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Sanguinarine is reduced by NADH through a covalent adduct

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ABSTRACT

Sanguinarine is a benzo[c]phenanthridine alkaloid with interesting cytotoxic properties, such as induction of oxidative DNA damage and very rapid apoptosis, which is not mediated by p53-dependent signaling. It has been previously documented that sanguinarine is reduced with NADH even in absence of any enzymes while being converted to its dihydro form. We found that the dark blue fluorescent species, observed during sanguinarine reduction with NADH and misinterpreted by Matkar et al. (Arch. Biochem. Biophys. 2008, 477, 43–52) as an anionic form of the alkaloid, is a covalent adduct formed by the interaction of NADH and sanguinarine. The covalent adduct is then converted slowly to the products, dihydrosanguinarine and NAD^+, in the second step of reduction. The product of the reduction, dihydrosanguinarine, was continually re-oxidized by the atmospheric oxygen back to sanguinarine, resulting in further reacting with NADH and eventually depleting all NADH molecules. The ability of sanguinarine to diminish the pool of NADH and NADPH is further considered when explaining the sanguinarine-induced apoptosis in living cells.

1. Introduction

Sanguinarine (SA) belongs to a family of plant secondary metabolites called quaternary benzo[c]phenanthridine alkaloids (QBAs). These compounds have been extensively studied for their numerous biological activities, such as antitumor, antimicrobial, antifungal, and anti-inflammatory. While the results have been summarized in several works, the greatest attention is given to the anticancer activity of QBAs (Slaninova et al., 2014; Gaziano et al., 2016). It has been reported that SA is *in vitro* cytotoxic preferentially toward cancer cells than normal cells at concentrations that are comparable to those of the current clinically used anticancer agents (Ahmad et al., 2000). Under physiological conditions, a hydroxide anion (OH^-) is reversibly attached to the iminium bond of SA to give a 6-hydroxy product called an alkanolamine or a pseudobase (Fig. 1). The alkanolamine form, which is a nonpolar uncharged molecule, can easily enter a cell to establish a new, pH-dependent equilibrium between the iminium and alkanolamine form inside the cell. The alkaloid toxicity depends on the ability of the planar, charged quaternary form of SA to produce a stable complex with DNA, which subsequently could affect the cell viability (Slaninova et al., 2001; Vacek et al., 2011). Treatment of cells with SA led to a rapid production of reactive oxygen species (ROS) (Burgeiro et al., 2013), fast and severe glutathione depletion (Debiton et al., 2003), oxidative DNA damage and very rapid apoptosis that was not mediated by p53-dependent DNA damage signaling (Matkar et al., 2008a; Hammerova et al., 2011). The first step in the metabolism of SA in rat liver is the reduction of the quaternary form to dihydrosanguinarine (DHSA) (Fig. 1). The conversion might be mediated by several NAD(P)H dependent oxidoreductases (Deroussent et al., 2010; Wu et al., 2013). In cell cultures of *Eschscholzia californica*, SA is reabsorbed and reduced to DHSA by sanguinarine reductase, which was isolated (Weiss et al., 2006) and characterized (Vogel et al., 2010). Additionally, it has been observed that SA underwent the conversion to its inactive reduced form even when incubated with NADH in the absence of any enzyme (Kovar et al., 1986; Matkar et al., 2008b), however; physiologically important reducing agents, such as glutathione and L-ascorbic acid,

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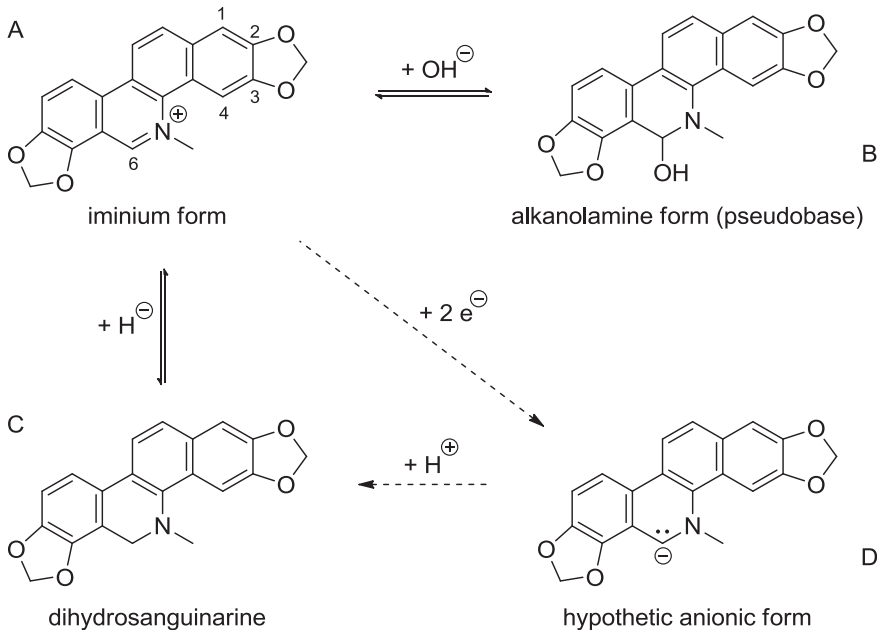

