MAJOR PHASE I BIOTRANSFORMATION PATHWAYS OF TRICHOSTATIN A IN RAT HEPATOCYTES AND IN RAT AND HUMAN LIVER MICROSOMES

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ABSTRACT:

Phase I biotransformation of Trichostatin A (TSA), a histone deacetylase inhibitor with promising antifibrotic and antitumoral properties, was investigated in rat and human liver microsomes and in suspensions of rat hepatocytes. TSA (50 μM) was readily and completely metabolized by rat hepatocytes in suspension (2 × 10⁶ cells/ml), whereafter its phase I metabolites were separated by high-performance liquid chromatography and detected with simultaneous UV and electrospray ionization mass spectrometry (ESI-MS). ESI tandem mass spectrometry (ESI-MS/MS) was used to identify the metabolites. Two major phase I biotransformation pathways in rat hepatocytes were shown to be N-demethylation and reduction of the hydroxamic acid function to its corresponding amide. N-monomethylated TSA and TSA amide were preferentially formed during the first 20 min of exposure, and N-monomethylated TSA amide appeared as the main metabolite after a 30 min incubation period. At this time, virtually all TSA had been metabolized. Trichostatic acid, N, N-monomethylated Trichostatic acid, and N-dimethylated TSA were identified as minor metabolites. Longer incubation led to the formation of N-dimethylated TSA amide as the main metabolite. Lower concentrations of TSA (5 and 25 μM) formed relatively higher amounts of N-demethylated, nonreduced metabolites. Incubations of TSA with rat and human microsomal suspensions, however, led to an incomplete biotransformation with the formation of two major metabolites, N-mono- and N-dimethylated TSA. Traces of Trichostatic acid, TSA amide, N-mono- and N-dimethylated TSA amide were also detected. This study is the first to show that TSA undergoes intensive phase I biotransformation in rat hepatocytes. This has important consequences for its potential development as a drug, since rapid biotransformation resulting in a short exposure to the pharmacologically active parent compound, and a complex mixture of metabolites is usually not desired. Further biotransformation studies of TSA and structural analogs with antitumoral and antibacterial properties need to be performed in cultured intact hepatocytes, in particular since one of the major phase I biotransformation pathways is catalyzed by nonmicrosomal enzymes.

In 1976, the hydroxamic acid Trichostatin A (TSA) (Fig. 2) was isolated from Streptomyces hygroscopicus and identified as an antifungal antibiotic, exhibiting anti-Trichophyton activity (Tsujii et al., 1976). About 10 years later, TSA was rediscovered as a potent, specific, and reversible inhibitor of histone deacetylase (HDAC) showing activity both in vivo and in vitro (Yoshida et al., 1990b). The equilibrium between HDAC and histone acetyltransferase activities determines the acetylation level of the N-terminal tails of core histones. Histone acetylation is an important mechanism for the regulation of eukaryotic gene expression. In general, transcriptionally active genes are associated with hyperacetylated histones, whereas hypoacetylation can result in transcriptional repression and gene silencing (Krajewski, 1999; Magnaghi-Jaulin et al., 1999, 2000; Cress and Seto, 2000; Mahlknecht et al., 2000). In a number of mammalian cell lines, nanomolar concentrations of TSA induce histone hyperacetylation, accompanied by various cellular phenotypic changes and a characteristic blockage of the cell cycle at G₁ and G₂ phases (Yoshida and Beppu, 1988; Yoshida et al., 1990b; Hoshikawa et al., 1994). The hydroxamic acid function and the natural R-(+)-configuration of the chiral center at the 6 position of TSA seem to be crucial in this respect, since Trichostatic acid and S-(-)-TSA are shown to be inactive (Yoshida and Beppu, 1988; Yoshida et al., 1990a).

