Biocatalytic synthesis and structure elucidation of cyclized metabolites of the deacetylase inhibitor Panobinostat (LBH589)

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Abbreviations: ACN, acetonitrile; CID, collision-induced dissociation; COSY, correlation spectroscopy; CYP, cytochrome P450; DAC, deacetylases; DAD, diode array detection; EI, electron ionization; IPTG, isopropyl β-D-thiogalactopyranoside; HDAC, histone deacetylases; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; LB broth, Luria-Bertani broth; NOE, nuclear Overhauser effect; OD600, optical density at 600 nm; ROESY, rotational frame nuclear Overhauser effect spectroscopy; rh, recombinant human; TFA, trifluoroacetic acid; TXI, triple resonance, heteronuclei inverse detected.
Abstract

Panobinostat (LBH589) is a novel pan-deacetylase inhibitor that is currently being evaluated in phase III clinical trials for treatment of Hodgkin’s lymphoma and multiple myeloma. Under catalysis of recombinant human cytochrome P450 3A4 and 2D6 co-expressed with human P450 reductase in *E. coli* JM109, five metabolites of panobinostat were produced via whole cell biotransformation. The structures of the metabolites were elucidated with the spectroscopic methods MS and NMR and revealed an oxidative cyclization of the ethyl-amino-group to the methylindole moiety. The MS² spectrum of the cyclized metabolite showed a base peak, where the closed ring is reopened and that – taken as sole base for structure proposals - would have lead to wrong conclusions. The metabolites were substantially less potent deacetylase inhibitors than the parent compound.
Introduction

Histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups from an ε-N-acetyl lysine and thereby increase the ability of histones to bind to DNA. In healthy cells there is a balance between activities of histone deacetylases and histone acetylases regulating the transcriptional activity. There is in vitro evidence in malignant cells that increased HDAC activity leads to prolonged survival through various mechanisms, such as disruption of cell cycle regulation or of apoptosis (Lee et al 2008; Marks et al, 2000). Therefore, inhibition of HDAC is beneficial for treatment of cancer and currently two HDAC inhibitors are approved by the U.S. FDA against cutaneous T-cell lymphoma, a rare form of non-Hodgkin's lymphoma: Vorinostat (Zolinza®) and the natural product and cyclodepsipeptide romidepsin (Istodax®, Zain et al 2010). Recent evidence shows that deacetylases also regulate diverse non-histone proteins such as P53 or HSP90 and it is therefore more appropriate to speak about deacetylase inhibitors rather than histone deacetylase inhibitors (Bolden et al 2006). Pan-deacetylase inhibitors that inhibit both HDAC and non-histone targets have the potential to be applicable in the clinic to a wider set of tumor types. HDACs are divided into four classes: The zinc-dependant classes I, IIa, IIb and IV and the NAD-dependent class III.

Panobinostat (LBH589) is an orally active pan-deacetylase inhibitor that inhibits in vitro class I, II and IV HDAC with IC₅₀ in low nanomolar concentration (Atadja 2009). It is currently being evaluated in phase III clinical trials for treatment of Hodgkin lymphoma and multiple myeloma (Prince et al 2009). Its structure features a hydroxamic acid and a 2-methylindole moiety linked with cinnamic acid and an aliphatic secondary amine.

Preclinical DMPK results of the drug candidate will be published in due time and a manuscript about the human metabolic pathways of panobinostat is in preparation (Clive S, Woo MM, Stewart M, Nydam T, Kelly L, Squier P, Kagan M: Characterizing the disposition, metabolism, and excretion of an orally active pan-deacetylase inhibitor, panobinostat, via trace radiolabeled ¹⁴C
material in advanced cancer patients, in preparation). *In vivo* studies with rats revealed a systemic metabolite that had a significantly later maximum of concentration ($T_{\text{max}}$) than the one of the parent compound panobinostat after p.o. administration suggesting a slow elimination or, less likely, a delayed formation (Dr. Mark Kagan, personal communication). The accurate mass suggested the same elemental composition as the parent panobinostat and smaller MS/MS fragments suggested an oxygenated amide. It was, however, not possible to find a meaningful explanation of the base peak in its MS/MS spectrum. These findings and the start of a back-up chemistry program in research, where all potential liabilities should be addressed, prompted the desire for an unambiguous structure elucidation of the aforementioned metabolite.

To assess DMPK results, it is highly advantageous to elucidate the structure of critical or unusual metabolites by $^1$H- and $^{13}$C-NMR and to evaluate their biological activity. Whole-cell biotransformation is the method of choice to produce the milligram quantities necessary for such tasks (Schroer *et al* 2010). Thanks to an academia - industry collaboration between the Biomedical Research Centre of the University of Dundee, Scotland, and nine pharmaceutical companies (LINK I consortium) 14 different recombinant human (rh) CYPs functionally co-expressed with rh P450-reductase in *Escherichia (E.) coli* are available (Blake *et al* 1996, Pritchard *et al* 1997, Pritchard *et al* 1998).

In the present study rh P450 CYP3A4 and 2D6 were used to prepare milligram amounts of oxidative metabolites of panobinostat for unambiguous structure elucidation and testing for their *in vitro* biological activity.

**Materials and Methods**

**General**

The following NMR instruments were used: Bruker DRX500 spectrometer (Bruker, Fällanden, Switzerland), equipped with a 5mm TXI-cryoprobe (triple resonance, heteronuclei inverse
detected), a Bruker AV600, equipped with a 1.7 mm TXI-cryoprobe, or a Bruker DPX400 spectrometer with a 5mm BBI probe. The compounds were solved in DMSO-d₆ in a 5 mm or in a 1.7 mm diameter NMR tube. All spectra were recorded at 298 °K. From M4 and M8, ¹H COSY, ROESY (rotational frame nuclear Overhauser effect spectroscopy), HSQC and HMBC spectra were accumulated, using a 1.7mm TXI-cryoprobe at 600 MHz, in the case of M8 with only approximately 30 µg.

