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Bioorganic & Medicinal Chemistry 14 (2006) 1558–1564
Bioorganic & Medicinal Chemistry

In vitro oxidative metabolism study of (−)-rhazinilam

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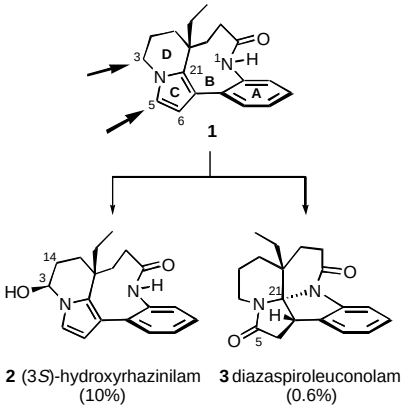
Received 15 June 2005; revised 30 September 2005; accepted 4 October 2005
Available online 27 October 2005

Abstract—Metabolism studies were conducted in order to investigate the reasons for the in vivo lack of activity of (−)-rhazinilam **1**, an original poison of the mitotic spindle. Bioconversion by *Beauveria bassiana* strains, rat and human liver microsomes allowed the identification of metabolites **2**, **3**, and **4** oxidized in positions 3 and 5 of rhazinilam. Further experiments indicated that CYP2B6 was the main CYP responsible for the oxidation of **1** by human liver microsomes. All isolated metabolites were markedly less active than rhazinilam in vitro, which might explain its in vivo inactivity.

1. Introduction

(−)-Rhazinilam **1** (Scheme 1) is a natural compound isolated from various *Apocynaceae*, whose tetracyclic structure possesses an axially chiral phenyl-pyrrole subunit bridged by a nine-membered lactam ring.[1] It was found to have unique antimitotic properties, with in vitro inhibition of both microtubule assembly and disassembly, and the formation of abnormal tubulin spirals.[2,3] As a consequence of these tubulin-binding properties, rhazinilam showed in vitro cytotoxicity toward various cancer cell lines in the low micromolar range,[3] but no activity was found in vivo.[4] Regarding the original in vitro properties of rhazinilam, several groups have undertaken structure–activity relationships (SAR) aimed at increasing the in vitro activity and solving the problem of in vivo inactivity.[1] To date, only one analogue was found to be more active than rhazinilam on tubulin, however its cytotoxicity toward cancer cells was nearly identical.[5,6] These rather disappointing results prompted us to understand the reason for the in vivo lack of activity of rhazinilam. This inactivity may of course have many different origins. Among factors which could be ruled out, it was found that rhazinilam easily crossed Caco-2 cell monolayers, predicting a satisfying intestinal absorption in human.[7] Besides, significant compound solubility issues were not encountered in the in vivo assays.[4] In this paper, we would like to address another possible cause for the in vivo inactivity of rhazinilam. To this purpose, we describe the biotransformation of rhazinilam by cytochromes P450 (CYP) expressed in the fungi *Beauveria bassiana* or present in rat and human liver microsomes. The microbial system was chosen in order to produce rhazinilam metabolites on a larger scale for identification and biological testing purposes. These metabolites were then compared to those obtained from incubations with mammalian microsomes. Experiments using transfected human cell lines were also conducted in order to determine which particular CYP isoforms are responsible for the biotransformation of rhazinilam by human liver



Scheme 1. Biotransformation of (−)-rhazinilam **1** by *Beauvaria bassiana*.

Keywords: Rhazinilam; Metabolism; Tubulin; CYP.
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doi:10.1016/j.bmc.2005.10.015

microsomes. The results shed light on one possible reason for the in vivo inactivity of rhazinilam and suggest further interesting developments in medicinal chemistry.[8]

2. Results and discussion

2.1. Microbial metabolism

A screening of potent oxidative microorganisms in resting cell experiments showed that the fungi *B. bassiana* exhibited the highest rhazinilam bioconversion.[9] The crude extract obtained from incubation of 180 mg rhazinilam **1** with *B. bassiana LMA* (ATCC 7159), followed by extraction with dichloromethane, was directly purified by preparative HPLC (C18 symmetry Shield), giving two major metabolites **2** and **3** in 10% and 0.6% yield, respectively (Scheme 1). The low overall yield was ascribed to a loss of material during workup and purification. At this stage the process was not further optimized. The structure of the major metabolite (3*S*)-hydroxyrhazinilam **2** was deduced from usual 2D NMR experiments.

