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Original article

Quantitative assessment of the metabolic activation of alicyclic amines via aldehyde

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ABSTRACT

Introduction: Recently it has been reported that some drugs that produce reactive intermediates may cause clinical adverse effects following covalent binding to biomacromolecules. For example, Schiff base production mediated by aldehyde is a possible mechanism of drug–protein adducts. However, because thiols do not trap aliphatic aldehydes via hemiacetal or hemiaminal, the glutathione-trapping method cannot be used to determine the covalent bindings of the Schiff base. **Methods:** We established a quantitative method to determine covalent binding mediated by aldehydes via hemiaminal or hemiacetal using non-radiolabeled compound and [^{14}C]semicarbazide as a hard-trap agent with unique post-incubation. **Results:** The trapped aldehyde obtained from the post-incubation was almost equivalent to the covalent binding of the radiolabeled tool compound. Our novel method showed its usefulness in quantitative detection of aldehyde's covalent binding ability by several reagents with alicyclic amine and launched drugs as control. **Discussion:** The post-incubation method is useful for screening newly synthesized compounds to quantitatively assess the bioactivation of aldehydes descending from alicyclic amines.

1. Introduction

Idiosyncratic adverse drug reactions (IADRs) are adverse drug reactions which occur in only a small percentage of the population, but are often serious and account for many drug-induced deaths. The IADRs include hepatotoxicity, hematological toxicity, and cutaneous reactions (Park, Pirmohamed, & Kitteringham, 1992). They typically have a delayed onset of weeks to months following the initial exposure to a drug (Uetrecht, 1999, 2000). Since the mechanisms of IADRs remain poorly understood (Liguori and Waring, 2006; Ju et al., 2002), it is currently impossible to accurately predict which new drugs will be associated with a significant incidence of IADRs, and this poses a significant challenge in drug discovery/development (Park et al., 1992; Bauman et al., 2009). Several hypotheses have been proposed (Shaw, Ganey, & Roth, 2009; Shenton, Popovic, Chen, Masson, & Uetrecht, 2005), and drugs involved in IADRs produce reactive intermediates which play important roles in occurring IADRs (Kalgutkr et al., 2005; Evans, Watt, Nicoll-Griffith, & Baillie, 2004; Zhou, Chan, Duan, Huang, & Chen, 2005). For example, idiosyncratic hepatotoxicity may be due to direct toxicity of a chemically reactive metabolite or secondary to an immune reaction (Park et al., 1992). In cases of fatal IADRs, liver injury is the most frequent cause for drug withdrawal, and accounts for 50% of the cases of acute liver failure mimicking all forms of acute and chronic liver dysfunction (Abboud and Kaplowitz, 2007).

Among such reactive intermediates, those which bind easily and covalently to biomacromolecules may cause idiosyncratic reactions (Evans et al., 2004). Thus, especially when reactive intermediates are suspected of binding covalently to proteins or other biomacromolecules, such new drug candidates should be assessed carefully before phase I clinical studies.

One of the most common screening ways for reactive intermediates is glutathione-trapping method using non-protein thiols (glutathione, glutathione ester or cysteine) to trap reactive intermediates, such as quinones, quinone imines, epoxides, nitrenium ions, arene oxides or Michael acceptors because these thiols can scavenge many kinds of reactive intermediates (Kalgutkr et al., 2005; Soglia et al., 2004; Zhou et al., 2005). However, glutathione-trapping methods cannot be used to determine the covalent binding with proteins in a Schiff-base form, for example, for reactive intermediates such as aliphatic aldehydes because these aldehydes are slowly generated via hemiacetal or hemiaminal and thus not trapped by soft-trap agents such as non-protein thiols (Evans et al., 2004; Xu et al., 2005).

Aldehyde-mediated Schiff base production is one of the possible mechanisms of drug–protein adducts (Evans et al., 2004; Xu et al., 2005). Hemiacetal- or hemiaminal-form metabolites from cyclic compounds gradually decompose to ring-opened aldehyde form under physiological conditions (Xu et al., 2005; Zhang, Naue, Arison, & Vyas, 1996). The aldehydes, hemiacetals or hemiaminals are relatively stable and may be distributed to systemic circulation (Xu

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et al., 2005; Zhang et al., 1996). The aldehydes may produce Schiff bases with biomacromolecules by a similar mechanism to acyl-glucuronidation and may cause some adverse effects (Bolze, Bromet, Gay-Feutry, Massiere, Boulieu, & Hulot, 2002). It is known that aliphatic aldehydes resulting from cyclic hemiacetal or hemiaminal produce stable adducts to semicarbazide (Chauret et al., 1995; Zhang et al., 1996; Xu et al., 2005). To our knowledge, however, aldehyde-mediated covalent binding has not been reported as a quantitative analysis but only as a qualitative analysis (Xu et al., 2005). Unless a radiolabeled drug is prepared prior to assessment, it is impossible to accurately determine the covalent binding of aldehydes generated from new molecular entities of alicyclic amine structure (Evans et al., 2004). Therefore, a new quantitative method without the use of a radiolabeled test compound is necessary to assess covalent binding mediated by aldehydes generated via hemiacetal or hemiaminal.

