Journal of Pharmaceutical and Biomedical Analysis 121 (2016) 174–180

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Identification of metabolites of selected benzophenanthridine alkaloids and their toxicity evaluation

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ARTICLE INFO

Article history:
Received 27 October 2015
Received in revised form 11 January 2016
Accepted 12 January 2016
Available online 15 January 2016

Keywords:
Benzophenanthridine alkaloids
Metabolism
LC MS
Microsomes
Biotransformation

ABSTRACT

Selected benzo[c]phenathridine alkaloids were biotransformed using rat liver microsomes and identified by liquid chromatography and mass spectrometry. While the metabolites of commercially available sanguinarine and chelerythrine have been studied in detail, data about the metabolism of the minor alkaloids remained unknown. Reactions involved in transformation include single and/or double *O*-demethylation, demethylenation, reduction, and hydroxylation. Two metabolites, when isolated, purified and tested for toxicity, were found to be less toxic than the original compounds.

1. Introduction

Quaternary benzo[c]phenanthridine alkaloids (QBAs) belong to a group of isoquinoline alkaloids widely distributed in the family Papaveraceae and, to a limited extent, in some species of the families Fumariaceae and Rutaceae. Sanguinarine (SA) and chelerythrine (CHE) are the best-known QBAs. In some plants, SA and CHE are accompanied by less-abundant pentasubstituted derivatives sanguilutine (SL), chelilutine (CL), sanguirubine (SR), chelirubine (CR), and hexasubstituted macarpine (MA) (Fig. 1).

The effects of QBAs on biological systems have been studied extensively, especially for commercially available SA and CHE. The results have been summarized in several reviews [1–4]. Research has primarily focused on antitumor effects of both alkaloids, but they also display antimicrobial, antifungal and anti-inflammatory activities. They may affect many targets on the molecular level and modify the activity and function of a wide variety of enzymes. The fractions rich in QBAs from *Sanguinaria canadensis* or *Macleaya cordata* have been used in toothpastes and mouthwashes as antiplaque agents [1,3].

While it seems that low concentrations of QBAs have no observable toxic effects [5–7], epidemiological case studies have shown an association between SA use and oral premalignant lesions [8,9] and a study with higher doses of SA discovered that SA displayed signs of significant liver damage in mice [10]. Further, it has been shown that SA creates complexes with DNA which could potentially lead to the changes in DNA structure and subsequently affect cell viability [11]. From a medical standpoint, the most interesting is the ability of QBAs to cause apoptosis. While SA causes apoptosis in cancer and normal cells alike, a few studies have shown that cancer cells are significantly more sensitive to the effects of SA than normal cells [12,13]. In contrast, Debiton et al. did not find any difference in effect of SA between cancer and normal cells [14]. Still, because of their medicinal potential, both SA and CHE have been substantially studied in this respect [15–18]. Nevertheless, the exact biological paths by which both substances affect cells are still not entirely known. Although the commercially available SA and CHE have been extensively studied, data about the effects of other minor QBAs on cells are still limited. For instance, it has been shown that MA and SR show a considerable fluorescence shift when bound to DNA, a property, which make them useful as fluorescent probes [19]. While

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http://dx.doi.org/10.1016/j.jpba.2016.01.024
0731-7085/

SL and CL exhibit antiproliferative and anti-microtubular activities [20], SR, CR, and MA are antiproliferative and pro-apoptotic, which has been well documented [21]. SL has been found to preferentially induce necroptosis over apoptosis in melanoma cells [22]. Their limited availability is the major challenge to be overcome when assessing their full potential and/or risks.

One of the reasons for inconsistent results of various studies could be the difference in metabolism of QBAs by different cells. There are substantial differences in the metabolism and enzymes present even between various healthy cell types and tissues, which can lead to different metabolite profiles. This could explain why some studies found a significant ROS production, leading to apoptosis [23–25] while others found no increase in ROS production when studying the pro-apoptic properties of QBAs [14,26]. The rapid apoptotic response induced by a glutathione depletion effect could be a part of a mechanism of SA cytotoxicity [14].