The (3*S*) configuration was determined in light of the small coupling constants ^{3}J(H-3–H-14) ≈ 2 Hz measured on the ^{1}H spectrum in acetone-d_6/D_2O, pointing to an equatorial position for H-3.[10] Despite the very small amount of metabolite **3** obtained (ca. 1 mg), it could be crystallized from acetone-d_6 and its structure could be assigned to diazaspiroleuconolam by X-ray crystallographic analysis (Fig. 1).[11] This intriguing pentacyclic molecule arises from oxidation of the pyrrole ring at C-5 and cyclization of the N-1 nitrogen onto the C-21 atom. A related compound, 6-chlorodiazaspiroleuconolam, was obtained previously by treatment of (−)-leuconolam **4** (Scheme 2) with concentrated HCl in methanol.[12] Leuconolam **4** is a natural product related to rhazinilam **1**, which was isolated from the same Malaysian Apocynaceae *Leuconotis eugenifolia*.[12]

Beauveria bassiana is known to induce a wide range of biooxidations through CYP enzymes and to mimic to

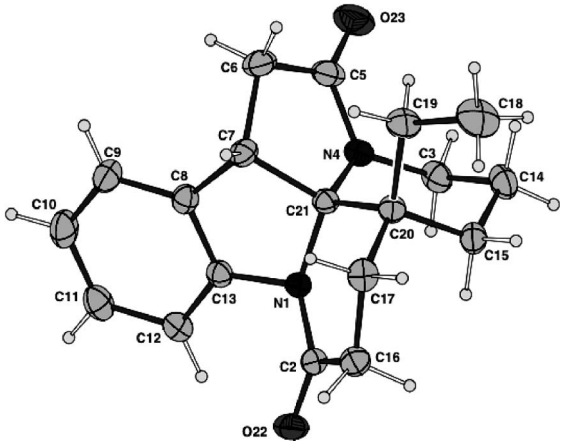
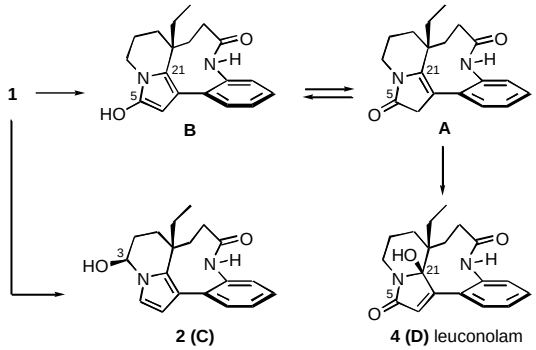


Figure 1. X-ray crystal structure of diazaspiroleuconolam **3** (30% thermal ellipsoids plot).



Scheme 2. Probable metabolites obtained by microsomal oxidation of (−)-rhazinilam **1**.

a certain extent the mammalian metabolism of xenobiotics.[13] In the present case, the bioconversion gives rise mainly to compounds mono-oxidized at the C-3 and C-5 positions α to the pyrrole nitrogen (black arrows, Scheme 1). The distances between the C-3 and C-5 positions, and the amide oxygen of rhazinilam are 5.4 and 4.8 Å, respectively, as measured from the X-ray structure.[14] These distances correspond to those reported in the literature for the biohydroxylation of nitrogen heterocycles containing amide groups by *B. bassiana*, which suggests that rhazinilam amide may also act as an anchoring point to the CYP, positioning the C-3 and C-5 centers in the vicinity of the enzyme porphyrin.[15] The stereoselectivity of the C-3 hydroxylation furnishing compound **2** can also be related to previous studies;[15] however, much caution should be exercised with regard to the interpretation of the regio and stereoselectivity of such biohydroxylations.[16]

2.2. Metabolism by rat liver microsomes

In parallel, rhazinilam biotransformation experiments were conducted first with rat and then with human liver microsomes. A small quantity of rhazinilam (25 nmol) was incubated with non-induced rat liver microsomes as well as liver microsomes from rats induced with phenobarbital (CYP2B inducer) or β-naphthoflavone (CYP1A inducer).[17] The conversion of rhazinilam was very fast with all microsomes, as indicated by the low amount of microsomes needed (0.7 mg of microsomal protein) and the short incubation time (15 min) necessary for these reactions. It is noteworthy that rhazinilam was rapidly metabolized by non-induced rat liver microsomes (33% degradation of 50 μM rhazinilam in 15 min, Fig. 2A′). In the absence of NADPH, no transformation was observed, which indicates that CYP are the enzymes responsible for rhazinilam conversion. Metabolites were analyzed by LC/MS under electrospray ionization (ESI). Most metabolites showed a *m*/*z* value of 311, corresponding to rhazinilam hydroxylation ($[M+O+H]^+$). Of the three major hydroxylation metabolites noted **A**, **B**, and **C**, metabolite **B** was by far the most abundant. Metabolites with a *m*/*z* value of 309, corresponding to the oxidation of a methylene to a ketone group ($[M+O-2H+H]^+$), were also found, albeit with a much lower abundance. Rat liver microsomes induced by phenobarbital were very active (78% degradation of rhazini-