The promotion of the transition of dedifferentiated, proliferating cells into more differentiated and less proliferative ones by TSA could potentially be employed to treat a variety of tumorous and fibroproliferative diseases. In vitro antitumoral effects of TSA have been shown in a wide variety of human transformed cell lines including neuroblastoma, leukemia, melanoma, and colon carcinoma cells (Li et al., 1996; Lin et al., 1998; Kosugi et al., 1999; Saunders et al., 1999; Marks et al., 2000). In vivo, TSA shows potent antitumor activity against breast cancer without apparent toxicity, as evaluated in a...
N-methyl-N-nitrosourea carcinogen-induced rat mammary carcinoma model (Vugusin et al., 2001). Promising pharmacological results were also obtained in a Lewis lung carcinoma model (Kim et al., 2001). Niki et al. (1999) found that TSA suppresses myofibroblastic differentiation of rat hepatic stellate cells in primary culture by hyperacetylation of histones (H4). These in vitro antifibrogenic properties have also been seen in vivo, in a CCl4-induced Balb/C mouse model of hepatic fibrosis with no apparent toxic effects (A. Geerts, personal communication).

Up to now and to the best of our knowledge, no data on the metabolic fate of TSA in humans or animals have been published in the current literature. However, biotransformation is of key importance in the understanding of the pharmacological value of a candidate drug such as TSA. In the early phase of drug development, biotransformation studies are often performed through the use of in vitro technology based on human and animal cells and tissues. Such in vitro studies provide information on the rate of metabolism, the kind of metabolites formed, and about the biotransformation (iso)enzymes involved (Tarbit et al., 1993; Wrighton et al., 1993; Ball et al., 1995; Maurel, 1996). In the present paper, the major in vitro phase I biotransformation pathways of TSA in rat and human liver microsomal suspensions and in freshly isolated rat hepatocytes are described. Separation and detection of the phase I metabolites were carried out using high-performance liquid chromatography (HPLC) and simultaneous electrospray ionization mass spectrometric (ESI-MS)/UV detection, respectively. Structural assignments of the metabolites for which no reference standards were available were based on the interpretation of the spectrum obtained from HPLC/MS and from collision-induced dissociation (CID) MS/MS experiments, both with ESI. A reference standard was available for Trichostatic acid.

### Materials and Methods

#### Chemicals and Reagents.

TSA or 7-[4-(dimethylamino)phenyl]-4,6-di-methyl-7-oxo-hepta-2,4-dienoic acid hydroxamide (purity ≥98%), crude col-lagenase type I, bovine serum albumin fraction, 7-ethoxyresorufin, resorufin, HEPES, NADP⁺, glucose 6-phosphate and acetonitrile were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium). Glucose 6-phosphate dehydrogenase (Grade I) was obtained from Roche Diagnostics (Mannheim, Germany). Trifluoroacetic acid (TFA) of analytical reagent grade was obtained from Trichostatic acid were performed under identical conditions, and samples were prepared as described (Hales and Neims, 1977), suspended in 0.1 M sodium potassium phosphate buffer either at pH 7.8 (for 7-ethoxyresorufin-0-deethylation (EROD)/7-pentoxyresorufin O-dealkylyase activity determinations) or at pH 7.4 (for incubations with TSA), and stored in liquid nitrogen.

Microsomes (0.0–2.0 mg of microsomal protein/ml) were incubated with 140 μM TSA and with a NADPH-generating system (0.5 mM NADP⁺, 10 mM glucose 6-phosphate, 2mM glucose 6-phosphate dehydrogenase and 0.6 mM MgCl₂) in 100 mM sodium potassium phosphate buffer (pH 7.4) (final volume 1 ml). The addition of substrate initiated the biotransformation reactions. Incubations were carried out at 37°C for 150 min and terminated by dilution of 300-μl aliquots with 750 μl of ice-cold acetonitrile. Control incubations, containing microsomes that had been boiled for 2 min, were performed under identical conditions. Microsomes were removed by centrifugation at 2000g at 4°C during 45 min, and the clear supernatant was injected onto a HPLC column as described below.