The liquid chromatograph consisted of a Waters UPLC Acquity (Waters, Milford, USA) equipped with a Waters Acquity PDA detector. Column: HSS T3 C18, 1.7 µm; 1.0 x 150 mm (Waters); flow rate 0.1 ml / min.; eluent A: H₂O / TFA 100 : 0.02; eluent B: ACN / TFA 100 : 0.02; gradient: 0 min. 2 % B; 15 min. 30 % B; 17 - 18 min. 95 % B; column temperature 40 °C; UV-detection: 200 - 330 nm, resolution 2.4 nm; injection volume 3 µl.

An ion trap mass spectrometer LTQ Velos (Thermo Scientific, San Jose, CA, USA) equipped with heated electrospray interface was operated in the positive mode with Xcalibur software version 2.1 as follows: A sheath gas setting of 20 units and auxiliary gas of 3 units was used and a spray voltage of 3.5 kV applied. The heated metal capillary was maintained at 300 °C with a mass range of 200 to 400 Da. The system was optimized for m/z 549 [M + H]⁺ of antimycin A₁ in the positive mode. Typical parameters: S-Lens 62 %; multipole 00 offset – 4 V; gate lens -35 V; front lens -5.25 V. MS/MS parameters: Isolation width 2.4 Da; without wide-band excitation activated; normalized collision energy 35 %; activation time 10 ms. Alternatively a TSQ Quantum AM (Thermo) mass spectrometer equipped with electrospray interface in the positive mode was used and operated with Xcalibur software version 2.0. A sheath gas setting of 20 units and auxiliary gas of 5 units was used and a spray voltage of 3 kV applied. The heated metal capillary was maintained at 280 °C; mass range 100 to 1000 Da. MS/MS parameters: Collision gas 1.5 mTorr argon; collision energy 17 V.

For accurate mass measurements an Orbitrap (Thermo Scientific) equipped with electrospray interface was operated in the positive mode at high resolution mode (30,000 Dalton). A sheath gas
setting of 15 units and auxiliary gas of 1 unit was used and a spray voltage of 4 kV applied. The
heated metal capillary was maintained at 275 °C. Typical parameters: tube lens 80 V, multipole 00
offset – 5 V; gate lens -80 V; front lens -6.5 V. MS/MS parameters: Isolation width 2.0 Da;
normalized collision energy 25 %; activation time 30 ms. Data acquisition and evaluation was done
with Xcalibur 2.0.7 SP1.

Luria-Bertani broth (Miller’s modification, L3397), antifoam 204 and isocitric dehydrogenase (from
porcine heart, type IV) were purchased from Sigma-Aldrich, Buchs, Switzerland, LB agar
(Vegitone; BioChemica #19344) from Fluka, Buchs, Switzerland, Amberlite XAD16 (industrial
grade) from Rohm and Haas, the Dow Chemical Company, Frankfurt, Germany, peptone from
casein (pancreatic 1.02239) and yeast extract (1.03753), both for microbiology, from Merck,
Darmstadt, Germany and supersomes™ from Becton Dickinson AG (Allschwil, Switzerland).
Panobinostat and synthetic standards of metabolite M5 were obtained from Novartis
Pharmaceuticals Corporation.

**Solutions and growth media**

The PSE-buffer (50 mM KH₂PO₄, 250 mM sucrose, 0.25 mM EDTA-Na₂·H₂O) was adjusted with 2
N NaOH to pH 7.4.

Stock solution of ampicillin (100 mg/ml in deionized water), chloramphenicol (25 mg/ml in
ethanol), thiamine hydrochloride (1 M in deionized water), δ-aminolevulinic acid (83.8 mg/ml in
deionized water) or isopropyl β-D-thiogalactopyranoside (IPTG, 1 M in deionized water) were
sterile filtered into aliquots and stored at -20 °C. To make 200 ml of the trace elements solution,
firstly the iron(III) citrate (1.22 g) was added to 100 ml of water and stirred over heat until
dissolved. After cooling concentrated HCl (37 %; 5 ml) was added to the solution and the solution
turned to a straw-yellow color. The rest of the compounds (ZnCl₂: 65.5 mg; CoCl₂·6 H₂O: 100 mg;
Na₂MoO₄·2 H₂O: 100 mg; CaCl₂·2 H₂O: 50 mg; CuCl₂·2 H₂O: 63.5 mg; H₃BO₃: 25 mg) was added
to the solution, which was finally made up to 200 ml with water, sterile filtered and stored at room temperature.

All media for the cultivation of *E. coli* expressing CYP3A4 contained 100 µg/ml of ampicillin, those for expressing CYP2D6 100 µg/ml of ampicillin plus 25 µg/ml of chloramphenicol. Immediately before inoculation, liquid media were spiked with 1 ml/l each of the relevant antibiotic(s) stock solution(s). To the agar medium, the antibiotics stock solution(s) were added after cooling to 50 °C just before pouring them into the petri dishes. LB broth and LB agar were dissolved in deionized water, the pH adjusted to 7.2 and autoclaved at 121 °C for 20 minutes.