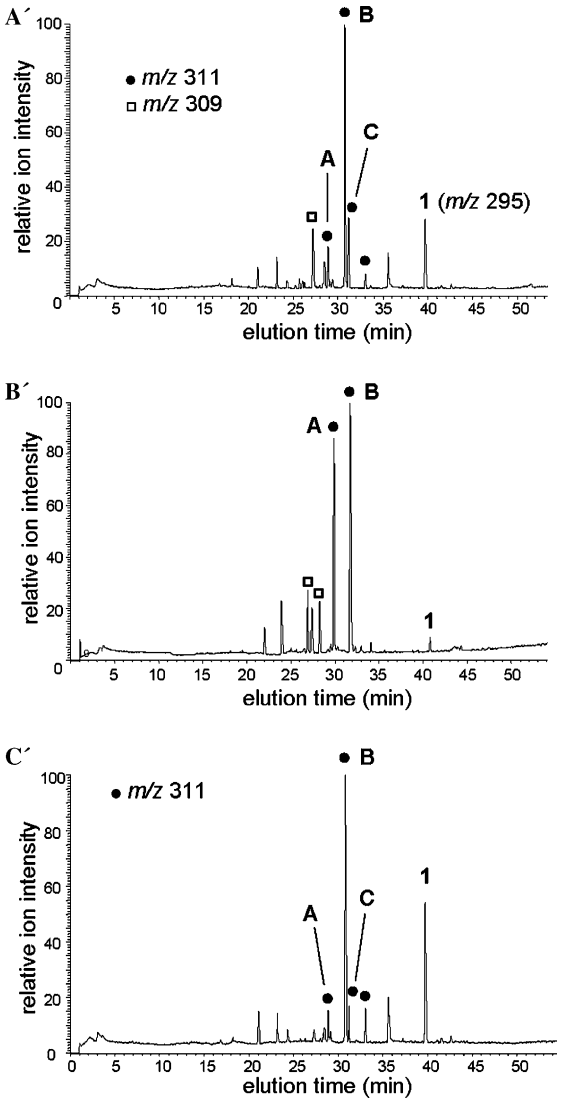


Figure 2. LC–MS analysis of the oxidation of rhazinilam by rat liver microsomes. (A′) Non-induced microsomes. (B′) Phenobarbital-induced microsomes. (C′) β-Naphthoflavone-induced microsomes. (C18 symmetry column, H_2O/CH_3CN gradient.) **A**, **B**, and **C** refer to selected metabolites.

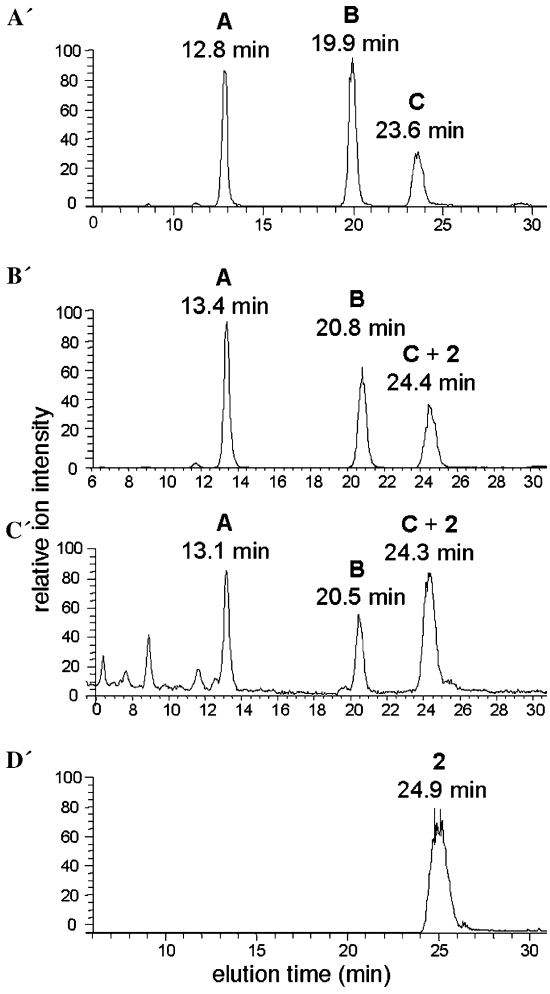


Figure 3. LC–MS analysis of the oxidation of rhazinilam by rat liver microsomes: co-injection experiments. (A′) Initial LC–MS profile. (B′ and C′) Co-injection with increasing amounts of (3*S*)-hydroxyrhazinilam **2**. (D′) Injection of (3*S*)-hydroxyrhazinilam **2** alone. C18 symmetry shield column, H_2O/CH_3CN 7/3.

