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^{1}H NMR study of monocrotaline and its metabolites in human blood

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ABSTRACT

Monocrotaline (MCT) is a naturally occurring hepatotoxic pyrrolizidine alkaloid found in plants. This investigation is aimed at furthering the understanding of the role of blood in mediating the transport of MCT and its reactive metabolites in humans. Reactions of monocrotaline and its metabolites, dehydromonocrotaline (DHM), retronecine (RET) and dehydroretronecine (DHR) with human blood plasma, red blood cells (RBCs), and whole blood were studied *in vitro* by proton nuclear magnetic resonance spectroscopy. In plasma MCT remained intact and weakly associated with plasma proteins, and DHM was rapidly hydrolyzed releasing necic and lactone acids, and the reactive pyrrolic metabolite. MCT and its metabolite DHM were internalized in RBCs to the extent of 46.0% and 48.9% respectively in 30 min. No polymerization of DHR was observed when incubated with plasma and RBCs. The data clearly showed that both human plasma and RBCs could be the carriers for the transportation of MCT and its metabolites, DHM, RET and DHR between organs and could stabilise the reactive MCT metabolite DHR.

1. Introduction

Monocrotaline (MCT) is a phytotoxin and belongs to a class of pyrrolizidine alkaloids obtained from *Crotalaria spectabilis* and *Crotalaria retusa* (Smith and Culvenor, 1981). The toxicological effects of pyrrolizidine alkaloids are such that a significant risk to human health is presented on accidental exposure (Mohabbat et al., 1976; Tandon et al., 1976, 1978; Kumana et al., 1985; Zuckerman et al., 2002).

MCT is oxidized by cytochrome P450 in the liver to generate dehydromonocrotaline (DHM) which is considered to be its primary toxic metabolite (Fig. 1) (Mattocks, 1968). It has been reported that both DHM and its hydrolyzed product DHR, are hepatotoxic (Huxtable, 1989), pneumotoxic (Huxtable, 1990) and mutagenic (Roeder, 2000). The oxidizing process in the metabolism of MCT activates both C-7 and C-9 carbons of the necine ring that can then react with nucleophilic cellular macromolecules to give relatively stable products. Products of reaction with soluble nucleophilic substances may be excreted through bile or kidney (White, 1977; Eastman and Segall, 1982; Candrian et al., 1985; Lafranconi et al., 1985; Mattocks et al., 1991). However, products with tissue-bound nucleophilic groups, such as RNA, DNA and proteins containing thiol groups, may stay in the liver for a considerable time to express their toxicity (Lame et al., 1990; Yan and Huxtable, 1995). Polymerization can also occur to both DHM and DHR in the liver, because they have a half-life in aqueous solution of only a few seconds (Mattocks, 1978). Since dehydro-metabolites of MCT contain two electrophilic (C-7 and C-9) sites and one nucleophilic site (C-3), they can form polymers rapidly, a feature partially responsible for occlusion in blood veins in the liver.

MCT is reported to produce pathological injury not only to liver, but also to other organs. Several cases of pulmonary poisoning suggested that dehydro-PAs can escape into the bloodstream and be transported into the lung (Valdivia et al., 1967; Meyrick et al., 1980; Candrian et al., 1985; Shubat et al., 1987; Wilson et al., 1989; Wilson and Segall, 1990), heart (Lafranconi and Huxtable, 1983), kidney (Chan et al., 2003), pancreas (Hooper and Scanlan, 1977), skin (Johnson et al., 1978) and brain (Liu et al., 2010). The question arises how toxic metabolites, presumed to include DHM, are transported from the liver, where they are generated, to other organs. To address this question previous studies have strongly implicated RBCs playing an important role in facilitating the circulation, delivery and pathology of the toxins. After intravenous or subcutaneous administration of ^{14}C-labeled MCT ([^{14}C]MCT), Estep et al. (1990) reported that RBCs contained levels of radioactivity approximately 10-fold higher than that of plasma.

Abbreviations: DHM, dehydromonocrotaline; DHR, dehydroretronecine; HSA, human serum albumin; MCT, monocrotaline; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; RET, retronecine.