Fig. 1. A molecule of sanguinarine in A) an iminium (a quaternary) form, B) an alkanolamine form, C) dihydrosanguinarine, and D) a hypothetic anionic form suggested by Matkar et al. (2008b). The iminium form exhibits yellow fluorescence while the forms B and C glow dark blue under the UV light.

were unable to effectively reduce SA (Kosina et al., 2011). On top of that, SA, as well as other QBAs, may undergo a complex series of metabolic changes, some of which may contribute to their biological effects (Sandor et al., 2016).

When studying a relationship between the cell death and the ability of SA to deplete the cellular antioxidant capacity Matkar et al. (2008b) observed a novel, dark blue fluorescent, anionic form of SA formed by the reduction with NADH in the absence of any enzyme. The authors detected a new form of SA by reversed phase thin layer chromatography (RP-TLC) and determined the negative charge of species by gel electrophoresis. While a new form of SA would be somewhat fascinating, an alternative explanation of these findings could be presented and interpreted.

In this study, we have employed chromatographic and spectrometric techniques to give a novel interpretation of SA being reduced with NADH to DHSA. We found that the dark blue fluorescent anion produced during SA reduction with NADH, originally observed by Matkar et al. (2008b), is a covalent adduct formed by the reaction of NADH and SA. The adduct is then converted slowly to products, DHSA and NAD^+, in the second step of reduction. DHSA is continuously re-oxidized back to SA by the atmospheric oxygen resulting in eventual NADH depletion.

2. Results

2.1. Reduction of SA with NADH forms an unexpected spot on thin-layer chromatography

First, we have aimed to reproduce the RP-TLC performed by Matkar et al. (2008b). Resulting chromatograms might be seen in Fig. 2. The retention factor (*Rf*) for NADH was one as it traveled along with the polar mobile phase with no retention on the non-polar solid phase. Visualization under UV light (340 nm) showed a fluorescence band of NADH broadened over the entire chromatogram concentrating at the head. The same band at the head of the chromatogram was clearly visible in the line where NADH was mixed with SA. This was in accordance with our expectations as the NADH molecule was negatively charged and thus highly polar. Dark blue fluorescence was clearly visible in the lines where SA was mixed with NADH (*Rf* ~ 0.75) and with $NaBH_4$ (*Rf* ~ 0.35). After four hours in dark, both dark blue spots turned their fluorescence to the same orange-yellow color as it has been observed in the sole SA line (Fig. 2B). A lower *Rf* of the dark blue spot in the SA + $NaBH_4$ line allowed for identification as DHSA as it was characterized by blue fluorescence and a decrease in polarity, which had resulted from a loss of the charged quaternary form. The same DHSA band would have been observed in the SA + NADH line if the mixture had been applied onto a TLC plate after a longer time (data not shown). A spot between SA and DHSA (*Rf* ~ 0.45) observed at lines of pure SA and SA + NADH probably represented an alkanolamine form of SA. Another dark blue fluorescent spot in the SA + NADH line with *Rf* ~0.75 could not have been attributed to any known species of SA and was then studied comprehensively by liquid chromatography with mass spectrometric detection (LC-MS).

2.2. Sanguinarine forms a covalent adduct with NADH

LC-MS was employed as a next step in revealing the origin of the interaction between SA and the NADH molecule. A representative chromatogram of an LC-MS method at very similar conditions to those used for RP-TLC might be seen in Fig. 3. The product, formed from the reaction between SA and NADH, was then expected to elute around the tenth minute. Detection at 280 nm and by MS showed a few distinct peaks around that retention time. In order to be able to identify the product, positive mode MS and MS/MS spectra were acquired (Fig. 4). Peaks observed in Fig. 4 might be accordingly assigned to the pure reactants (SA at 332 Da and NADH at 666 Da) as well as to the product of their reaction (997 Da for a singly charged and 499 Da for a doubly charged ion). The MS/MS spectrum of the doubly charged ion produces only SA as a major fragment. This fragmentation might be explained by a favorable formation of an aromatic quaternary form of SA and a neutral residue of NADH. In other words, SA is a good leaving fragment while retaining its charge as it contains quaternary nitrogen. In negative mode, the adduct was observed at 995 Da (data not shown) indicating that the mass of the neutral species was 996 Da.