lam in 15 min), and showed a different metabolism profile (Fig. 2B′), with essentially the production of metabolites **A** and **B** in a 1:1 ratio. Microsomes induced by β-naphthoflavone were less efficient (22% degradation in 15 min), with a profile similar to that of non-induced microsomes (Fig. 2C′), compound **B** being the major metabolite. These data strongly suggest that phenobarbital induces a CYP isoform with a high activity toward rhazinilam.

Co-injection experiments indicated that (3*S*)-hydroxyrhazinilam **2**, previously isolated from the bioconversion with *B. bassiana*, is identical to metabolite **C** (Fig. 3). These compounds also showed the same UV–vis (λ_{max} = 201 nm) and mass spectra (*m*/*z* = 311 for $[M+H]^+$, 293 for $[M-H_2O+H]^+$). In order to characterize the major metabolites **A** and **B** by NMR spectroscopy, a larger quantity of rhazinilam (1.9 mg in 66 fractions) was incubated with phenobarbital-induced microsomes. After HPLC separation and storage for 12 h at −22 °C, compounds **A** and **B** were re-injected individually, giving again a ca. 1:1 mixture of **A** and **B**, together with a small amount of a third compound **D** (Fig. 4A′). This behavior indicates that **A** and **B** are in equilibrium. Moreover, different experiments with the same or with different types of microsomes gave different ratios of **A** and **B**, **B** being always the major metabolite (see, for instance, Fig. 2). This indicates that **B** might be formed first and then might equilibrate slowly into the **A** + **B** mixture. The ^1H NMR of this mixture could not provide us with useful structural information, given the low amount of material. On prolonged storage at −22 °C (about 1 month), the **A** + **B** mixture transformed quantitatively into compound **D** having a *m*/*z* value of 327 ($[M+2O+H]^+$). The latter co-eluted with an authentic sample of leuconolam **4** (Fig. 4B′, see structure in Scheme 2) and both compounds showed the same UV–vis and mass spectra, suggesting that they were identical.

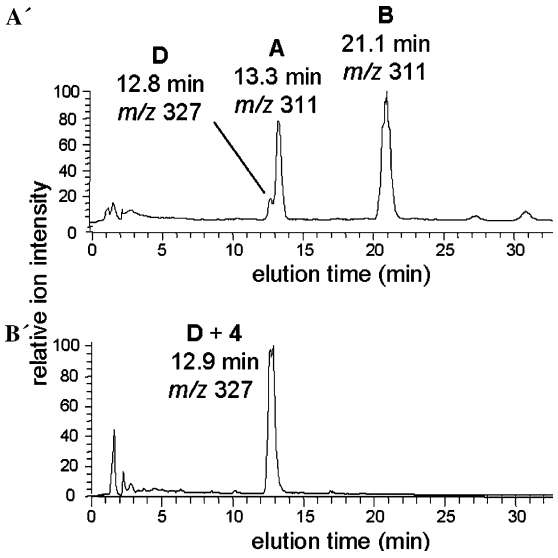


Figure 4. LC–MS analysis of metabolites **A** or **B** produced by phenobarbital-induced rat liver microsomes. (A′) Re-injection of compound **B** 12 h after separation. (B′) Co-injection of metabolite **D** with leuconolam **4** (C18 symmetry shield column, H_2O/CH_3CN 7/3).

The above results are summarized in Scheme 2. The oxidation of rhazinilam **1** by non-induced rat liver microsomes produces metabolite **C**, assigned to (3*S*)-hydroxyrhazinilam **2**, and a mixture of metabolites **A** and **B** which is converted upon storage to metabolite **D**, assigned to leuconolam **4**. We propose that compounds **A** and **B** could be keto-enol tautomeric forms of 5-hydroxyrhazinilam (the enol form **B** being formed first),[18] which is further oxidized upon storage to leuconolam **4**. Pentacyclic compound **3** obtained from oxidation of rhazinilam by *B. bassiana* (Scheme 1) could also originate from compounds **A**-**B** by cyclization of the amide N-1 nitrogen onto carbon C-21, which would reinforce the similarity between the fungal and rodent microsomal biotransformation routes.