For the 25 l main culture modified terrific broth (MTB) was used as follows: Peptone (300 g) and yeast extract (600 g), the latter together with 25 ml of trace element solution, were each dissolved in 2 l of water containing 1 ml of antifoam 204 agent. After adjusting the pH to 6.8 and autoclaving at 121 °C for 20 min, the solutions were pumped into a sterile 50 l polyethylene bag used for cultivation (Biostat Cultibag RM 50, Sartorius BBI Systems GmbH, Melsungen, Germany) under sterile conditions using a peristaltic pump. A second solution was prepared by dissolving K₂HPO₄ (235 g), KH₂PO₄ (55 g), glycerol (250 g), ampicillin stock solution (25 ml) and thiamine stock solution (25 ml) in 5 l of water and, after adjusting the pH to 6.8, it was pumped into the wave bag through a sterile filter capsule type Sartobran 150 containing two sequential membranes, pore diameters 0.45 and 0.2 µm, (Sartorius, Göttingen, Germany, no. 5231307H4-00) using a peristaltic pump. Finally, the medium was completed by pumping in 16 l of demineralized water through the sterile filter capsule used before.

**Fermentative production of *E. coli* cells with CYP3A4 activity**

From frozen glycerol stocks (-80 °C, see below) of *E. coli* JM109 co-expressing rh CYP3A4 and rh P450-reductase some material was streaked onto a LB agar plate containing ampicillin. After incubation at 37 °C for 16 h, single colonies were used to inoculate the preculture consisting of 400 ml of LB broth with pH 6.8 distributed into two 500 ml shake flasks containing ampicillin as
mentioned above. The preculture was placed in an orbital shaker set at 37 °C and a rate of 220 rpm until it reached an OD600 between 0.7 and 1. Then it was stored overnight in a refrigerator at +4 °C.

The main culture was performed in a BioWave 50SPS bioreactor. In this system, a disposable polyethylene bag, here with 50 l of total and 25 l of working volume, serves as the reactor which is rocked on a temperature controlled table at 30 °C. Oxygen is supplied via a stream of sterile air through the headspace of the bag. Under conditions recommended for the cultivation of E. coli (42 rocks / min, 10.5° rocking angle) and an airflow of 0.5 l/min under supplementation of 10 % (v/v) of pure oxygen, the CYP3A4 expressing E. coli-cell line provides cell densities (OD600 = 14 - 16) and CYP3A4 activities comparable to the ones obtained in shake flasks.

The main culture volume was 25 l of modified terrific broth with ampicillin inoculated with 1% v/v of preculture. Induction was performed at an OD600 of 0.7 - 1 by addition of 1 mM of IPTG and 0.5 mM of δ-aminolevulinic acid. The total cultivation time was around 24 h. The cells were centrifuged at 5000 rpm and 4 °C for 15 minutes using a GS-3 rotor in a Sorvall RC-5B refrigerated superspeed centrifuge (Sorvall, Kendro Lab. Products AG, Zürich, Switzerland). The pellet was resuspended in one tenth of the original main culture volume using PSE-buffer. The cells were sedimented by centrifugation and resuspended another two times. For adsorbing indole and other potential CYP inhibitors, 150 g of XAD16, regenerated with methanol and water before use, was added and the suspension was stirred with an overhead stirrer in an ice bath at 0 °C for 30 – 60 min and kept there overnight. Prior to use as biocatalyst, the XAD16 resin was removed from the cell suspension by filtration over gauze.

**Bioconversion on preparative scale and purification**

The preparative bioconversion was again performed applying the BioWave 50SPS bioreactor under the same incubation conditions as for the fermentation. The 10-fold concentrated cell suspension (2.5 l) was pumped into the 50 l wavebag and was supplemented with 100 ml of an EDTA solution (100 g/l, pH 7.5). After warming to 25 °C, a solution of the lactate salt of panobinostat (205 mg) in
200 µl of DMSO, mixed rigorously with 200 ml of a suspension (5 % w/v) of highly dispersed silicon dioxide (Merck # 113126) in water, was added. Since no difference in metabolite formation was observed between the samples taken after 90 min and 210 min by HPLC, the reaction was stopped by addition of 200 g of XAD16 adsorber resin. After 2 h all metabolite had adsorbed (HPLC analysis) and the resin was recovered from the combined suspensions by filtering over gauze followed by washing with 2 l of water.

The suspended resin was extracted 4 times with methanol (1 l) and 2-propanol (1 l) at room temperature under gentle shaking for 0.5 – 1 h followed by vacuum filtration through a glass fiber filter. The combined solvents were removed in vacuo, the residue suspended in methanol, mixed with 15 g of diatom granulate (Isolute HM-N, Separtis AG, Grellingen, Switzerland), and by evaporating the solvent under reduced pressure the substances to be separated were absorbed to the Isolute material. The compounds were separated on a Labochrom AMC glass column (28 x 350 mm, Labomatic Instruments AG, Allschwill, Switzerland) filled with Lichroprep RP-18, 40 - 63 µm. For the first chromatographic run, the crude extract absorbed on Isolute was dry filled in a pre-column (20 x 250 mm, Büchi Labortechnik AG, Flawil, Switzerland), which was pre-filled to 50 % height with Lichroprep RP-18. The metabolites were purified in two consecutive runs, each linear gradient from 3 % mobile phase B to 30 % B in 50 min. with 30 ml/min flow rate. The first run was under acidic conditions using 10 mM aqueous formic acid (A) / ACN (B) as mobile phases, the second runs (separate runs for all compounds) with mobile phase A: 5 mM aqueous ammonium formate / ammonia, pH 7.1; mobile phase B: ACN. The solvents of the metabolite containing fractions were removed in vacuo, and the residues were dried in high vacuum. A total of 5 mg M1 and 12 mg M2 were obtained.

