Metabolism of Boswellic Acids In Vitro and in Vivo

Phillip Krüger, Rambod Daneshfar, Gunter P. Eckert, Jochen Klein, Dietrich A. Volmer, Ute Bahr, Walter E. Müller, Michael Karas, Manfred Schubert-Zsilavecz, and Mona Abdel-Tawab

Central Laboratory of German Pharmacists, Eschborn, Germany (P.K., M.S.-Z., M.A.-T.); Institutes of Pharmacology (G.P.E., J.K., W.E.M.) and Pharmaceutical Chemistry (U.B., M.K., M.S.-Z.), J. W. Goethe-University, Center for Drug Research, Development and Safety, Frankfurt, Germany; Institute for Marine Biosciences, National Research Council, Nova Scotia, Halifax, Canada (R.D.); and Medical Research Council, Department of Human Nutrition Research, Cambridge, United Kingdom (D.A.V.)

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ABSTRACT:

*Boswellia serrata* resin dry extract is among the few herbal remedies designated with an orphan drug status for the treatment of peritumoral brain edema. In addition, boswellic acids (BAs), the main active ingredients of *B. serrata* extracts, have potent anti-inflammatory properties, and may represent promising agents for the treatment of inflammatory diseases. Pharmacokinetic studies, however, revealed poor bioavailability, especially of 11-keto-β-boswellic acid (KBA) and 3-acetyl-11-keto-β-boswellic acid (AKBA), the most potent BAs. To address the question of whether BAs are extensively metabolized, we determined the metabolic stability of KBA and AKBA in vitro, investigated the in vitro metabolism of BAs, and compared the metabolic profiles of KBA and AKBA with those obtained in rats in vivo. In rat liver microsomes and hepatocytes as well as in human liver microsomes, we found that KBA but not AKBA undergoes extensive phase I metabolism. Oxidation to hydroxylated metabolites is the principal metabolic route. In vitro, KBA yielded metabolic profiles similar to those obtained in vivo in rat plasma and liver, whereas no metabolites of AKBA could be identified in vivo. Furthermore, AKBA is not deacetylated to KBA. This study indicates that the efficacy of *B. serrata* extract may be enhanced by increasing the bioavailability of AKBA.

Pentacyclic triterpenes have gained significant importance because their chemical structures resemble those of steroids. In particular, boswellic acids (BAs), the main active components of the gum resin extract of *Boswellia serrata* (Salai guggal), have received considerable attention. Based on positive data on the treatment of peritumoral brain edema accompanying gliomas (Winkling et al., 2000), the European Medicines Agency designated an orphan drug status to *B. serrata* dry resin extract in 2002, thus boosting the status of *B. serrata* extract as an herbal remedy.

Traditionally, *B. serrata* extract is used in Indian ayurvedic medicine for the treatment of inflammatory and arthritic diseases (Gupta et al., 1998; Culioli et al., 2003). BAs possess potent anti-inflammatory properties by inhibiting 5-lipoxygenase, human leukocyte elastase and the nuclear factor-κB pathway, without exerting the adverse effects known for steroids (Safayhi et al., 1992, 1997; Gupta et al., 1997; Syrovets et al., 2005; Poeckel and Werz, 2006). Among the six most important derivatives of BAs (see Fig. 4), KBA and AKBA are the most potent inhibitors of 5-lipoxygenase with IC₅₀ values of 2.8 and 1.5 μM, respectively (Safayhi et al., 1992; Sailer et al., 1996). In several clinical trials the efficacy of *B. serrata* extract was comparable with that of sulfasalazine and mesalazine for the treatment of Crohn’s disease and ulcerative colitis, with a risk-benefit analysis in favor of BAs (Gupta et al., 1997, 2001; Gerhardt et al., 2001). Efficacy, however, could not be clearly demonstrated for other inflammatory diseases such as asthma and polyarthritis (Gupta et al., 1998; Sander et al., 2005). Moreover, *B. serrata* extract also exhibited antiproliferative and cytotoxic effects (Poeckel and Werz, 2006).