2.3. Metabolism by human liver microsomes and CYP-expressing cells

The metabolism of rhazinilam by liver microsomes of six different human individuals displayed a profile similar to

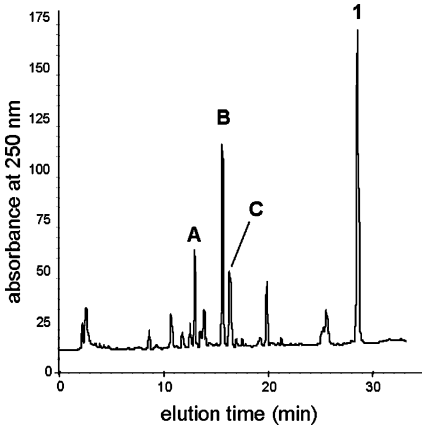


Figure 5. LC/UV analysis of the oxidation of rhazinilam by human liver microsomes (C18 symmetry column, H_2O/CH_3CN gradient).

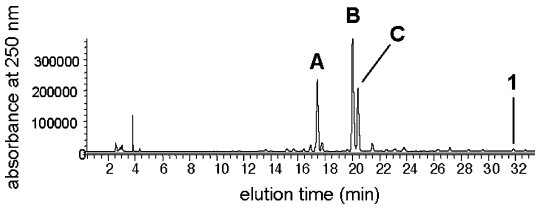


Figure 6. LC/UV analysis of the oxidation of rhazinilam by human Ad293 cells expressing CYP2B6 (C18 symmetry column, H_2O/CH_3CN gradient).

that of non-induced rat liver microsomes (Fig. 5). Control experiments again showed that no reaction occurred in the absence of NADPH. Metabolites **A**, **B**, and **C** were again the major metabolites produced by all human microsomes.[19] The relative amount of metabolites as well as the extent of rhazinilam conversion differed from individual to individual due to the variability of CYP expression in humans and ranged from 9 to 58% degradation of 50 μM rhazinilam in 15 min (mean value ± SD = 33.5 ± 24.5%, $n = 6$). Kinetic parameters were calculated and compared with those of rat liver microsomes. Comparable values were obtained: K_m values ranged from 30 to 50 μM for rat liver microsomes with V_{max} varying from 0.1 to 1.2 nmol metabolites formed in 1 min by 1 mg protein, whereas K_m and V_{max} in human liver microsomes were 20 to 170 μM and 0.3 to 3.1 nmol min^{-1} mg^{-1} protein, respectively. In order to identify the CYP isoforms responsible for the oxidation of rhazinilam by human microsomes, rhazinilam was incubated with Ad293 cell lines individually expressing the major human CYP isoforms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7), and compared with control Ad293 cells expressing no CYP.

Among these cell lines only those expressing CYP2B6, 3A4, and 3A7 showed significant oxidation activity, CYP2B6 being the most active and giving metabolites **A**, **B** and **C** (Fig. 6).[19] In all other cell lines no metabolite was formed. The conversion of rhazinilam into metabolites **A**, **B** and **C** by recombinant CYP2B6 expressed in cells was 32.7, 70.5, and 34.5 nmol $(24\ h)^{-1}$ by 5×10^6 cells. Similarly, CYP3A4 and 3A7 expressed in cells produced, respectively 16.8, 29.5, and 11.8, and 9.9, 36, and 15 nmol metabolites, indicating that CYP2B6 is roughly twice more active than CYP3A4 and 3A7 in the bioconversion of rhazinilam. The CYP3A subfamily is one of the most abundant in human, whereas CYP2B6 is expressed at variable levels.[20] This variability may account for the interindividual differences in rhazinilam metabolism observed among human microsomes. The high metabolism efficiency of CYP2B6 can be correlated with those of phenobarbital-induced microsomes, with minor qualitative differences in the metabolite profiles. Indeed, phenobarbital increases the expression of the rat CYP2B1 and 2B2, orthologous to the human CYP2B6.[21]

2.4. Biological activity

Two of the three metabolites identified in this study, (3*S*)-hydroxyrhazinilam **2** and leuconolam **4**, were

Table 1. Antitubulin activity and cytotoxicity of rhazinilam metabolites

Compound	Inhibition of microtubule assembly IC_{50} (μM)[a]	Inhibition of microtubule disassembly IC_{50} (μM)[a]	Cytotoxicity KB cell line IC_{50} (μM)[b]
1	18	2.0	0.7
2	110	16	8.1
4	Inactive[c]	Inactive[c]	Inactive[d]

[a] IC_{50} is the concentration of compound required to inhibit 50% of the rate of microtubule assembly or disassembly.
[b] IC_{50} is the concentration of compound corresponding to 50% growth inhibition after 72 h incubation.
[c] No inhibition up to 10 mM.
[d] No inhibition up to 100 μM.