In the same way, using E. coli JM109 co-expressing rh CYP2D6 and rh P450 reductase, the metabolites M4 (268 mg) and M7 (22 mg) were produced, also with a biotransformation time of 210 min.

**Analytical bioconversion experiments**
Suspensions of *E. coli* cells expressing 14 different CYP isoenzymes were diluted with PSE buffer to constant wet biomass (100 mg/ml). These suspensions (0.5 ml) were mixed with 25 µl of an DL-isocitrate solution (0.46 g Na$_3$C$_6$H$_5$O$_7$ / ml) and 10 µl of substrate stock solution (5 mg/ml in ACN / DMSO 19 : 1) in 2 ml Eppendorf caps, closed with stoppers to ensure aeration (steristoppers no. 10 from Herenz, Hamburg, Germany) and incubated at 30 °C and 1100 rpm in a temperature-controlled Eppendorf mixer (Eppendorf-Vaudaux- AG, Schönenbuch, Switzerland) for 0.5 h or 1 h. The reactions were stopped by mixing with 0.5 ml of ACN / methanol 1 : 1 for 5 min, centrifuged in an Eppendorf 5424R-centrifuge and the supernatant was subjected to LC-MS analysis.

The reaction conditions for the incubation with supersomes were: HEPES (185 mM, pH 7.5), LBH589 (10µM, stock solution 1 mM in ACN), MgCl$_2$ (6.65 mM, including the quantity from the NADPH-regeneration system), NADP$^+$·Na$_2$ (0.5 mM), DL-isocitrate·Na$_3$ (2.54 mM), isocitric dehydrogenase (5 µl/ml), total volume 1 ml, incubation in an Eppendorf Thermomixer at 30 °C without shaking. The reactions were started by addition of the NADPH regeneration system in form of a 20-fold concentrated stock solution (NADP$^+$·Na$_2$, MgCl$_2$ (58 mM), DL-isocitrate·Na$_3$, isocitric dehydrogenase) and initial gentle vortexing. After incubation for 1 h the assays were extracted by shaking in the presence of one volume of ACN for 10 min and centrifugation at 21000 g in an Eppendorf 5424R-centrifuge for 3 min. The supernatants were subjected to LC-MS analytics.

**Mini-preparative biotransformation and micro-preparative isolation of M8**

For small-scale isolation of metabolite M8 an incubation with CYP2D6 expressing *E. coli* cells was performed in the same way as in the analytical bioconversion experiments, but using eight 10 ml aliquots of cell suspensions with 200 g/l wet biomass in 100 ml Erlenmeyer flasks incubated at 30 °C on an orbital shaker (220 rpm; 50 mm shaking amplitude) and applying citrate solution instead of isocitrate. The reaction was stopped after 30 min by addition of an equal volume of ACN. Then it was centrifuged, concentrated *in vacuo* to a volume of approximately 10 ml at 40 °C using a Cyclone high speed evaporator (Prolab Instruments GmbH, Reinach, Switzerland) yielding in average 5 % of M8.
The residue was diluted with an equal volume of water and centrifuged at 4000 g for 20 min in an Eppendorf centrifuge 5810R. Solid phase extraction was performed with Plexa 200 mg/6 cc cartridges (Varian Inc. Palo Alto, CA, USA) preconditioned with 3 ml MeOH and conditioned with 3 ml H2O. Aliquots of 10 ml sample were applied to the cartridges which were washed twice with 3 ml H2O / MeOH 95 : 5 (v/v). The product mixture was eluted with 3 ml ACN, pooled, evaporated to dryness with the Cyclone high speed evaporator and reconstituted in 1.2 ml H2O / ACN 90 : 10 (v/v) for micro-preparative isolation of M8.

The micro-preparative HPLC/MS system consisted of a Prominence UFLC system (SIL-20AC autosampler, LC-20AB pumping system, DGU-20A online solvent degasser, CBM-20A system controller; Shimadzu Corp., Reinach, Switzerland) with a column heater (Portmann, Biel, Switzerland) using the following LC conditions: Column XBridge BEH130 C18 3.5 µm; 4.6 x 150 mm (Waters); mobile phase A: H2O + 0.1 % HCOOH; mobile phase B: ACN + 0.1 % HCOOH; gradient 0 min 5 % B, 2 min 5 % B, 13 min 35 % B, 15 – 18 min 95 % B; 30 °C; 0.5 ml/min; injection volume: 100 µl. The chromatographic flow was splitted with a static T-union. The major portion (95 %) was directed to a valve switching system composed of a Cheminert 6-port bi-position divert valve (VICI AG international, Schenkon, Switzerland) used for fraction collection. The minor part of the chromatographic flow (5 %) was introduced directly into the ion source of an ion trap mass spectrometer LTQ XL (Thermo Scientific) equipped with a heated electrospray interface operating in the positive ion mode as follows: sheath gas / auxiliary gas / sweep gas: 10/1/3 units; spray voltage 4.0 kV; heated metal capillary 275 °C; scan range 150 to 2000 Da; microscans: 2; max. inject time 50 ms. The system was optimized for m/z 350 [M + H]+ of parent drug LBH589. The complete micro-preparative HPLC/MS system was controlled by Xcalibur software version 2.0 integrating the specific Shimadzu instrument driver version 5.4. This gave a programmed external event to the CBM-20A instrument controlling the cutoff process by switching the divert valve. The isolation of metabolite M8 was controlled by monitoring 366 [M + H]+ and also specific MS² fragments (CID with nitrogen gas; normalized collision energy 25 % and isolation...
width 1.5 Da) and the LC effluent was collected during a defined time-window of 10.60 to 10.95 min. The collected sample was evaporated to dryness under vacuum at 43 °C for 2.5 h with a Speedvac plus SC210A concentrator (Savant Instruments, Holbrook, NY, USA). A standard solution of LBH589 was used for semi-quantification of the isolated metabolite M8 (selected ion trace at m/z 366 and m/z 350 respectively), considering equal MS response factors of the two compounds. The estimated amount of M8 was approximately 30 µg.