#### Hepatocytes (Rat).

The isolation of rat hepatocytes was performed as described by De Smet et al. (1998). Cell integrity was assessed by trypan blue exclusion and only suspensions with a minimum of 84% observed viability were used. Incubations were carried out in HEPES buffer (pH 7.65, 37°C) at a final cytocrit of 2×10⁶ cells/ml (3 ml). Concentrations of TSA were 5, 25, and 50 μM, and incubations were conducted up to 6 h. Control samples were obtained by boiling the hepatocyte suspension for 2 min prior to incubation with TSA. Cell-free control incubations contained the substrate in the incubation medium without hepatocytes. Incubations of rat hepatocytes with 50 μM Trichostatic acid were performed under identical conditions, and samples were taken every 1 h up to 3 h.

At the selected time intervals, a 1.0-ml aliquot was removed from the incubation mixture and the biotransformation reaction was stopped by submersion in liquid nitrogen. Samples were stored at −80°C until further analysis.

Membrane damage, following exposure to TSA, Trichostatic acid, and/or to solvent [0.0833% (v/v) methanol], was checked by measuring lactate dehydrogenase (LDH) leakage from the cells into the medium using a Merckotest (LDH index = 100 × LDH activity in the supernatant divided by the sum of LDH activity in the supernatant and in the cells) (Merck, Darmstadt, Germany).

#### Microsomal Proteins.

Microsomal protein concentration was determined according to the Bradford procedure (Bradford, 1976) using a Bio-Rad protein assay kit (Bio-Rad, Brussels, Belgium) with bovine serum albumin as a standard.

#### Cytochrome P450-dependent Activities.

Microsomes were incubated with 5 μM 7-ethoxyresorufin. The formation of resorufin was measured fluorimetrically according to a modified procedure of Burke and Mayer (1974). EROD activity was expressed versus microsomal protein content.

#### Sample Preparation.

Samples from incubations with hepatocytes were thawed on ice and centrifuged at 120g for 2 min. With the aim of sample clean-up, the supernatants (extracellular medium) were subjected to solid

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**TABLE 1**

<table>
<thead>
<tr>
<th>Donor Number</th>
<th>Age</th>
<th>Sex</th>
<th>Cause of Death</th>
<th>Drug History*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>45</td>
<td>F</td>
<td>Brain hemorrage</td>
<td>Cyclandelate, dopamine, furosemide, heparine, insulin, minocycline, methylprednisolon, piracetam</td>
</tr>
<tr>
<td>H2</td>
<td>4</td>
<td>F</td>
<td>Cerebral anoxia</td>
<td>Adrenalin, ceftaxim, dobutamine, flacoxacillin, phenobarbital, phenytoin, ranitidine</td>
</tr>
<tr>
<td>H3</td>
<td>35</td>
<td>M</td>
<td>Cerebrovascular accident</td>
<td>Cefuroxim, desmopressin acetate, digoxine, flucloxacillin, insulin, labetalol, methylprednisolon, netilimicin, thyroxine, ranitidine</td>
</tr>
</tbody>
</table>

F. Female; M. Male.

* Drugs received during reanimation.
phase extraction (Waters Oasis HLB cartridges; Waters Corporation, Milford, MA). After prewashing with 1 ml of methanol followed by 1 ml of milliQ-water, the cartridges were loaded with 0.8 ml of sample and washed [5% (v/v) methanol, 1 ml]. TSA and its metabolites were eluted with 1.2 ml of methanol. HPLC analysis after centrifugation (2000 g, 30 min, 4 °C) of the ultrasonicated pellet in 1 ml of methanol revealed the intracellular biotransformation profile.

