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Human metabolism of nebicapone (BIA 3-202), a novel COMT inhibitor: characterization of *in vitro* glucuronidation

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Human metabolism of nebicapone: *in vitro* glucuronidation

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COMT, catechol-*O*-methyltransferase; Nebicapone, BIA 3-202; 1-[3,4-dihydroxy-5-nitropheny]-2-phenyl-ethanone; BIA 3-467, 1–(5-acetamido-3,4-dihydroxyphenyl)–2-phenyl-ethanone; BIA 3-476, 1–(3-*O*-ß-D-glucopyranuronosido-4-hydroxy-5-nitrophenyl)–2-phenyl-ethanone; BIA 3-465 1-(4-hydroxy-5-nitro-3-*O*-sulphatophenyl)-2-phenyl-ethanone; BIA 3-270, 1–(4-hydroxy-3-methoxy-5-nitrophenyl)-2-phenyl-ethanone; L-DOPA, L-3,4-dihydroxyphenylalanine; AADC, aromatic L-amino acid decarboxylase; HPLC/MS, High performance liquid chromatography/Mass spectrometry; AP-ESI, Atmospheric pressure- electrospray ionization; UGT, UDP-glucuronosyltransferase; SPE, Solid phase extraction; HLM, human liver microsomes; HIM, human intestinal microsomes; UDPGA, uridine 5'-diphosphoglucuronic acid; SIM, selected ion monitoring.

Abstract

Nebicapone (BIA 3-202; 1-[3,4-dihydroxy-5-nitropheny]-2-phenyl-ethanone), a novel catechol-*O*-methyltransferase (COMT) inhibitor, is mainly metabolised by glucuronidation. The purpose of this study was to characterise the major plasma metabolites of nebicapone following oral administration of nebicapone to healthy volunteers and to determine the human UGT enzymes involved in nebicapone glucuronidation. Plasma samples were collected as part of a clinical trial at different time points post-dose and were analysed for nebicapone and its metabolites using a validated method consisting of a solid phase extraction (SPE) followed by high performance liquid chromatography/mass spectrometry (HPLC/MS) detection. The primary metabolic pathways of nebicapone in humans involve mainly 3-*O*-glucuronidation, the major early metabolite, and 3-*O*-methylation, the predominant late metabolite. Among nine commercial available recombinant UGT enzymes studied (UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15), only UGT 1A9 exhibited high nebicapone glucuronosyltransferase specific activity (24.3±1.3 nmol mg $prot^{-1}$ min^{-1}). UGTs 1A6, 1A7, 1A8, 1A10, 2B7 and 2B15 exhibited low activity (0.1–1.1 nmol mg $prot^{-1}$ min^{-1}) and 1A1 and 1A3 showed extremely low activities (below 0.03 nmol mg $prot^{-1}$ min^{-1}). The results show that nebicapone is mainly glucuronidated in humans and that multiple UGT enzymes are involved in this reaction.

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Introduction

L-3,4-dihydroxyphenylalanine (L-DOPA) therapy has revolutionized treatment of idiopathic Parkinson's disease by providing an orally administered source of dopamine precursor with ready access to the brain, and it remains the most widely used palliative drug for Parkinson's Disease. However, L-DOPA is metabolised in the periphery by aromatic L-amino acid decarboxylase (AADC) and catechol-*O*-methyltransferase (COMT) enzymes, reducing brain availability. Therefore an improvement in L-DOPA therapy is provided by a combination treatment of L-DOPA with an AADC inhibitor plus a COMT inhibitor, effectively increasing its availability to the brain (Backstrom et al., 1989).

Second generation COMT inhibitors entacapone and tolcapone (Figure 1), have proven beneficial when used together with L-DOPA and an AADC inhibitor such as carbidopa or benserazide in the medical treatment of patients with PD (Dingemanse, 1997; Dingemanse et al., 1995; Heikkinen et al., 2001; Heikkinen et al., 2002). When metabolised, these nitrocatechol derivatives are extensively conjugated, usually undergo direct glucuronidation catalysed by UDP-glucuronosyltransferases (UGT) and are then rapidly excreted in the urine (Lautala et al., 1997). Additionally, both methylation and sulfation compete with glucuronidation for conjugation of the adjacent phenolic hydroxyls (Lautala et al., 1997). Major metabolites of tolcapone in human plasma are the 3-*O*-ß-glucuronic acid (~18.6%) and the 3-*O*-methyl conjugate (~2.1%). In urine the 3-*O*-ß-glucuronic conjugates of tolcapone (~13%) and its derivative N-acetyl amino (~5.7%) are the predominant metabolites found (Jorga et al., 1999). Regarding entacapone, the only metabolite described in human plasma is

the Z-isomer (~5%) (Keranen et al., 1994; Wikberg et al., 1993), however, in human urine besides the Z-isomer (~25%), the 3-*O*-ß-glucuronic acid (~70%) is the prevalent metabolite (Wikberg et al., 1993).