**HDAC Inhibition**

The *in vitro* assay was performed as described by Sambucetti (Sambucetti *et al* 1999) and modified for isoform selectivity. With the exception of HDAC4 that was purchased from BPS Bioscience (San Diego CA, USA) the isoenzymes were prepared in-house: HDAC1, HDAC3 and HDAC6 were expressed in HEK-293, flag-tagged, HDAC2 in SF21, his-strep-tagged and HDAC8 in SF9, tag cleaved.

**Results**

Occasionally CYP enzymes, which are of low abundance in the liver or not present in this organ at all (e.g. CYP1A1), are the most efficient biocatalysts for drug metabolites synthesis (Schroer *et al* 2010). Therefore, all 14 different rh CYPs functionally co-expressed with rh P450-reductase in *Escherichia (E.) coli* were screened for metabolite production on an analytical scale (whole cell biotransformations) and investigated by LC-MS/MS (Table 1). The UV curve at 280 nm was used for a first quantification, as no standard compounds were available at that time. UV detection is commonly used in early metabolism, as the response factors of metabolites are mostly similar to the parent compound (Ramanathan *et al* 2010). Panobinostat reacted in high yields with the host strain JM109 and even in higher yields with some CYPs. After prolonged exposure – as typically used for preparative conversion – a complete conversion of panobinostat to metabolites was observed. To differentiate between the host and CYP reactions short reaction times were applied for the analytical
experiment. Metabolites were identified by similar UV spectra, appropriate mass difference to the parent compound and related MS/MS spectra.

A total of 8 major metabolites were found by LC-MS/MS (Scheme 1), three of which were produced by the host as well. Hence, the host *E. coli* strain JM109 performed two reactions and the combination of these two: The faster reaction was the reduction of the hydroxamic acid to the amide forming M4 from LBH589 as well as M2 starting from M1. The considerably slower reaction in the host was the ring closure forming M1 from LBH589 or M2 from M4. This ring closure reaction yielding M1 and M2 ran at a higher rate in several CYPs, notably 2A6, 3A4 and 3A5. The enzymes responsible for the *E. coli* host reactions are unknown, the more since native *E. coli* carries no CYPs (Kelly *et al* 2003). The enzyme CYP2D6 and, to a lower extent, 2C19 was responsible for the production of several metabolites with hydroxylation at the indole moiety (M6, M7 and M8).

Supersomes™ are microsomes prepared from insect cells infected by baculovirus for co-expressing individual rh CYP isoenzymes plus rh P450 reductase and therefore, have no interfering *E. coli* enzymes. Incubation with CYP3A4 supersomes revealed M1 as the sole metabolite in 6 % yield (Table 1). In contrast to the whole cell biotransformation with CYP3A4 in *E. coli* no reduction of the hydroxamic acid group occurred. Incubation with CYP2D6 supersomes showed M6 in 6 % yield and M1 in 1 % yield.

The CYP isoform 3A4 was selected for preparative synthesis of metabolites, as the oxidative metabolism of LBH589 in humans is mainly mediated by this CYP (Clive S, Woo MM, Stewart M, Nydam T, Kelly L, Squier P, Kagan M. Characterizing the disposition, metabolism, and excretion of an orally active pan-deacetylase inhibitor, panobinostat, via trace radiolabeled 14C material in advanced cancer patients. in preparation). Whole cell biotransformation with CYP3A4 and subsequent purification yielded metabolites M1 and M2 in 5 mg to 12 mg quantities.

Additionally CYP2D6 was selected for the remaining hydroxyl metabolites not covered by CYP3A4 and this experiment gave M4 (268 mg) and M7 (22 mg). The minimal threshold for
isolation of metabolites from biotransformations in the scale applied (200 mg) is about 5% conversion. Therefore, it was not possible to isolate some minor metabolites.

The isolated metabolites were compared to panobinostat for enzymatic HDAC activity and the results were compiled in Table 5.

Structure elucidation

The structures of the metabolites M1, M2, M4, M7 and M8 were elucidated on the basis of different homo- and heteronuclear 2D spectra (Tables 2 & 3) and HR-MS results (Table 4). The MS/MS fragmentation of the other, not isolated, metabolites were analog either to the parent compound panobinostat or to M1 (Table 4) and allowed to propose structures for metabolites M3 and M6. Compound M5 was identified by comparison with synthetic standard.

Structure of M4. The compound M4 had one oxygen less than panobinostat according to HR-MS. This had to be the oxygen of the hydroxamic acid, as the molecule had only two oxygens and as the $^{13}$C chemical shift of the carbonyl-23 was well in range for an amide. All other $^{13}$C- and $^1$H-NMR data are very similar to the parent compound panobinostat (data not shown).

Structure of M2. HR-MS revealed the same elemental composition as the parent compound panobinostat. The $^{13}$C- and $^1$H-NMR data of the cinnamic amide part were virtually identical to M4. The aromatic protons in positions 4 to 7 and the methyl group showed a high field shift due to the reduction of the parent indole ring into an indoline type ring system. Moreover the geminal CH$_2$ protons of C-11, C-12 and C-14 were splitted, as they possessed adjacent chiral centers. The structure of M2 was supported by $^1$H, COSY, HSQC and HMBC experiments (Fig. 1).