Preliminary pharmacokinetic studies found only very low concentrations of KBA in human plasma after oral administration of *B. serrata* extract, ranging from 0.17 μM after a single dose administration of 786 mg (Sterk et al., 2004), to 1.6 μM after taking 1600 mg (Abdel Tawab et al., 2001), to 2.7 μM subsequent to the intake of 333 mg (Sharma et al., 2004). AKBA, the most potent BA, was determined at a concentration of 0.1 μM after a multiple-dose administration of 786 mg of *B. serrata* extract (Büchele and Simmet, 2003). In the pharmacokinetic study carried out by Sharma et al. (2004), AKBA was not detected in plasma, possibly because of the deacetylation of AKBA to KBA in vivo. In rats dosed with 240 mg/kg of *B. serrata* extract, plasma levels of KBA and AKBA were determined to be 0.4 and 0.2 μM, respectively, whereas they reached concentrations of 0.3 μM in the brain (corresponding to 99 and 95 ng of KBA and AKBA per g of brain, respectively) (Reising et al., 2005). The studies mentioned above clearly suggest a substantial potential of BAs for the treatment of inflammatory diseases and central nervous system malignancies, if sufficient systemic concentrations can be achieved. Among many factors affecting bioavailability, poor absorption,
and/or extensive metabolism may play a crucial role in limiting the systemic availability of BAs. The present study will focus on the contribution of hepatic metabolism to the low bioavailability observed with KBA and AKBA. To date, no data describing the metabolism of pentacyclic triterpenes, including BAs, is available. To our knowledge, only the metabolism of oleandric acid, leading to the formation of hydroxylated metabolites upon incubation with rat liver microsomes, has been described (Jeong et al., 2007). Furthermore, the fate of the most potent BA, AKBA, is not known. It is unclear whether AKBA is predominantly deacetylated to the pharmacologically active KBA or whether it is metabolized via other pathways. Based on the particular therapeutic importance of B. serrata extracts, the present study has three objectives: first, to investigate the metabolic stability of KBA and AKBA in rat liver microsomes (RLM), rat hepatocytes, and human liver microsomes (HLM); second, to determine whether AKBA is deacetylated to KBA in vivo; and finally, to identify the metabolites of BAs in vitro and to compare the in vitro metabolic profiles of the most potent BAs, KBA and AKBA, with those in rat plasma, liver, and brain after oral administration.

Methods and Materials

Chemicals and Reagents. Boswellic acids [α-boswellic acid (αBA), (3α)-3-hydroxy-olean-12-en-23-oic acid; β-boswellic acid (βBA), (3α,4β)-3-hydroxyurs-12-en-23-oic acid; 3-acetyl-α-boswellic acid (AcBA), (3α)-3-acetoxy-olean-12-en-23-oic acid; 3-acetyl-β-boswellic acid (AcβBA), (3α)-3-acetoxy-urs-12-en-23-oic acid; 3-acetyl-11-keto-β-boswellic acid (AKBA), (3α)-3-acetoxy-urs-12-en-11-keto-23-oic acid; and 11-keto-β-boswellic acid (KBA), (3α)-3-hydroxy-urs-12-en-11-keto-23-oic acid] (purity > 99%) were purchased from Phytoplan (Heidelberg, Germany). All solvents were of analytical grade. Methanol, acetonitrile, tetrahydrofuran, n-hexane, 2-propanol, and ethyl acetate were from Caledon (Georgetown, ON, Canada). Water was collected from a Milli-Q organic free water system (Millipore Corporation, Bedford, MA). Extrelut NT was obtained from OASIS (Waters Corporation, Milford, MA). The solutions were incubated at 37°C in a water bath. The NADPH-regenerating system was used in this study because it provides constant NADPH levels over the entire incubation period. The final incubation concentration of NADPH was 10 μM in methanol-water (50:50, v/v). Based on the method described by Büchele and Simmet (2003), 0.8 g of Extrelut NT/ml of plasma was used for sorbent assisted liquid-liquid extraction in Extrelut glass columns. For the extraction, 6 ml of brain or liver homogenate were passed through the Extrelut NT3 glass columns containing 5 g of Extrelut. After 15 min, KBA and AKBA were eluted with a solvent mixture consisting of tetrahydrofuran-n-hexane-ethyl acetate-2-propanol (160:160:160:15, v/v/v/v). The plasma samples were eluted with 6 ml of solvent, whereas 30 ml was used for the elution of brain and liver samples. The eluates were dried under a stream of nitrogen at 40°C, and the residues were reconstituted in 150 μl of methanol and cooled on ice for 15 min before centrifugation at 2000g (4°C) for 10 min. The clear supernatants were used for LC-MS/MS analysis.

