administered intravenously in N,N-dimethylformamide, were found to affect pulmonary function in rats (83). With a half-life in aqueous solutions of ~3 s (6), a significant portion of the dose would also be expected to be lost before exacting an effect. Therefore proteins that are abundant and structural in nature may not receive enough adduct to significantly alter cellular function. However, in the case of actin, adducts to actin alone may not present problems to the cell but actin cross-links to DNA may have more dire consequences (84). Proteins that are found in low levels or function in enzymatic or signaling processes are potentially better candidates. Alterations in a small percentage of the PDI pool may be amplified if adducted PDI can still participate in enzymatic processes resulting in the incorrect formation of disulfides or fails to act as a proper chaperone causing the aggregation of unfolded proteins. ER60 has previously been found to be associated with the internal nuclear matrix and participates in the anchorage of the DNA loops at the matrix (85). Such matrix DNA interactions have been shown to be important in the control of gene expression (86). Adducts that result in hyper-reactivity reactions may also be important. PDI has been implicated in halothane hepatitis along with other adducted proteins (87–89). Autoantibodies to PDI were detected in rats exposed to d-galactosamine and acetalaminophen or carbon tetrachloride when the latter two were combined with diethylmaleate (90). A similar mechanism may be relevant to MCTP toxicity.

The proteins observed to form covalent adducts with MCTP and their resulting partial or total loss of function could be important elements in the puzzling model MCT-induced PH. Further exploration of these proteins and their role in both chemically precipitated or natural occurring PH warrant future investigation.

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Protein Targets of Monocrotaline Pyrrole in Pulmonary Artery Endothelial Cells
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