evaluated on tubulin and on KB cancer cells (Table 1). Compound **2** was 6 and 8 times less active than rhazinilam on the inhibition of microtubule assembly and disassembly, respectively. It was also 12 times less cytotoxic than **1** toward KB cells. Besides, leuconolam **4** was inactive both on tubulin and on KB cells. These results are consistent with previous SAR data showing that the introduction of substituents on the pyrrole ring of **1** is deleterious to the binding to tubulin.[1] It is tempting to extrapolate the sharp decrease or the loss of in vitro activity of compounds **2** and **4** to the in vivo inactivity of rhazinilam observed in mice models.[4] Since different mammalian (rat and human) models were used for the present in vitro study, it is not certain that the same metabolites were responsible for the in vivo inactivity in mice. However, the similarity between the rat, human, and even microbial metabolisms suggests that the extrapolation to mouse is valid.

3. Summary and conclusions

In conclusion, we have shown that rhazinilam is easily oxidized at positions 3 and 5 by *B. bassiana* and by rat and human liver microsomes. The fungal and microsomal routes showed a number of similarities, such as the production of the same metabolite **2** and a similar putative pathway leading either to diazaspiroleuconolam **3** or to leuconolam **4**. We have also shown that the metabolism of rhazinilam by human microsomes is mostly due to the CYP2B6 isoform, which correlated with the high activity of phenobarbital-induced rat liver microsomes. Finally, we have shown that rhazinilam metabolites **2** and **4** are, respectively, much less active than rhazinilam and inactive in vitro. Therefore, the in vivo inactivity of rhazinilam might originate in a fast biotransformation leading to a number of metabolites which are both much less active than the parent compound and possibly more rapidly eliminated. These results suggest further research directions such as the introduction of substituents on positions 3 and 5 of rhazinilam in order to prevent its fast oxidation.[8]

4. Experimental

NMR spectra were recorded on Bruker AMX-300, AMX-500 or AMX-600 instruments at 295 K and calibrated using tetramethylsilane as an internal reference. Attributions were made on the basis of 2D experiments. The following abbreviations were used to designate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; ar, aromatic ring; eq, equatorial; ax, axial; Cq, quaternary carbon. IR spectra were recorded on a Perkin Elmer BX FT-IR spectrometer. High resolution mass spectra (HRMS) were recorded under ESI conditions at the Laboratoire de Spectrométrie de Masse (ICSN). HPLC purifications were performed on Waters systems equipped with a photodiode array detector (monitoring at 200–400 nm), using a C18 symmetry shield column (30 cm × 1.9 cm) by Waters, Inc. LC/MS spectra were obtained on a Surveyor Apparatus by Thermo Electron, Inc. equipped with a Finnigan LCQ Deca spectrometer under ESI conditions. LC conditions: C18 symmetry (25 cm × 0.46 cm) or C18 symmetry shield (15 cm × 0.46 cm) columns and a flow rate of 1 mL min^{-1}. Analyses were performed under UV detection or mass detection with scanning from 50 to 1000 Da. (−)-Rhazinilam **1** was synthesized from (+)-vincadifformine following a described procedure.[22] An authentic sample of leuconolam **4** was obtained from A. Razak Mohd Ali, University of Malaysia at Kuala Lumpur.

4.1. Biotransformation of (−)-rhazinilam by *B. bassiana* LMA (Laboratoire de Microbiologie Appliquée, ICSN)

The *B. bassiana* strain ATCC 7159 was maintained on a nutritive agar composed of 25 g L^{-1} glucose, 5 g L^{-1} pancreatic peptones, 5 g L^{-1} yeast extracts, 5 g L^{-1} malt extracts, and 25 g L^{-1} agar. The strain was inoculated in a 3 L culture and incubated for 72 h at 27 °C and 200 rpm. The culture medium consisted of 0.5 g L^{-1} KH_2PO_4, 1 g L^{-1} K_2HPO_4, 1 g L^{-1} $MgSO_4{\cdot}7H_2O$, 2 g L^{-1} $NaNO_3$, 0.5 g L^{-1} KCl, 0.02 g L^{-1} $FeSO_4$, 30 g L^{-1} glucose, and 10 g L^{-1} corn steep. The biomass was recovered by filtration (60 g wet weight). The bioconversion was conducted in a 2 L NewBrunswick fermentor containing distilled water (1 L) and rhazinilam 1 (180 mg, 0.61 mmol) dissolved in dichloromethane (2 mL). The mixture was stirred at 27 °C and 200 rpm under aeration at 1 VVM. Rhazinilam conversion was monitored by HPLC. After 96 h, the mixture was filtered and extracted with dichloromethane (5 × 500 mL). The organic layer was dried over $MgSO_4$ and evaporated under vacuum to give 210 mg crude product, which was purified by preparative HPLC (C18 ymmetry shield, H_2O/CH_3CN 75/25, 25 mL min^{-1}), giving compound **3** (t_R 18.8 min, 1.2 mg, 0.6%) and (3*S*)-hydroxyrhazinilam **2** (t_R 24.8 min, 18.7 mg, 10%) as white films.