Preparation of in Vivo Samples. Based on the method described by Büchele and Simmet (2003), 0.8 g of Extrelut NT/ml of plasma was used for sorbent assisted liquid-liquid extraction in Extrelut glass columns. For the extraction, 6 ml of brain or liver homogenate were passed through the Extrelut NT3 glass columns containing 5 g of Extrelut. After 15 min, KBA and AKBA were eluted with a solvent mixture consisting of tetrahydrofuran-n-hexane-ethyl acetate-2-propanol (160:160:160:15, v/v/v/v). The plasma samples were eluted with 6 ml of solvent, whereas 30 ml was used for the elution of brain and liver samples. The eluates were dried under a stream of nitrogen at 40°C, and the residues were reconstituted in 150 μl of methanol and cooled on ice for 15 min before centrifugation at 2000g (4°C) for 10 min. The clear supernatants were used for LC-MS/MS analysis.

LC-MS/MS. For the experiments on the metabolic stability of KBA and AKBA, isocratic separation was performed on a PerkinElmer series 200 high-performance liquid chromatography system with a reversed-phase 18 column (HyperSil BDS, 150 × 4 mm, 5 μm particles; MZ-Analysentechnik, Mainz, Germany) at 40°C and a flow rate of 1 ml/min. The mobile phase consisted of methanol-water-glacial acetic acid (8:1:0.4, v/v/v). Mass spectrometry

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Results

Metabolic Stability of KBA and AKBA. After 15 min of incubation with RLM, more than 80% of the initial KBA concentration was metabolized and less than 1% of the starting concentration remained after 120 min. Extensive phase I metabolism was also observed in HLM. After 15 min, more than 60% of the initial KBA concentration was metabolized and less than 10% of the starting concentration remained after 120 min (Fig. 1). On the other hand, AKBA was less susceptible to phase I metabolism than KBA, with approximately 80% of the starting concentration still remaining 120 min after incubation with RLM and HLM, respectively (Fig. 1).

The direct glucuronidation experiments performed in RLM and HLM in parallel revealed no decrease in the initial concentrations of KBA and AKBA. Furthermore, KBA and AKBA glucuronides could not be detected upon screening with LC-MS/MS (data not shown). This finding clearly indicates that KBA and AKBA are subjected mainly to phase I metabolism. Moreover, no substantial differences between RLM and HLM were observed in their metabolic turnover rates.

Upon incubation of KBA and AKBA with rat hepatocytes, metabolic turnover rates similar to those described above for RLM and HLM were obtained (Fig. 1). Also, for rat hepatocytes more than 80% of the initial KBA was metabolized after 30 min, whereas approximately 80% of the starting AKBA concentration still remained after 120 min.

Identification of the KBA Metabolites in Vitro. The initial screening of the in vitro RLM reaction medium revealed the formation of metabolites with mass shifts of +16, +32, and +14 u in comparison to KBA. Upon screening for m/z 485.4 (corresponding to mass shifts of +16 u), three major metabolites eluting earlier than KBA.

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(Fig. 2A) were detected (Fig. 2B), indicating the presence of derivatives with more hydrophilic properties than the parent compound. Because these peaks were absent in the control incubations, they can be clearly assigned to metabolic conversions of KBA.

This assumption is further supported by comparing the MS/MS spectra of KBA and its metabolites, providing consistent structure-specific fragmentation patterns. Whereas the MS/MS of KBA produced fragments at \( m/z \) 376.4, 391.3, 407.3, and 451.3 (Fig. 2E), the three metabolites yielded the same fragment species with a characteristic mass shift of 16 u at \( m/z \) 392.3, 407.2, 423.2, and 467.3 (Fig. 2F). The peaks at 10.9 and 11.1 min could not be related to KBA, as they were also present in the control group and did not show the characteristic fragmentation pattern of KBA.