**Metabolite Separation and Identification.** Separations were performed by reversed-phase HPLC using a Kontron chromatographic system, which consisted of a low-pressure gradient pump type 325, a UV detector type 332, and an autosampler type 465 (Kontron Interments, Milan, Italy). The HPLC system was coupled to a VG Quattro II triple quadrupole mass spectrometer with an ESI interface (Micromass, Manchester, UK). Data collection and processing was done by Masslynx software version 2.22 (Micromass). Separations (20 μl of samples) were achieved at room temperature on a Discovery C 18,5 μm, 250 × 4.6 mm column (Supelco; Sigma-Aldrich NV/SA). The mobile phase consisted of component A [0.1% (v/v) aqueous TFA] and component B [0.1% (v/v) TFA in acetonitrile]. A linear gradient from 3 to 80% (v/v) B was used over 30 min at a flow rate of 1.0 ml/min. The HPLC effluent was split (ACURATE by LC Packings, Amsterdam, The Netherlands) 9:1 to direct 100 μl/min into the mass spectrometer. UV detection was at 266 nm, whereas ESI was performed in the positive mode. The operating parameters of the mass spectrometer were set as follows: capillary voltage 3kV, cone potential 50V, source temperature 70°C. Nitrogen was used as nebulizer and drying gas at a flow rate of 20 and 250 l/h, respectively. The data from the HPLC/MS analysis were collected over a mass range from m/z 110 to 850 at a scan rate of 1 s/scan. The full scan ESI-MS spectrum from the HPLC/MS analysis allowed us to determine the protonated molecular ions [M + H]+ of the metabolites. However, for structural identification of the metabolites, the mass spectrometer was operated off-line in the MS/MS mode (product-ion scanning mode). Samples were directly introduced to the mass spectrometer by continuous-flow injection using a syringe pump (Harvard Apparatus model 55-2222; Harvard Apparatus, Holliston, MA). Water/acetonitrile (50:50 v/v) was used as a carrying solvent, and the sample flow rate was set at 10 μl/min. Argon was used as collision gas at a pressure of 2.5 × 10⁻³ mbar and collision energy was set to 35eV. The first quadrupole of the mass spectrometer, operated in the static mode, was set to monitor the selected molecular ions of the metabolites, and the third quadrupole was used in the scan mode, over a mass range of m/z 75 to 350 at a scan rate of 1 s/scan, to detect all the fragments obtained from the CID of the selected molecular ions. Other parameters of the mass spectrometer were set as described above. Structural assignments of the metabolites were based on the interpretation of the spectra obtained from HPLC/MS and from the CID MS/MS experiments, both with ESI.

Degradation of TSA and formation of the metabolites were evaluated semiquantitatively by comparing the peak areas detected at 266 nm. The detection limit for TSA was determined to be 1 μM, the quantification limit 5 μM, and linearity in UV-absorption was seen up to concentrations of 80 μM TSA.

**Statistical Analysis.** Results of LDH leakage in suspensions of freshly isolated rat hepatocytes and microsomal EROD activity measurements were subjected to a paired Student’s t-test.

**Results**

**Phase I Biotransformation of TSA in Rat and Human Liver Microsomes.** Enzymatic activities in rat and human microsomes. EROD (rat and human CYP 1A1/2-dependent) activity was measured as a marker enzyme for quality control of the microsomes used. Based on values before and after storage (results not shown), we assumed that the microsomal phase I biotransformation activity had remained intact.

TSA metabolism in rat liver microsomes. Microsomal samples (n = 3) containing 0.0, 0.5, 1.0, 1.5, and 2.0 mg of microsomal protein/ml were exposed to 140 μM TSA during 150 min. In all cases, the breakdown of TSA was incomplete. No degradation of TSA occurred when incubated without microsomes or with boiled microsomes. An...
increase in protein concentration resulted in an increased breakdown of TSA during the first 30 min, an earlier “reaction stop”, and a reduced percentage of unmetabolized TSA (Fig. 1). Addition of higher concentrations of NADPH-generating system or MgCl₂ to the incubation mixtures had no effect on the metabolic rate (results not shown).

Comparison of the chromatograms obtained after incubation of rat microsomes with 140 μM TSA with the blank chromatogram (microsomal sample without TSA) led us to conclude that two major metabolites, 2 (17.42 min) and 1 (20.23 min), and four minor metabolites [3 (26.53 min), 5 (23.46 min), 6 (21.09 min), and 7 (18.20 min)] of TSA were formed. Since they were present in only very small amounts, the minor metabolites were identified later on through incubations of TSA with rat hepatocyte suspensions. Metabolite structures are depicted in Fig. 2.