Structure of M1. The metabolite M1 had one oxygen more than M2. The $^1$H-NMR spectral data of the two compounds were very similar with the exception of the missing amide N-H signals and an additional OH signal at 5.28 ppm. These findings left only the amide nitrogen as an attachment
point of this oxygen leading to the hydroxamic acid analog of M2. Therefore this moiety remained unchanged from the parent compound panobinostat.

**Structure of M8.** The compound M8 had one oxygen more than M2 according to HR-MS. The $^{13}$C- and $^1$H-NMR data were identical except for the aromatic ring at the left part of the molecule suggesting a hydroxylation. This aromatic hydroxylation was deduced by $^1$H- and $^{13}$C-shifts of the aromatic protons and carbons. An oxidation at C-5 or C-6 would have provided the same aromatic coupling pattern, but the $^1$H- and $^{13}$C-shifts were in accordance only with an H-6 substitution. Homo- and heteronuclear 2D spectra supported unambiguously the determined tricyclic structure (Fig. 1). Because the 3-OH proton was very broad, an NOE could not be seen to CH$_3$-10. The relative stereochemistry at C-2 and C-3 had to be cis, as a trans connection of the two 5-membered rings could be excluded due to the enhanced rigidity of the indoline ring by its endocyclic aromatic bond. In conclusion, there was no evidence seen in the NMR spectra for the entropically unfavorable trans-isomer.

**Structure of M7.** The compound M7 had one oxygen more than M4 according to HR-MS and the two metabolites had very similar $^1$H-NMR spectra with the exception of the indole ring. The similar coupling pattern of that moiety to the one of M8 and the chemical shifts suggested a hydroxylation at carbon-6.

**Discussion**

The product ion spectrum of M1 is displayed in Figure 2. The dominant ion at m/z 144.1 had an elemental composition of [C$_{10}$H$_{10}$N]+ and was presumably formed by a rearrangement (Figure 4). The rearrangement of indole derivatives to form very stable protonated quinoline ions has been reported with EI ionization and CID fragmentation (Stagno d’Alcontres et al 1973; Prokai et al 1993). The analogous ion to m/z 144.1 was also formed in fragmentation of 6 in low intensity (Ronsein et al 2009). Therefore, the ring that had been closed during CYP3A4 metabolism reopened in CID fragmentation, so that a simple interpretation of the fragmentation pattern might
have lead to erroneous metabolite structures. Notably the fragment $m/z$ 144 of M1 corresponded formally to a loss of CH$_2$ compared to $m/z$ 158 of panobinostat suggesting -wrongly – that hydroxylation at carbon-11 might have occurred. The proposed fragmentation mechanism was supported by HR-MS on an orbitrap and MS$^3$ experiments. The fragmentation of the parent compound panobinostat (Fig. 3) showed two characteristic fragments: $m/z$ 158 where cleavage of the bond 12-13 next to the secondary amino group had occurred and $m/z$ 176, as in Figure 4.

The proposed mechanism of formation of the metabolites is shown in Scheme 1. Epoxidation might be the first step catalyzed by CYP3A4 and other CYP enzymes. This epoxide, in turn, reacts via an intramolecular nucleophilic attack of the secondary amino group to the cyclized hydroxy-methyl-tetrahydropyrrolo-indole moiety. The reaction seems to proceed with several substitutions at the cinnamic acid. This epoxide should be very short living, as there are no indications of its presence neither in the in vitro experiments here nor by hepatotoxic adverse effects in the clinical studies (Prince et al 2009).

The biological activity of the isolated metabolites was compared to panobinostat (Atadja 2009) for enzymatic HDAC activity (Table 5): Metabolite M4 where the zinc-binding hydroxamic acid had been reduced to an amide was virtually inactive in all the tested HDACs. M2 and M7 inhibited HDACs in the low micromolar range. Such minor inhibition might be due to small unknown impurities in the isolated compounds. The inhibitory activity of M1 for HDAC8 and the class II enzymes HDAC4 and HDAC6 was in a similar range to panobinostat. Class III HDACs were not tested.

A cyclic melatonin metabolite 3 (Figure 5) with a very similar tricyclic structure to M1 was proposed by Tan based on 2D COSY $^1$H-NMR studies (Tan et al 1998). Later Agozzino showed that this structure was not correct, as it was not compatible with the $^{13}$C-NMR and that the metabolite had structure 4 (Agozzino et al 2003). Chemical oxidation of tryptophan to the tricyclic product 6 can be achieved by various methods including oxidation with peroxyacetic acid (Savige
W 1975), by photooxidation followed by reduction (Nakagawa et al 1981), by horseradish peroxidase (Nguyen et al 1986a) or electrochemically (Nguyen et al 1986b) and its $^{13}$C-NMR has been described (Yang et al 2003). The tricyclic moiety is furthermore quite common in natural products, for example brevianamide E (13) which belongs, together with the paraherquamides, to an unusual class of prenylated indole-derived alkaloids produced by *Penicillium* sp. and *Aspergillus* sp. (Williams 2002).

Cyclized metabolites are quite rarely described. An example is the anti-inflammatory drug indomethacin 7 (Figure 5) where an intramolecular lactonized product is formed upon incubation with rat microsomes and an 2,3-epoxide is proposed as an intermediate *in vitro* (Li et al 2005; Komuro et al 1996). The oxidative ring closure of the estrogen receptor modulator 9 proceeds in a CYP3A4 mediated reaction (Zang et al 2005) presumably via a radical coupling. Also for the major human metabolite 12 of the 5-hydroxytryptamine$_{1B}$ receptor antagonist elzasonan 11 a radical mechanism of formation is proposed (Kamel et al 2010). Other examples of cyclized metabolites are aminals, where the cyclized metabolite is formed from an aldehyde intermediate. A recent example is the inhibitor of the human epidermal growth factor BMS-690514 14 where a substituted pyrrolo[2,1-f][1,2,4]triazine ring is opened and the aldehyde formed binds to a different, more nucleophilic, nitrogen in the molecule with ring closure (Hong et al 2011).