4.2. (3*S*)-Hydroxyrhazinilam 2

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 (dd, J = 7.5, 1.8 Hz, 1H, H-9) ; 7.40 (ddd, J = 7.2, 7.2, 1.8 Hz, 1H, H-11), 7.33 (ddd, J = 7.5, 7.5, 1.8 Hz, 1H, H-10), 7.24 (dd, J = 7.5, 1.8 Hz, 1H, H-12), 6.69 (d, J = 2.6 Hz, 1H, H-5), 6.65 (br s, 1H, H-1), 5.83 (d, J = 2.6 Hz, 1H, H-6), 5.59 (br m, 1H, H-3), 3.02 (br d, 1H, O*H*), 2.44 (dd, J = 12.9, 12.9 Hz, 1H, H-17ax), 2.40 (m, 1H, H-14a), 2.39 (dd, J = 12.9, 12.9 Hz, 1H, H-16ax), 2.22 (ddd, J = 13.5, 13.5, 2.4 Hz, 1H, H-15ax), 1.99 (m, 1H, H-14b), 1.93 (m, 1H, H-16eq), 1.52 (m, 1H, H-19a), 1.47 (m, 1H, H-17eq), 1.37 (ddd, J = 12.9, 2.7, 2.7 Hz, 1H, H-15eq), 1.28 (m, 1H, H-19b), 0.79 (t, J = 7.5 Hz, 3H, H-18) ppm; ^{1}H NMR (500 MHz, acetone-d_6/D_2O) δ 5.58 (dd, J = 2 Hz, H-3) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 177.6 (C-2), 139.8 (C-8), 138.0 (C-13), 131.4 (C-9), 129.9 (C-21), 128.4 (C-11), 127.5 (C-10), 127.0 (C-12), 119.0 (C-5), 118.1 (C-7), 111.4 (C-6), 76.9 (C-3), 39.0 (C-20), 36.0 (C-17), 29.9 (C-19), 28.0 (C-16), 27.0 and 26.6 (C-14 and C-15), 8.2 (C-18) ppm; IR (film) nu(tilde) 3074, 1680 cm^{-1}; HRMS (ESI) calcd for $C_{19}H_{22}N_2NaO_2$ [(M+Na)$^+$]: 333.1579, found: 333.1587.

4.3. Diazaspiroleuconolam 3

A monocrystal was obtained by recrystallization from acetone. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.78 (d, J = 7.9 Hz, 1H, H-12), 7.26 (ddd, J = 7.5, 7.5, 1.6 Hz, 1H, Har), 7.17 (d, J = 6.8 Hz, 1H, H-9), 7.14 (dd, J = 7.3, 7.3 Hz, 1H, Har), 3.95 (m, 1H, H-3eq), 3.82 (d, J = 7.4 Hz, 1H, H-7), 2.88 (dd, J = 16.9, 7.4 Hz, 1H, H-6a), 2.80 (m, 1H, H-3ax), 2.76 (dd, J = 13.2, 6.3 Hz, 1H, H-16a), 2.70 (d, J = 16.9 Hz, 1H, H-6b), 2.51 (dd, J = 19.1, 4.6 Hz, 1H, H-16b), 1.97 (ddd, J = 13.2, 13.2, 6.5 Hz, 1H, H-15a), 1.86 (ddd, J = 13.5, 6.5, 1.3 Hz, 1H, H-17a), 1.79 (dq, J = 13.5, 7.5 Hz, 1H, H-19a), 1.67 (m, 1H, H-15b), 1.63 (m, 1H, H-17b), 1.59–1.52 (m, 2H, H-14), 1.37 (dq, J = 13.5, 7.5 Hz, 1H, H-19b), 0.93 (t, J = 7.4 Hz, 3H, H-18) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 173.0 (C-2), 171.0 (C-5), 141.6 (C-13), 135.3 (C-8), 128.2 (CHar), 125.7 (CHar), 124.0 (CHar), 120.3 (CHar), 92.2 (C-21), 42.1 (C-7), 38.3 (C-20), 37.8 (C-6), 37.0 (C-3), 29.6 (C-16), 27.1 (C-17), 26.9 (C-15), 26.4 (C-19), 20.3 (C-14), 7.5 (C-18) ppm.