Upon screening for \( m/z \) 501.4 (corresponding to a +32 u mass shift), various signals were observed between retention times \( t = 2.4 \) and 7.8 min (Fig. 2C), exhibiting characteristic fragment ion shifts of
+32 u, in comparison with those of KBA at m/z 408.2, 423.1, and 439.3 (Fig. 2G). Furthermore, the two peaks at 32 u, in comparison with those of KBA at m/z 483.4 were not present in the control, suggesting metabolites corresponding to a mass shift of +14 u. As expected, the fragmentation pattern of these products (fragment ions at m/z 390.2, 405.2, and 421.3) again resembled that of KBA, indicating a link to KBA. The peak at 12.7 min could not be related to KBA.

Incorporation of KBA into hepatocytes yielded only phase I metabolites, represented by KBA derivatives with mass shifts of +16 u (t = 5.6, 7.7, and 7.9 min) and +32 u (t = 2.5, 4.4, and 7.8 min) as well as +48 u (t = 2.0 min). No phase II metabolites could be identified, either by screening for glucuronides, sulfates, and amino acid conjugates or by precursor ion scanning.

**Metabolites of KBA in Vivo in Rat Plasma, Liver, and Brain in Comparison to Its Metabolites in Vitro.** After the identification of the KBA metabolites in vitro, the metabolites of KBA were further explored in vivo in the course of a feeding study. To ensure sufficient metabolic conversion of KBA, screening for the in vivo metabolites in rats was carried out 2 h after oral administration of KBA suspension. This time interval was chosen on the basis of previous pharmacokinetic studies (Reising et al., 2005) and the rapid metabolism of KBA observed in vitro upon incubation with RLM.

In rat plasma, two derivatives with a mass shift of +16 u (at t = 5.7 and 8.1 min) and one derivative with a mass shift of +32 u (t = 7.8) were detected, in addition to the parent compound KBA. All MS/MS spectra of these metabolites correlated well with the corresponding spectra of KBA and its metabolites obtained in vitro after microsomal incubation, indicating that these metabolites were identical.

The analysis of the rat liver revealed the formation of various metabolites showing the same fragmentation pattern as KBA and its metabolites in vitro. In addition to KBA, three metabolites with a mass shift of +16 u (t = 5.7, 7.7, and 8.0 min) and four KBA derivatives exhibiting a mass shift of +32 u (t = 2.8, 3.4, 4.4, and 7.8 min) were detected as well as one derivative corresponding to KBA +48 u (t = 1.8 min). These results suggest that the liver plays an important role in the metabolism of KBA. The analysis of the rat brain revealed the presence of KBA, but no metabolites could be detected.

Moreover, the rat plasma, liver, and brain were screened for the occurrence of phase II metabolism of KBA and/or KBA metabolites but no conjugates were found. In the control group consisting of rats fed with agaroar suspension only, neither KBA nor any metabolite were identified in rat plasma, liver, or brain. Table 1 presents an overview of all metabolites of KBA detected in vivo in comparison with those identified in vitro.

**Identification of AKBA Metabolites in Vitro.** The experiments on the metabolic stability of AKBA in the liver microsomes described in the previous section showed that the transformation rate of AKBA is much lower than that of KBA. Hence, the initial screening of the RLM in vitro reaction medium revealed the formation of derivatives with mass shifts of only +16 u. Upon screening for m/z 527.3, corresponding to a +16 u mass shift, three different metabolites with retention times of t = 7.7, 8.2, and 9.4 min were detected, which were absent in the control incubations. As the MS/MS spectra of AKBA as well as those of the metabolites observed showed a characteristic fragment at m/z 59 (acetic acid anion), these metabolites can be assigned to AKBA. In hepatocytes, AKBA yielded only one AKBA derivative with a mass shift of +16 u (t = 8.1). Again, no phase II metabolites could be detected.

**Metabolites of AKBA in Vivo in Rat Plasma, Liver, and Brain in Comparison to Its Metabolites in Vitro.** Only AKBA but no metabolites were detected in rat plasma, liver, and brain 2 h after oral administration of AKBA suspension. A representative selected ion monitoring (SIM) chromatogram and product ion spectrum of AKBA (t = 12.3) in the rat liver is shown in Fig. 3, A and B. The peaks at t = 12.8, 10.6, and 9.5 are not derived from AKBA, as they were also present in the control animals and did not show the characteristic fragmentation pattern of AKBA. Screening the plasma, liver, and brain samples for the occurrence of phase II metabolism of AKBA and AKBA metabolites, no evidence for phase II metabolism could be found, as in the case of KBA.