In conclusion the isolation of five panobinostat metabolites produced under catalysis of rh CYP3A4 and 2D6 allowed us to establish their unusual and unexpected structures. The amount isolated was sufficient not only to elucidate the structure unambiguously, but also to investigate their biological activity *in vitro*. Furthermore, they were provided as reference material to clarify animal or human metabolism *in vivo*. The metabolite M2 corresponded to the one that had a prolonged $T_{\text{max}}$ in rat plasma as mentioned in the introduction. The human metabolic pathways of panobinostat have been established recently (Clive S, Woo MM, Stewart M, Nydam T, Kelly L, Squier P, Kagan M: Characterizing the disposition, metabolism, and excretion of an orally active pan-deacetylase inhibitor, panobinostat, via trace radiolabeled $^{14}$C material in advanced cancer patients; in
preparation): It is very complex with about 80 distinct metabolites that include oxidation of the methyl indole ring as described in this publication, a number of distinct glucuronidations, and multiple biotransformations of the hydroxamic acid containing side chain, some of which were not observed in this study. These multiple pathways occurring alone and in multiple combinations lead to the observed complexity. Knowledge of the soft spot of metabolism contributed to the design of novel DAC inhibitors with improved metabolic properties and no dose-limiting cardiac effects (Shultz et al 2011).
Acknowledgements

We thank Thomas Lochmann for the NMR measurements and interpretation of the spectra, to Fabian Eggimann for technical assistance and to Dr. Eric Francotte, Dr. Oreste Ghisalba and Dr. Stephan Lütz for the continuous support of this work. Thanks are also due to Dr. Mark Kagan for sharing his DMPK results with us.

Authorship contributions

Participated in research design: Fredenhagen, Kittelmann, Atadja, Shultz

Conducted experiments: Fredenhagen, Kuhn, Kühnöl, Délémonté, Wang

Contributed new reagents or analytic tools: nobody

Performed data analysis: Fredenhagen, Kittelmann, Oberer, Délémonté, Aichholz, Atadja

Wrote or contributed to the writing of the manuscript: Fredenhagen, Kittelmann, Oberer, Kuhn, Délémonté, Aichholz
References


Legend for Schemes

**Scheme 1**: Structure and atom numbering of panobinostat (LBH589) and CYP3A4 or 2D6 metabolites and putative epoxide intermediate. **M3** and **M6** are proposed structures. The structures represent the relative configuration at centers 2 and 3. The naming of the metabolites (**M1** …) is different from the one used internally in Novartis and therefore also from other publications on panobinostat metabolism.

Legend for Figures

**Fig 1**: Significant HMBC connectivities (→) and ROESY correlations (dashed double arrows) for compounds **M2** (left) and **M8**.

**Fig 2**: CID product ion spectrum of metabolite **M1**

**Fig 3**: CID product ion spectrum of parent panobinostat

**Fig 4**: Proposed CID fragmentation mechanism of metabolite **M1** and accurate mass measurements

**Fig 5**: Ring forming metabolism and the natural product brevianamide E (13)
Table 1: Percentage of uncorrected peak areas of Panobinostat (LBH589) and metabolites after exposure to rh CYPs for 1 hour (DAD at 280 nm)

<table>
<thead>
<tr>
<th>rh CYPs in E. Coli</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
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<td>3A4 + Cyt b₅ a)</td>
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</table>

| rh CYPs in supersomes |    |    |    |    |    |    |    |    |        |
|-----------------------|----|----|----|----|----|----|----|----|        |
| 3A4                   | 6.1|    |    | 94 |    |    |    |    |        |
| 2D6                   | 0.8|    |    | 94 |    |    |    |    |        |

a) Cyt b₅ = Cytochrome b₅ (Voice et al 1999)
Table 2: $^{13}$C assignments and HMBC correlations for metabolites M2, M4 and M8

<table>
<thead>
<tr>
<th>Atom Nr.</th>
<th>M2 shift in ppm</th>
<th>HMBC correlations</th>
<th>M4 shift in ppm</th>
<th>HMBC correlations</th>
<th>M8 shift in ppm</th>
<th>HMBC correlations</th>
</tr>
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<td>2</td>
<td>88.4 H-10</td>
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<td>133.1 NH-1, H-10, H-11</td>
<td></td>
<td>88.7 NH-1, H-10, H-11</td>
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<td>87.7 H-N1, H-4, H-10, H11</td>
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<td>117.7 H-6</td>
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<td>120.7 H-4</td>
<td>158.6 H-4, H-5, H-7</td>
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<td></td>
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<tr>
<td>7</td>
<td>107.3 H-5</td>
<td>111.0 H-5, H-4</td>
<td>95.3 H-5</td>
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<td>8</td>
<td>150.4 H-4, H-6</td>
<td>135.8 H-N1, H-4, H-6</td>
<td>152.0 H-4</td>
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<td>132.7 H-N1, H-7</td>
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<td>123.4 NH-1, H-5, H-7, H-11</td>
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<td>48.5 H-11, H-14</td>
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<tr>
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a) for atom numbering see Scheme 1

b) The $^{13}$C shifts were extracted from HSQC and HMBC correlation peaks, except those of M4
Table 3: $^1$H assignments for metabolites M1, M2, M4, M7 and M8. Coupling constants are given in Hertz.