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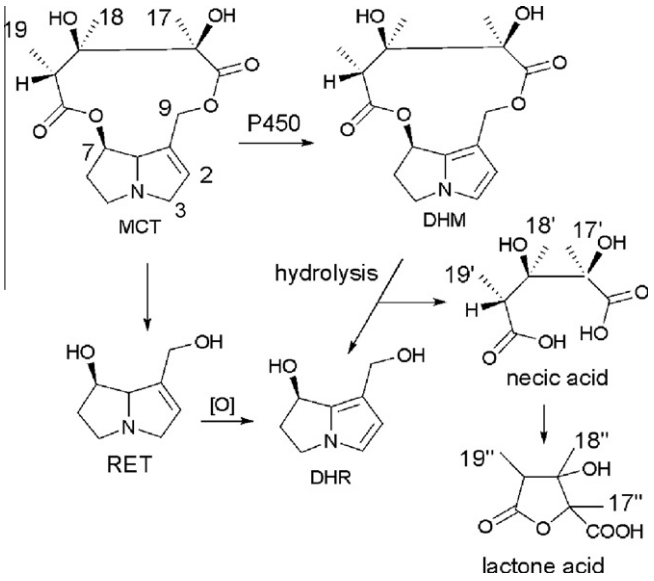

Fig. 1. Metabolic pathways of monocrotaline.

Studies on the tissue distribution and covalent binding of [^{14}C]DHM following intravenous administration in rats showed that the association of [^{14}C]DHM with RBC contents, such as ghosts, globins and heme played a role in the transport of this toxic metabolite of MCT (Lame et al., 1997). RBCs significantly facilitated the transport of toxic metabolites of MCT from the liver to the lung in tandem liver–lung preparations (Pan et al., 1991). The radio-labeled MCT metabolites were also observed to be capable of covalent interaction with lung tissue.

Increasing interest has been expressed in the application of nuclear magnetic resonance (NMR) spectroscopy in natural product analysis over the last 40 years. ^{1}H NMR spectroscopy has been proven to be one of the most useful and common methods employed to identify a variety of compounds in complex mixtures such as biological fluids, and for qualitative and quantitative determination of toxic compounds (Foxall et al., 1993a; Pauli et al., 2005). The technique was effective for the selective attenuation of signals associated with low molecular-weight mixtures of drug and endogenous metabolites in human urine and blood plasma (Foxall et al., 1993b). ^{1}H NMR spectroscopy has been employed for identification and quantitation of a wide range of xenobiotics that cause poisoning in humans, such as paraquat, tetrahydrofuran and salicylate, and their metabolites in biological fluids (Imbenotte et al., 2003). NMR can also be used to study the complexation of a ligand with a macromolecular target in solution for both tightly and weakly binding complexes (Fielding, 2003; Carlomagno, 2005). Observed lineshapes in NMR spectra can be used as indicators of complex formation. Perturbation of the NMR spectroscopic profile of the ligand in the presence of a protein target generally involves broadening of NMR ligand signals (Foster et al., 2007). NMR spectroscopy has advantages over other techniques in that it does not require separation and derivatisation procedures, the setting up of any target-specific assay, exhaustive and expensive isotope labeling of MCT and its metabolites, or pre-treatment of target proteins. To complement *in vivo* studies we have adopted the use of ^{1}H NMR spectroscopy to study the interaction of MCT and metabolites with blood, and blood components *in vitro*.

The present study investigates for the first time the state of MCT and its metabolites DHM, RET and DHR in the presence of blood plasma, red blood cells and whole human blood by ^{1}H-nuclear magnetic resonance spectroscopy. The uptake of MCT and DHM by red blood cells has also been estimated.

2. Materials and methods

2.1. Chemicals

Monocrotaline (MCT) isolated from *Crotalaria assamica* Benth was kindly supplied by Professor Zhiben Tu (Wuhan Botanical Institute, Academia Sinica, Wuhan PR China). The methods of preparation of RET, DHM and DHR were adopted from the literature (Culvenor et al., 1970; Hoskins and Crout, 1977; Mattocks et al., 1989). They were characterized by NMR and MS analysis, and the purity of all compounds was greater than 98% by HPLC. Human blood (anti-coagulant: citrate phosphate dextrose) was supplied by the UK National Blood Service. Human serum albumin (HSA) and 3-(trimethylsilyl) propionic-(2,2,3,3-d_4)-acid sodium salt (TSP-d_4) were from Sigma–Aldrich (Poole, UK). Dimethyl sulfoxide (DMSO) and d_6-dimethyl sulfoxide (d_6-DMSO) were obtained from Fisher Scientific (Loughborough, UK). Deuterium oxide (D_2O) was manufactured by Cambridge Isotope Laboratories Inc (Cheshire, UK). NMR Stock solutions of MCT (60 mM), DHM (60 mM), RET (130 mM) and DHR (60 mM) were prepared in d_6-DMSO.