4.4. Biotransformation of (−)-rhazinilam by rat and human liver microsomes

Rat liver microsomes were prepared from non-induced, phenobarbital-treated (80 mg kg^{-1}, 3 days) or β-naphthoflavone-treated (80 mg kg^{-1}, 3 days) animals as previously described.[23] Human microsomes were processed from six human livers obtained from kidney transplant donors with the informal consent of relatives.[24] Liver microsomes were kept at −80 °C until use. Microsomes (0.6–0.8 mg microsomal proteins) were incubated for 15 min at 37 °C with 25 nmol rhazinilam in the presence of a NADPH generating system consisting of 25 mM glucose 6-phosphate and 1.5 mM NADP in a final volume of 500 μL under a mild agitation. For the determination of kinetic parameters, incubations were carried out with 0–200 μM rhazinilam. Control incubation was carried out under the same condition without NADPH. After addition of CH_2Cl_2 and extraction, the dry residue was analyzed by LC.

4.5. Biotransformation of (−)-rhazinilam by CYP-expressing human cells

Human embryonic kidney cells Ad293 which do not express CYP and display no basal monooxygenase activity were stably transfected with pMT2 or pCIneo containing human CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 cDNA as detailed previously, and clones expressing at a high level the CYP protein were selected as detailed elsewhere.[24–26] The level of expression of CYP proteins was roughly equivalent as demonstrated by immunoblots of cellular microsomes with appropriate antibodies and calibration with baculovirus-insect cell-expressed CYP (BD Gentest) and measurement of monooxygenase activities using fluorescent substrates (methoxy-, ethoxy-, benzyloxy-trifluoromethylcoumarin or cyanoethoxycoumarin). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37 °C under 5% CO_2. Subconfluent Ad293 cell cultures in 75 cm^3 flasks were added with 3 mL medium containing 150 nmol rhazinilam. After 24 h, the incubation was stopped by the addition of 5 mL CH_2Cl_2 and extracted twice before LC analysis. In that case, controls were Ad293 cells transfected with plasmids containing no CYP insert.

4.6. Inhibition of microtubule disassembly

The drug, dissolved in DMSO at different concentrations, was added to a solution of microtubules (tubulin concentration ca. 20 μM, freshly prepared from sheep brain) at 37 °C. Then the solution was placed in a temperature-controlled cell at 9 °C (microtubule disassembly) and the decrease of the optical density was monitored in a UV spectrophotometer at 350 nm for 1 min. The maximum rate of disassembly was recorded and compared to a sample without drug. The IC_{50} of the compound was calculated from the effect of several concentrations and compared to the IC_{50} of rhazinilam obtained within the same day with the same tubulin preparation.

4.7. Inhibition of tubulin assembly

The assay was conducted in a reverse manner as above: the drug, dissolved in DMSO at different concentrations, was added to a solution of free tubulin at 0 °C. Then the solution was placed in a temperature-controlled cell at 37 °C (microtubule assembly) and the increase of the optical density was monitored in a UV spectrophotometer at 350 nm for 1 min. The maximum rate of assembly was recorded and compared to that of a sample without drug. The IC_{50} of the compound was calculated from the effect of several concentrations and compared to the IC_{50} of rhazinilam obtained within the same day with the same tubulin preparation.

4.8. Cytotoxicity assays

The effect of the drugs on the growth of KB human cell lines was monitored at the Laboratoire de Cultures Cellulaires, ICSN, Gif-sur-Yvette, France. The IC_{50} value refers to the concentration of drug corresponding to 50% growth inhibition after 72 h incubation.[27]

Acknowledgments

We thank M.-T. Martin for assistance with NMR experiments, M.-T. Adeline for assistance with HPLC separations, S. Thoret for tubulin-binding assays, and C. Gaspard and G. Aubert for cytotoxicity assays. We also thank the University of Malaysia at Kuala Lumpur, for a generous gift of leuconolam **4**. This work was financially supported by the Centre National de la Recherche Scientifique (France).

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10. In $CDCl_3$, H-3 appears as a broad multiplet due to couplings with both H-14 and with OH. Suppression of the H-3–OH coupling, best effected in acetone-d_6 with one drop of D_2O, allowed the estimation of the ^3J(H-3–H14) coupling constants. A ^3J(axial-axial) value of ca. 13 Hz would have been expected for an axial position of H-3.
11. CCDC257644 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB 1EZ, UK; fax: (+44)1223/336-033; e-mail: deposit@ccdc.cam.ac.uk].
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