The HPLC/MS spectrum and the CID MS/MS pattern of TSA (Fig. 3A) were important for the subsequent identification of the metabolites. The protonated molecular ion [M + H]⁺ of TSA at m/z 303, as well as a significant fragment ion at m/z 148 were observed both in the HPLC/MS full scan and in the CID MS/MS spectra. This fragment ion corresponds to the 4-dimethylaminobenzoyl moiety. In the MS/MS spectrum of TSA, two other fragment ions were detected at m/z 285 [M + H-18]⁺ and m/z 270 [M + H-33]⁺ and resulted from the loss of water and hydroxylamine, respectively. The spectrum lacks fragment ions in the region between m/z 148 and 270, indicating that the 4,6-dimethyl-2,4-hexadiene chain is not fragmented. In addition, no fragmentation at this part of the molecule could be observed by varying the collision energy and collision gas pressure.

The protonated molecular ions [M + H]⁺ of metabolites 1 and 2 were detected at m/z 289 and 275, respectively. The proposed molecular weights (mol. wt. = 288 and 274, respectively) were even-numbered, like TSA (mol. wt. = 302), which indicated that the metabolites retained both nitrogen atoms. Furthermore, these values were 14 and 28 atomic mass units (amu) smaller than the value of the molecular ion of TSA, suggesting demethylation of TSA. The site of demethylation, which could occur either at the 4-dimethylaminobenzoyl function or at the alkene chain at carbon 4 and/or carbon 6, was investigated by MS/MS (the MS/MS spectrum of 2 is shown in Fig. 3B). The HPLC/MS spectra and the MS/MS fragmentation profiles of these metabolites were similar to those of TSA. Fragment ions at m/z 271 (1) and at m/z 257 (2) [M + H-18]⁺ corresponded to the loss of water. Other fragments at m/z 256 and 242 for 1 and 2, respectively, resulted from the loss of hydroxylamine [M + H-33]⁺. These observations suggested the intact nature of the hydroxamic acid function.

**Fig. 2. Proposal for phase I biotransformation pathways of TSA and structures of the identified phase I metabolites formed in suspensions of rat and human liver microsomes and in freshly isolated rat hepatocytes.**

Phase I metabolites of R(+)-TSA or 7-[4-(dimethylamino)phenyl]-4,6-dimethyl-7-oxo-hepta-2,4-dienoic acid hydroxamide were identified by ESI CID MS/MS as N-monodemethylated TSA (1), N-didemethylated TSA (2), Trichostatic acid (3), N-monodemethylated Trichostatic acid (4), TSA amide (5), N-monodemethylated TSA amide (6), and N-didemethylated TSA amide (7).
The major fragment ions were observed at m/z 134 (1) and 120 (2). Since these were 14 and 28 amu less than the corresponding 4-dimethylaminobenzoyl fragment of TSA (m/z 148), TSA demethylation occurred at this site. According to these results, we propose that metabolites 1 and 2 are N-mono- and N-didemethylated hydroxamates: N-mono- and N-didemethylated TSA, respectively (Fig. 2).

N-demethylation of TSA formed the major phase I biotransformation pathway in rat liver microsomes. The concentration of N-didemethylated TSA (2) gradually increased during the incubation to reach a constant level at the time of the TSA "reaction stop", whereas N-monodemethylated TSA (1) first showed a slight increase and then a decrease. The minor metabolites 3, 5, 6, and 7 were detected at very low concentrations throughout the incubation period.

TSA metabolism in human liver microsomes. The biotransformation patterns of TSA in rat and human microsomes were similar and the breakdown of TSA was incomplete in both species. Interindividually, the three human samples showed no qualitative differences.