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<tr>
<th>Atom Nr. $^a$</th>
<th>M1</th>
<th>M2</th>
<th>M4</th>
<th>M7</th>
<th>M8</th>
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<tr>
<td>1</td>
<td>5.74 s</td>
<td>5.75 s</td>
<td>10.64 s</td>
<td>10.23 s</td>
<td>5.69 s</td>
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<td>7.09 d, 8.0</td>
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<td>6.95</td>
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<td>1.97 m</td>
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$a$) for atom numbering see Scheme 1


### Table 4: UPLC retention time, accurate mass data and MS/MS fragments

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<th>Compound</th>
<th>Retention Time</th>
<th>Composition</th>
<th>Measured Mass</th>
<th>Calculated Mass</th>
<th>error (ppm)(^a)</th>
<th>MS/MS Fragments (bold = base peak)</th>
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<td>366.1812</td>
<td>1.7</td>
<td>348.1, 219.1, 205.0, 176.0, 162.1, <strong>144.1</strong></td>
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<tr>
<td>M2</td>
<td>10.36</td>
<td>C(<em>{21})H(</em>{24})N(_3)O(_2)</td>
<td>350.1858</td>
<td>350.1863</td>
<td>1.4</td>
<td>332.1, 203.1, 189.1, 160.1, <strong>144.1</strong></td>
</tr>
<tr>
<td>M3</td>
<td>12.35</td>
<td>C(<em>{21})H(</em>{23})N(_2)O(_3)</td>
<td>351.1702</td>
<td>351.1703</td>
<td>0.3</td>
<td>333.2, 204.1, 190.1, 162.1, 158.1, <strong>144.1</strong></td>
</tr>
<tr>
<td>M4</td>
<td>13.26</td>
<td>C(<em>{21})H(</em>{24})N(_3)O</td>
<td>334.1911</td>
<td>334.1914</td>
<td>0.9</td>
<td>317.0, 189.0, <strong>158.0</strong></td>
</tr>
<tr>
<td>M5</td>
<td>15.25</td>
<td>C(<em>{21})H(</em>{23})N(_2)O(_2)</td>
<td>335.1750</td>
<td>335.1754</td>
<td>1.2</td>
<td>318.2, 294.0, <strong>158.2</strong></td>
</tr>
<tr>
<td>M6</td>
<td>9.35</td>
<td>C(<em>{21})H(</em>{24})N(_3)O(_3)</td>
<td>366.1812</td>
<td>366.1812</td>
<td>0.2</td>
<td>349.2, 337.2, 205.1, <strong>174.1</strong>, 162.1</td>
</tr>
<tr>
<td>M7</td>
<td>9.94</td>
<td>C(<em>{21})H(</em>{24})N(_3)O(_2)</td>
<td>350.1859</td>
<td>350.1863</td>
<td>1.0</td>
<td>333.2, 321.2, <strong>174.3</strong>, 162.2</td>
</tr>
<tr>
<td>M8</td>
<td>7.80</td>
<td>C(<em>{21})H(</em>{24})N(_3)O(_3)</td>
<td>366.1808</td>
<td>366.1812</td>
<td>1.0</td>
<td>348.2, 203.0, 185.2, <strong>160.1</strong></td>
</tr>
</tbody>
</table>

\(^a\) ppm: \((\text{measured mass} - \text{calculated mass}) \times 10^6 / \text{calculated mass}\)
Table 5: DAC inhibitory activity of major CYP metabolites and panobinostat (nanomolar IC<sub>50</sub>)

<table>
<thead>
<tr>
<th>HDAC Class</th>
<th>LBH589</th>
<th>M1</th>
<th>M2</th>
<th>M4</th>
<th>M7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>I</td>
<td>2.5</td>
<td>140</td>
<td>970</td>
<td>11,000</td>
</tr>
<tr>
<td>HDAC2</td>
<td>I</td>
<td>13</td>
<td>1,500</td>
<td>6,000</td>
<td>29,000</td>
</tr>
<tr>
<td>HDAC3</td>
<td>I</td>
<td>2.1</td>
<td>180</td>
<td>930</td>
<td>22,000</td>
</tr>
<tr>
<td>HDAC4</td>
<td>IIa</td>
<td>200</td>
<td>230</td>
<td>1,000</td>
<td>18,000</td>
</tr>
<tr>
<td>HDAC6</td>
<td>IIb</td>
<td>11</td>
<td>62</td>
<td>1,100</td>
<td>&gt; 30,000</td>
</tr>
<tr>
<td>HDAC8</td>
<td>I</td>
<td>280</td>
<td>920</td>
<td>23,000</td>
<td>22,000</td>
</tr>
</tbody>
</table>
Scheme 1

M5 → LBH589 → M3

Epoxide intermediate

M6 → M4 → M2

M7 → M8
Figure 1
Figure 2

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Figure 3
Figure 4

H transfer

$m/z$ 162.0918  
calc for $C_{10}H_{12}NO$: 162.0919

$m/z$ 205.0975  
calc for $C_{11}H_{13}N_{2}O_{2}$: 205.0977

$m/z$ 176.0710  
calc for $C_{10}H_{10}NO_{2}$: 176.0712

$m/z$ 144.0812  
calc for $C_{10}H_{10}N$: 144.0813
Figure 5

2 Melatonin \( \text{CH}_2\text{CO} \quad \text{H} \quad \text{CH}_3\text{O} \)

5 Tryptophan \( \text{H} \quad \text{COOH} \quad \text{H} \)

3 \( \text{CH}_3\text{CO} \quad \text{H} \quad \text{CH}_3\text{O} \)

4

7

8

9

10

11

12

13

14

15