In the present study, we first incubated an alicyclic amine alone in a typical liver microsome system, and post-incubated to optimally trap reactive aldehyde with radiolabeled semicarbazide. Then, after the adduct (i.e., trapped aldehyde) was extracted using solid phase extraction (SPE), the extract was quantitatively determined by using high-performance liquid chromatography–radiometric detection system (HPLC–RAD) or a liquid scintillation counter (LSC). Application of this method to various related compounds has proven to be that it is a useful tool for the screening of covalent binding generated from newly synthesized compounds with alicyclic amine moiety.

2. Methods

2.1. Radiolabeled materials

[^{14}C]Semicarbazide (Fig. 1a) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), accompanied by a certificate of analysis confirming the radiochemical purity (99%) and specific activity (40 mCi mmol^{-1}). [^{14}C]compound X (Fig. 1b) was synthesized by Amersham Biosciences Corp. (Piscataway, NJ, USA) accompanied by a certificate of analysis confirming the radiochemical purity (>98.5%) and specific activity (23.5 mCi mmol^{-1}). All radiolabeled materials were stored at −80 °C in the dark.

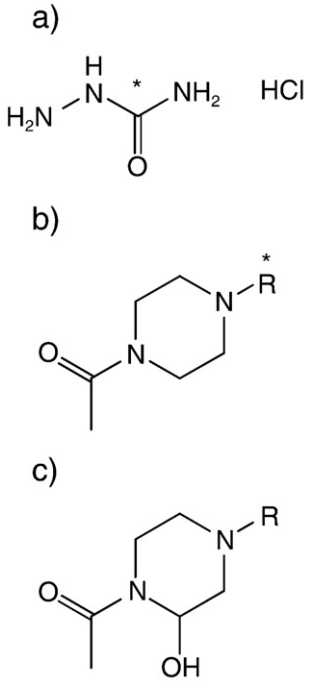

Fig. 1. Chemical structures of semicarbazide hydrochloride a), compound X b) and M1 c). *: ^{14}C-labeled position.

2.2. Chemicals

Authentic standards of compound X, its hydroxylated metabolite M1, compound Y and compound Z were synthesized at Kyorin Pharmaceutical Co., Ltd. (Tochigi, Japan). Non-radiolabeled semicarbazide was purchased from Sigma Chemical (St. Louis, MO, USA). All alicyclic amines were purchased from Bionet (Murcia, Spain), Maybridge (Cornwall, UK) or Enamine (Kiev, Ukraine). Dog liver microsome and rat liver microsome were purchased from XenoTech (Kansas City, KS, USA). Sildenafil and loratadine were purchased from LKT Laboratories (St. Paul, MN, USA). Aprepitant was purified from Emend® capsules purchased from Merck & Corp., Inc. (Whitehouse Station, NJ, USA).

2.3. Optimization experiments

2.3.1. Measurement covalent binding of [^{14}C]compound X

Experiments to measure the covalent binding of compound X were performed by a general microsomal incubation system. Microsomal samples were prepared in duplicate using 10 μM [^{14}C]compound X, 1 mg mL^{-1} rat liver microsomal or dog liver microsomal protein, potassium phosphate buffer 100 mM, $MgCl_2$ 5 mM, β-Nicotineamide adenine dinucleotide phosphate (β-NADPH) 1 mM, with or without 1 mM glutathione (soft-trap reagent), or, with or without 1 mM semicarbazide (hard-trap reagent) in a final volume of 200 μL in a micro-lab tube. After pre-incubation at 37 °C for 5 min, a metabolic reaction was initiated by the addition of β-NADPH. The micro-lab tubes were incubated at 37 °C for 1 h, and the incubation was terminated by the addition of 1 mL of ice-cold acetonitrile. After centrifugation for 5 min at 10,600×*g*, the precipitated protein pellet was washed with 0.8 mL of 80% methanol by sonication. The protein pellet was washed four times. The residue was dissolved in 1 mL of tissue solubilizer and mixed with 10 mL of liquid scintillant, Hionic-flour™ (PerkinElmer Inc., Waltham, MA, USA), to prepare a scintillation cocktail (final scintillation cocktail). The covalent binding of compound X was calculated from the residual radioactivity of the pellet.

2.3.2. Post-incubation (using [^{14}C]compound X)

To optimize the post-incubation method, the same microsomal incubation system without any trap reagents was used. After the termination of incubation and the centrifugation, 180 μL of the upper layer was transferred to another micro-lab tube for the following post-incubation procedure. Post-incubation at 37 °C was initiated by the addition of 5 mM semicarbazide and incubated for 0, 1, 2, 4, 6 or 24 h. After each of these periods, the samples were subjected to centrifugation for 10 min at 10,600×*g*. The supernatants were analyzed by a high-performance liquid chromatography–radiometric detection system (HPLC–RAD) to determine the production of the semicarbazide adduct of reactive metabolite descendant from [^{14}C]compound X. The post-incubation time was optimized in this procedure.

2.3.3. Co-incubation (using [^{14}C]semicarbazide)

Experiments to detect the adduct of compound X with [^{14}C] semicarbazide during co-incubation were performed in the same microsomal incubation system in triplicate using cold compound X in a final volume of 500 μL. After the termination by cooling on ice, 500 μL of ice-cold distilled water was added to the micro-lab tube to prepare 1 mL of crude sample solution for the following cleanup step (SPE procedure, Fig. 2). The micro-lab tubes were subjected to centrifugation for 10 min at 10,600×*g*, and the supernatants were collected. Then, 900 μL of the supernatant was applied to the SPE column primed with 3 mL of methanol and 3 mL of distilled water. The solid phase cartridges were flashed with 1 mL of distilled water