Biotransformation of TSA in Suspensions of Freshly Isolated Rat Hepatocytes. Cell viability. No significant toxic effects (LDH leakage) were seen in cell suspensions incubated with 5, 25, and 50 μM TSA for 6 h and with 50 μM Trichostatic acid for 3 h as compared with control suspensions without TSA/Trichostatic acid and suspensions containing only the solvent (results not shown).

Biotransformation of TSA. Preliminary experiments showed that incubation of 5, 25, and 50 μM TSA with 2 × 10⁶ rat hepatocytes/ml led to a complete degradation within the first hour. Since control suspensions showed no changes in the extracellular level of TSA, the degradation of TSA was completely catalyzed by liver biotransformation enzymes. In Fig. 4, an HPLC chromatogram of the extracellular medium of rat hepatocytes incubated with 50 μM TSA for 1 h is shown. Seven metabolites (1-7) could be chromatographically resolved and structurally identified.

The HPLC/MS full scan and CID MS/MS spectra obtained from microsomal incubations contained significant fragment ions at m/z 148, 134, and 120, corresponding to the N-dimethyl-, N-monomethyl-, and -aminobenzoyl moiety, respectively. Therefore, HPLC/MS single ion recording of these fragments could be used to detect metabolites with the same structural features in hepatocyte incubation samples. The HPLC/MS-single ion recording chromatograms showed that metabolites 5 and 3 both bore the N-dimethylaminobenzoyl moiety, that 1, 6, and 4 contained the N-monomethylaminobenzoyl function, and that 2 and 7 comprised the aminobenzoyl moiety. CID MS/MS product ion spectra gave us enough information to propose the chemical structures of these metabolites. The protonated molecular ion [M + H]⁺ of metabolite 3 was detected at m/z 288, and the proposed odd-numbered molecular weight (mol. wt. = 287) indicated the loss of one nitrogen atom by biotransformation (TSA has two nitrogen atoms and its molecular weight is even). In the CID MS/MS product ion spectrum of 3 (Fig. 3C), the most intense fragment at m/z 148 proved that the 4-dimethylaminobenzoyl moiety was intact. Therefore, the loss of nitrogen had occurred at the hydroxylamine function. Another fragment ion at m/z 270 [M + H-18]⁺ corresponding to the loss of water suggested the presence of a hydroxyl group in the metabolite. Based on these results, 3 was thought to be Trichostatic

**Fig. 3.** Representative CID MS/MS-product ion spectra for the structural elucidation of TSA metabolites.

Spectra of TSA (A), N-didemethylated TSA (2, B), Trichostatic acid (3, C), and N-monodemethylated TSA amide (6, D) are shown.
The protonated molecular ion $[M+H]^+$ of metabolite 4 was detected at $m/z$ 274. The odd-numbered molecular weight (mol. wt. = 273) indicated the loss of one nitrogen atom during TSA biotransformation. The fragment ion at $m/z$ 134 showed that this metabolite is an N-demethylation product of TSA and proved that the loss of nitrogen must have occurred at the hydroxylamine function. A fragment ion detected at $m/z$ 256 $[M+H-18]^+$, corresponding to the loss of water, suggested the presence of a hydroxyl group in the molecule. We propose that metabolite 4 is N-monodemethylated Trichostatic acid (Fig. 2). Comparison of the chromatograms of incubation samples with TSA and of those with Trichostatic acid underlined this observation. Like in the case of TSA, N-demethylation is a major phase I biotransformation pathway of Trichostatic acid (results not shown).

The protonated molecular ion $[M+H]^+$ of metabolite 5 was detected at $m/z$ 287, 16 amu less than that of TSA, and indicated the loss of an oxygen atom. The proposed even-numbered molecular weight (mol. wt. = 286) suggested the presence of both nitrogen atoms. Along with the most intense fragment ion at $m/z$ 148 corresponding to the 4-dimethylaminobenzoyl function, a fragment ion at $m/z$ 270 $[M+H-17]^+$ consistent with the loss of ammonia was detected in the CID MS/MS product ion spectrum of 5. We therefore suggest that the loss of the oxygen atom resulted from the reduction of the hydroxamic acid function to the corresponding amide. The metabolite (5) is believed to be TSA-amide (Fig. 2).