**Checking for Deacetylation as an Alternative Metabolic Pathway for AKBA.** Because AKBA represents an acetyl derivative of KBA, it could be metabolized to KBA via deacetylation. AKBA incubated with rat hepatocytes, however, yielded only very small amounts of KBA, not exceeding 2% of the initial AKBA concentration. Also, the plasma, liver and brain of the rats fed with AKBA were screened for the presence of KBA. In the plasma, KBA was almost undetectable. As shown in the SIM chromatogram of KBA in Fig. 3C, KBA (t = 11.2) was detected only in negligible amounts in the liver, not exceeding those determined in hepatocytes, indicating that deacetylation of AKBA to KBA takes place in the liver to a minor extent only. The peaks at t = 9.7 and 9.9 were also present in the control group and did not show the characteristic fragmentation pattern of KBA represented in Fig. 3D. Therefore, they cannot be related to KBA. Furthermore, no KBA was detected in the brain samples of the rats fed with AKBA.

**Identification of Metabolites of αBA, βBA and Their Acetylated Derivatives in Vitro.** The incubation of αBA and βBA with rat liver microsomal preparations and its subsequent analysis by LC-MS/MS revealed the formation of various metabolites. In addition to metabolites with +16 and +32 u mass shifts seen for both BA derivatives, metabolites with a mass shift of +48 u were observed for βBA. No metabolites were detected on incubation of AαBA and AβBA with microsomal enzymes.

**Discussion**

To date only one study exists on the microsomal incubation of *Boswellia* species, which focused on investigating the inhibitory effect of *Boswellia* extract on cytochrome P450 enzymes (Frank and Unger, 2006). Our study represents the first investigation of BA metabolism. An overview of all phase I metabolites obtained in vitro from different BAs and their respective retention times and mass shifts is given in Fig. 4.

In general, the incubation of BAs with phase I enzymes in vitro revealed that oxidation, yielding derivatives with mass shifts of +16 and +32 u and in some cases of +48 u, corresponding to mono-, di-, and trihydroxylated metabolites, respectively, is the principal metabolic route of KBA, AKBA, αBA, and βBA. The phase I metabolites are
of BAs identified in this study are in agreement with the phase I metabolites described for oleanolic acid, which have been characterized as hydroxyl- and dihydroxyoleanolic acid (Jeong et al., 2007). Together, this previous investigation and the present study represent the first detailed insight into the metabolism of pentacyclic triterpenes.

The incubation of KBA with RLM yielded three monohydroxylated and six dihydroxylated metabolites as well as two additional metabolites, exhibiting a +14 u mass shift. Taking the chromatographic elution behavior of these metabolites into consideration, indicating more hydrophilic properties in comparison to KBA, the mass shift of +14 u may be attributed to ketone formation or dehydrogenation and hydroxylation of KBA. The incubation of KBA with hepatocytes yielded similar metabolites represented by three mono- and three dihydroxylated KBA derivatives as well as one trihydroxylated metabolite.

In vivo hydroxylated derivatives of KBA were detected only in rat plasma and liver. The observed absence of KBA metabolites in rat brain may be attributed to the increased hydrophilicity of the hydroxylated derivatives, not allowing them to pass the blood-brain barrier. Both the in vitro experiments in RLM and hepatocytes as well as the in vivo feeding study revealed the formation of the same metabolic profiles suggesting that no phase II metabolism is taking place.

For AKBA, only derivatives with a mass shift of +16 u, corresponding to monohydroxylated metabolites, were detected in vitro, and no metabolites were identified in vivo. The limited metabolism of AKBA by phase I enzymes coincides with observations made for other acetylated BAs (AcαBA and AcβBA), for which no phase I metabolites were identified at all in vitro. Obviously, the acetyl group at position 3 is responsible for the impeded phase I metabolism. Nevertheless, some hydroxylated metabolites were identified for AKBA in vitro compared with AcαBA and AcβBA. This result may be attributed to the presence of a keto group at position 11.