Plant cells producing QBAs are able to reabsorb SA and CHE reducing it to dihydro derivatives, thereby protecting themselves from their own product of synthesis [27]. It seems that also in animals and humans the first step in biotransformation of CHE and SA is their conversion to the respective dihydro derivatives. Psotova et al. identified in experiments with rats dihydro SA (DHSA) as the main metabolite in liver and plasma after the oral administration of SA [28]. Kosina et al. showed that formation of the dihydro-metabolites, which may be followed by specific *O*-demethylenation/*O*-demethylation processes, is probably the main route of biotransformation (detoxification) of the benzo[c]phenanthridines in human hepatocytes [29]. Deroussent et al. studied oxidative biotransformation of SA using eight human recombinant CYPs; however, only CYP1A1 and CYP1A2 displayed any metabolic activity. Up to six metabolites were detected. The main metabolite M2 (*m*/*z* 320) resulted from a ring-cleavage of SA followed by demethylation; 5,6-dihydrosanguinarine was the prominent derivative formed from SA in cells expressing no CYP [30].

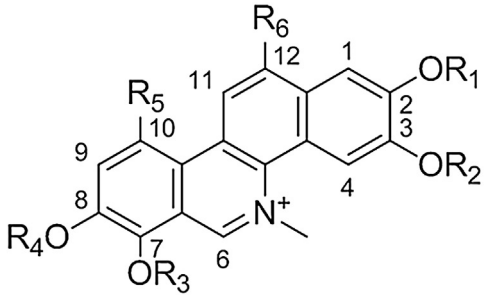
The enzyme cofactors NADH and NADPH can participate in the reduction of SA to DHSA in cells, and this conversion has been demonstrated *in vitro* by several authors in the absence of any enzyme [29,31,32]. Wu et al. demonstrated that the SA iminium bond can be reduced by rat liver microsomes and cytosol in the absence of NAD(P)H. The reductase activity of both fractions was significantly enhanced in the presence of NADH. The amount of DHSA formed in liver cytosol was 4.6-fold higher in the presence of NADH. In this case, NADH was more effective than NADPH as an electron donor for the SA iminium bond reduction [33]. These observations indicated that cytosolic enzymes are highly likely to be involved in SA reduction in the rat liver. DHSA and dihydrochelerythrine (DHCHE) was proved in other animal cells, however; details of such processes have not yet been clarified [28–30,32,34].

While the metabolic biotransformation of SA has been well studied [35], the exact properties of many of its metabolites are not known. Without the knowledge of the metabolic fate of QBAs and properties of their metabolites, it is impossible to understand or predict the potential therapeutic options and side effects. Vacek et al. have performed a well-designed study where they have produced both phase I and II metabolites of CHE and DHCHE *in vitro* using human hepatocytes [36]. Authors tried testing cytotoxicity of DHCHE and *O*-demethylated CHE (DMCHE); however, both were prepared *in vitro* by reduction with $NaBH_4$. As enzymatic, stereoselective *O*-demethylation of CHE might provide two isomers, C8 DMCHE (fagaridine) and C7 DMCHE (NK 109 or isofagaridine), they could correspondingly differ in toxicity [37]. Metabolic studies of minor QBAs have not been yet performed and herein presented approach comprising of drug biotransformation, isolation of its metabolites, and (cyto-) toxicity assessment is required for their comprehensive study.

2. Materials and methods

2.1. Reagents and materials

Methanol (MeOH, p.a.) was obtained from Penta (Czech Republic). Phosphoric acid (p.a.), formic acid (LC MS), ammonium hydroxide (28–30% NH_3 in water), sodium formate (LC MS), Tris, KCl, sucrose, EDTA, NADP, glucose-6-phosphate, $MgCl_2$, glucose-6-phosphate dehydrogenase, fetal bovine serum, glutamine, penicillin, streptomycin, MeOH (LC MS), acetonitrile (ACN, HPLC grade, LC MS) and dimethylsulfoxide (DMSO, p.a.) were purchased from Sigma–Aldrich (Czech Republic). All water used was of



Alkaloid	R_1	R_2	R_3	R_4	R_5	R_6
Sanguinarine (SA)	$-CH_2-$		$-CH_2-$		-H	-H
Chelerythrine (CHE)	$-CH_2-$		$-CH_3$	$-CH_3$	-H	-H
Sanguilutine (SL)	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-OCH_3$	-H
Chelilutine (CL)	$-CH_2-$		$-CH_3$	$-CH_3$	$-OCH_3$	-H
Sanguirubine (SR)	$-CH_3$	$-CH_3$	$-CH_2-$		$-OCH_3$	-H
Chelirubine (CR)	$-CH_2-$		$-CH_2-$		$-OCH_3$	-H
Macarpine (MA)	$-CH_2-$		$-CH_2-$		$-OCH_3$	$-OCH_3$

Fig. 1. Benzo[c]phenanthridine alkaloid structures.