In Fig. 4, we can see that 6 and 7 were the major metabolites formed after 1 h incubation of hepatocytes with 50 μM TSA. At that time, they were present in comparable amounts. However, after 2 h incubation, 7 was predominant. In hepatocyte suspensions with 25 μM TSA, this pattern could already be seen after the first hour of incubation. 2 was a major metabolite in rat hepatocyte suspensions incubated with 5 μM TSA during 1 h and was later reduced to 7. In
contrast, only small amounts of 2 were detected for higher concentrations of TSA.

During subsequent experiments, samples were taken every 10 min, as shown in Figs. 5A (5 μM), B (25 μM), and C (50 μM). The metabolites present at 0 min were formed during the short time interval between the addition of TSA to the hepatocytes and the snap-freezing in liquid nitrogen, as well as during thawing of the samples on ice prior to centrifugation and extraction. Although large amounts of 6 were detected after exposure to 25 and 50 μM TSA for 10 and 20 min, respectively, its presence in suspensions with 5 μM TSA was limited to those samples taken immediately after addition of TSA to the hepatocytes. At lower concentrations of TSA, the major metabolite 7 was formed earlier during the incubation period and in relative higher amounts.

Further experiments included incubations of 2 × 10⁶ rat hepatocytes/ml with 50 μM TSA up to 6 h. Samples taken from the extracellular and intracellular media were analyzed. In Fig. 6, the degradation of 50 μM TSA and the formation of its identified metabolites in the extracellular (A) and intracellular (B) medium of 2 × 10⁶ rat hepatocytes/ml during the first 20 min of incubation are shown. No metabolites additional to the ones detected in previous samples and no phase II metabolites could be identified. Comparison of the intra- and extracellular media showed that no metabolite was selectively retained in the hepatocytes, although about 30% of undegraded TSA was continuously present intracellular. The recovery of TSA, as evaluated by incubation of 50 μM TSA with boiled hepatocytes followed by extraction, was found to be 98 ± 3% (n = 3).
Analysis of the effluents collected during sample loading and washing steps of the solid-phase extraction procedure showed no significant loss of metabolites. In Fig. 2, a scheme of the major phase I biotransformation pathways observed for TSA in rat hepatocyte suspensions is proposed.

**Discussion**

Based on its chemical structure and knowledge of the biotransformation pathways of molecules with comparable functional groups, one could assume that the most likely phase I biotransformation pathways of TSA (Fig. 2) are N-demethylation of the dimethylaminogroup, reduction, hydrolysis, and carbonyl reduction of the hydroxamic acid function, together with oxidation of the conjugated double bonds (Gibson and Skett, 1994; Meyer, 1996; Parkinson, 1996). Thus, TSA can be expected to undergo extensive and rapid biotransformation. To the best of our knowledge, no specific data are available on the biotransformation of TSA.

Incubation of TSA with both human and rat liver microsomes led to the formation of two major metabolites, N-monodemethylated TSA (1) and N-didemethylated TSA (2) and several minor metabolites, as identified by CID MS/MS. Although relatively high concentrations of microsomal protein were used (up to 2.0 mg/ml), the biotransformation of TSA remained slow and incomplete. This cannot be explained by a decrease in biotransformation activity during storage (up to 5 years for human liver samples and 6 months for rat liver microsomes), as the microsomal phase I marker enzyme activity EROD was intact at the beginning of each experiment. Minor changes in the cytochrome P450 enzyme pattern, however, cannot be excluded.