2.2. Instrumentation

A Bruker Avance 500 MHz spectrometer (Bruker BioSpin, Germany) was used for all the NMR measurements in this study. 0.02% TSP-d_4 in D_2O was used as a reference (0.00 ppm). A 1D pulse sequence was used for water peak suppression, relaxation delay (2.00 s)-90°-fixed delay (10 μs)-90°-mixing time (100 ms)-acquisition time (2.34 s). The water resonances were irradiated during both relaxation time and mixing time. A total of 128 scans were acquired into 32 k data points. The spectral width was 14 ppm.

2.3. NMR study of plasma with MCT, DHM, RET and DHR

Plasma and RBCs were separated from human blood (10 mL) by centrifugation at 1000*g* for 5 min at 4 °C and aliquots of 565 μL of plasma were placed into four sample tubes. D_2O with 0.02% TSP-d_4 (100 μL) was added into each tube. ^{1}H NMR spectra of these four samples were recorded as negative controls. Then, 35 μL stock solutions of MCT, DHM, RET and DHR were added into separate tubes to final concentrations 3.0, 3.0, 6.5 and 3.0 mM respectively. ^{1}H NMR spectra were recorded again. All samples were incubated at 37 °C overnight and ^{1}H NMR measurements were repeated the next day.

2.4. NMR study of human serum albumin (HSA) with DHR

HSA (0.067 g) was dissolved in 1 mL PBS buffer as the stock solution. 70 μL of HSA stock solution was diluted up to 700 μL, followed by 100 μL D_2O. The sample was analyzed as negative control by ^{1}H NMR. DHR stock solution was added into the HSA solution to give a ratio of 50:1. The mixture was analyzed by ^{1}H NMR immediately and at every hour up to 11 h at 37 °C.

2.5. NMR study of RBCs with MCT, DHM, RET and DHR

RBCs were removed from 20 mL human blood by centrifugation at 1000*g* for 5 min at 4 °C and washed four times with 3 mL phosphate-buffered saline (PBS) solution. In five sample tubes 700 μL RBCs (4.27×10^{10} RBCs/mL counted by hemocytometer) were suspended in 700 μL fresh PBS buffer. RBCs were incubated with 35 μL of MCT, DHM, DHR, RET or d_6-DMSO at 37 °C for 30 min and then washed four times with 700 μL PBS. The supernatant and fourth washings were taken for ^{1}H NMR analysis. To extract contents, RBCs were subjected to freeze–thawing, centrifuged and 700 μL of the supernatant was transferred to an NMR tube. 100 μL D_2O with 0.02% TSP-d_4 (100 μL) was added into each tube for NMR analysis.

2.6. NMR study of MCT and DHM in whole blood

Human blood (10 mL) was mixed gently and three aliquots of 1.4 mL were transferred into NMR sample tubes. One was treated with 70 μL d_6-DMSO in 700 μL PBS buffer as negative control and the other two with 70 μL of MCT and DHM stock solution respectively. All three samples were incubated at 37 °C for 30 min. Then, plasma was separated from RBCs and was analyzed by ^{1}H NMR. The RBCs were washed four times with 700 μL PBS. The supernatant and fourth washings were submitted for ^{1}H NMR analysis. The RBC contents were extracted by freeze–thawing and 700 μL of each sample was removed and transferred into NMR tubes. D_2O with 0.02% TSP-d_4 (100 μL) was added into each tube.

2.7. Time study of MCT and DHM with RBCs

RBCs were removed from 30 mL of human blood and washed with (4×3 mL) PBS buffer solution. 700 μL of RBCs were suspended in 700 μL fresh PBS buffer in 8 sample tubes. A time study of MCT in the RBCs was carried out by incubating RBCs with four MCT stock solutions as reaction set and four DMSO solutions as control set at 37 °C. At each incubation time point of 0.5, 1.0, 1.5 and 2.0 h, one sample of reaction set as well as the control set was taken out and gently conducted under