In general, microsomes and hepatocytes are used not only for the elucidation of drug metabolism but are also typically applied during the drug-screening process for determining metabolic stability (Shearer et al., 2005). Because the pharmacological effects of B. serrata are attributed mainly to KBA and AKBA, this study focused on the metabolic stability of these two BAs in rat liver microsomes and hepatocytes. In addition, the metabolic stability was also determined in HLM to get a preliminary idea about the hepatic transformation rate of KBA and AKBA in humans.

The present study revealed that KBA undergoes extensive hepatic phase I metabolism. This might represent one of the major reasons for the low systemic KBA bioavailability observed in rats and humans after the oral administration of very high doses of Boswellia extract. In contrast, the pharmacokinetic behavior of AKBA raises several questions. Although the concentration of AKBA is equivalent or even exceeds that of KBA in Boswellia extracts, AKBA was detected in rat and human plasma at even lower concentration levels than KBA (Büchele et al., 2003; Reising et al., 2005). This finding is surprising, because AKBA is more lipophilic than KBA and should therefore be more easily absorbed. A possible explanation could be extensive first-pass metabolism. However, the present study revealed that although AKBA differs from KBA only by acetylation of the hydroxyl group at position 3, its metabolic behavior is completely different. AKBA was more stable toward phase I enzymes than KBA. Therefore, extensive hepatic metabolism could not explain the low systemic availability of AKBA.

Furthermore, the present study revealed that deacetylation of AKBA to KBA takes place only to a minor extent and thus cannot be responsible for the low systemic availability of AKBA as assumed previously (Abdel Tawab et al., 2001; Sharma et al., 2004).

A further reason for the lower plasma levels of AKBA compared with KBA might be the greater volume of distribution of AKBA associated with its greater lipophilicity. Indeed, the brain/plasma ratio determined for AKBA (0.8) in a previous study (Reising et al., 2005)
was higher than that of KBA (0.5), although only half the plasma levels of KBA were determined for AKBA. These are very promising results, as they suggest a higher brain penetration of AKBA compared with that for KBA. Based on these findings, the metabolic stability determined for AKBA is of particular importance. Finally, the influence of intestinal metabolism and poor absorption on limiting the systemic availability of BAs should not be underestimated. Initial results of subsequent in vitro permeability studies using Caco-2 cells, which are still in progress, suggest poor absorption of KBA and AKBA from the gastrointestinal tract. It seems that the poor systemic availability of AKBA may be attributed mainly to its poor absorption and the low bioavailability of KBA results from its metabolic instability and poor absorption (P. Krueger, J. Kanzer, J. Hummel, G. Fricker, and M. Abdel-Tawab, unpublished observations).

In a previous study, B. serrata extract, as well as KBA and AKBA, was identified as potent inhibitors of P-glycoprotein (Pgp) in porcine brain capillary endothelial cells (PBCECs) and human lymphocytic leukemia parenteral (VLB) cell lines (Weber et al., 2006). AKBA produced a significant inhibition of Pgp at concentrations of 3 and 10 μM in PBCECs and VLB cells, respectively, whereas KBA exerted...
significant Pgp inhibition at a concentration of 10 μM in PBCECs but not in VLB cells compared with untreated cells. As this study could not differentiate whether substrate inhibitor or allosteric effects were responsible for the observed Pgp inhibition, the relevance of Pgp efflux, as a possible factor affecting the bioavailability of KBA and AKBA, cannot be assessed at present.

For numerous drugs, the duration and intensity of action is determined by their metabolic rate. Characterizing the metabolic behavior is therefore an important issue to assess the therapeutic effect of drugs. Considering the results of this study, it can be concluded that the observed extensive hepatic metabolism of KBA strongly contributes to its low bioavailability. However, the low bioavailability of the more lipophilic AKBA, which may not be attributed to extensive hepatic metabolism, will surely represent a pivotal question in further studies addressing the permeability of boswellic acids.

References


Address correspondence to: Dr. Mona Abdel-Tawab, Zentrallaboratorium Deutscher Apotheker, Carl-Mannich-Strasse 20, D-65760 Eschborn, Germany. E-mail: m.tawab@zentrallabor.com