In contrast to microsomes, freshly isolated rat hepatocytes in suspension exhibited a rapid (complete in 40 min) and extensive phase I metabolism of TSA. Seven metabolites could be identified. N-demethylation and reduction of the hydroxamic acid function were shown to be the major phase I biotransformation pathways, whereas TSA hydrolysis to its corresponding acid was a minor pathway. Although N-mono- (6) and N-didemethylated TSA amide (7) were found to be the major metabolites formed in suspensions incubated with 25 and 50 μM TSA, 6 was present in relatively small amounts in experiments with 5 μM TSA, whereas N-didemethylated TSA (2) became one of the main metabolites together with 7. This leads us to conclude that, in the presence of 25 and 50 μM TSA, the cytochrome P450 enzymes responsible for the N-demethylation reaction are saturated, which has to be taken into consideration when in vitro results are extrapolated to the in vivo situation.

Since TSA reduction is a major phase I biotransformation pathway in hepatocytes, but not in microsomes, the reaction is catalyzed by nonmicrosomal enzymes. This is in agreement with the findings by other authors that hydroxamic acids undergo enzymatic reduction to their corresponding amides by cytosolic or mitochondrial liver enzymes such as aldehyde oxidase (Sugihara and Kiyoshi, 1986; Katsura et al., 1993; Kitamura et al., 1994; Beedham et al., 1995). The possibility of interspecies differences between rat and human in phase I biotransformation of TSA cannot be excluded based on our results with microsomal suspensions. Short-term cultures of hepatocytes or liver slices are the preferable model to study human biotransformation of TSA. Human in vivo experiments to further elucidate TSA biotransformation are quite difficult to perform because of the low pharmaceutically active dosage. Analyses of blood, urine, and liver samples of treated patients for traces of TSA metabolites are extremely difficult, and administration of higher dosages of TSA is unethical at this stage of the development of TSA as a drug candidate because limited data with respect to its general toxicological profile are available.

In this manuscript, we have shown that TSA undergoes rapid biotransformation in suspensions of freshly isolated rat hepatocytes. In vivo, this results in low plasma levels of the active mother compound for a limited time span and in a mixture of metabolites that can be either pharmacologically active or inactive. Furthermore, the existence of polymorphic phase I pathways can lay at the basis of interspecies differences and unwanted drug-drug interactions and should therefore be further investigated (Tarbit et al., 1993; Wrighton et al., 1993; Ball et al., 1995; Maurel, 1996). Whether TSA biotransformation results in pharmacological and/or toxicological inactivation is not yet known. We do know that TSA acid is not biologically active (Yoshida et al., 1990a). The activities of the other metabolites of TSA identified here remain to be further investigated. X-ray crystallographic studies of an archaeaerobic HDAC homolog have revealed the structure of the catalytic core of HDACs and the mode by which hydroxamic-acid based HDAC inhibitors like TSA bind the enzyme. Through its hydroxamic acid group, TSA chelates zinc at the bottom of the tube-like pocket and simultaneously forms multiple hydrophobic interactions with the walls of the pocket. The hydrophobic group on the other end of the TSA molecule (the dimethylaminobenzoyl fragment) is present in an open hydrophobic area where the histone core normally sits (Finnin et al., 1999). Jung et al. (1999) synthesized different structural analogs of TSA and compared their HDAC-inhibiting activity as well as their ability to induce terminal cell differentiation in Friend leukemic cells. The pharmacological activity was clearly dependent on aliphatic chain length, whereas modification of the para-substituent in the benzoyl moiety had little influence. Based on these findings, we can predict that the detected N-demethylated TSA metabolites, 1 and 2, are probably capable of inhibiting HDAC at potencies comparable with TSA. On the contrary, reduced metabolites (5, 6, 7) are probably not active because they lack the hydroxamic acid group capable of chelating zinc. Since 2 is a major metabolite in rat hepatocyte suspensions incubated with 5 μM TSA, in contrast to results at higher concentrations of TSA, it is quite possible that the in vivo effects of TSA are at least partially due to the action of 2.

Further experiments will focus on the design of structurally related analogs of TSA with comparable pharmacological activities but which are more metabolically stable, and the use of human hepatocytes to obtain more information on the biotransformation pathways of TSA and these analogs.

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**References**