No methylation products of entacapone were detected in human plasma or urine, possibly because the nitro group of entacapone hinders methylation of the cathechol (Wikberg et al., 1993). As an alternative to molecular conjugation with endogenous species like glucuronidation, sulfation, methylation, gluthatione conjugation and acetylation (phase II drug metabolism reactions), these drug could undergo oxidation, reduction and hydroxylation (phase I drug metabolism reactions), however such phase I metabolites are minor (Jorga et al., 1999; Wikberg et al., 1993).

Human UGT family presently comprise 16 individual distinct expressed enzymes, not including variant enzymes (Taskinen et al., 2003). UGTs are lumenally facing in the endoplasmatic reticulum and are expressed not only in liver but also in extrahepatic tissues, where the extent of glucuronidation can be substantial. Some UGT enzymes (e.g. UGT1A7, UGT1A8, UGT2A1 and UGT1A10) are expressed only in extrahepatic tissues (Kiang et al., 2005). Hepatic expression of UGTs shows little inter-individual variation, as opposed to the high inter-individual variability found in gastrointestinal (GI) tract (Strassburg et al., 2000; Strassburg et al., 1998), further accentuated by the differential enzyme expression within different segments of the GI tract (Antonio et al., 2003; Basu et al., 2004; Strassburg et al., 2000; Strassburg et al., 1998). Genetic polymorphisms have been described for several UGTs and some of them have been associated with inter-individual variations in drug effect (Chung et al., 2005) and toxicity (Marsh and McLeod, 2004; Massacesi et al., 2006). It is therefore important the identification of a specific enzymes involved in the metabolism of a drug .

There have been some studies identifying UGTs able of conjugating entacapone and tolcapone with glucuronic acid. Entacapone was described to be a substrate for UGT1A1, UGT1A3, UGT1A7, UGT1A8, UGT1A10, UGT2B7 and UGT2B15 and particularly good one for UGT1A9 (Lautala et al., 2000a). Tolcapone, on the other hand, was tested on limited number of enzymes and it was described to be efficiently conjugated by UGT1A9 and to a lesser extent by UGT2B7, UGT2B15 (Lautala et al., 2000a).

Nebicapone (BIA 3-202, Figure 1) is a new COMT inhibitor currently being developed for use as an adjunct to L-DOPA/AADC inhibitor therapy in the treatment of Parkinson's Disease. It is a reversible and mainly peripherally acting inhibitor that was shown to decrease the biotransformation of L-DOPA to 3-OMD by inhibition of COMT in clinical trials (Almeida and Soares-Da-Silva, 2003; Almeida et al., 2004; Silveira et al., 2003).

The purpose of the study presented herein was to quantify major metabolites of nebicapone in human plasma, using a sensitive and specific HPLC/MS assay and to investigate the UGT enzymes involved in nebicapone glucuronidation *in vitro*.

Materials and Methods

Chemicals

Nebicapone (BIA 3-202; 1-[3,4-dihydroxy-5-nitropheny]-2-phenyl-ethanone), its metabolites BIA 3-467 (1–(5-acetamido-3,4-dihydroxyphenyl)–2-phenyl-ethanone), BIA 3-476 (1–(3-*O*-ß-D-glucopyranuronosido-4-hydroxy-5-nitrophenyl)–2-phenyl-ethanone), BIA 3-465 prepared as a pyridinium salt (pyridinium-1-(4-hydroxy-5-nitro-

3-*O*-sulphatophenyl)-2-phenyl-ethanone), BIA 3-270 (1–(4-hydroxy-3-methoxy-5-nitrophenyl)-2-phenyl-ethanone) and tolcapone as internal standard, were synthesized in the Laboratory of Chemistry, BIAL (S. Mamede Coronado, Portugal), with purities >99.5%. All other chemicals were purchased from SIGMA-Aldrich (St. Louis, MO). Recombinant human UGTs expressed in baculovirus-infected insect cells were purchased from PanVera-Invitrogen (Carlsbad, CA, UGT: 1A1, 1A3, 1A6, 1A7, 1A10 and 2B7) and from BD Gentest (Woburn, MA, UGT: 1A8, 1A9 and 2B15). Pooled human liver microsomes (HLM, from 48 donors) and intestinal microsomes (HIM, prepared from the duodenum and jejunum sections of each of 5 donors) were purchased from BD Gentest (Woburn. MA). The protein contents were used as described in the data sheets provided by the manufacturers.

Human Administration

The assay of nebicapone and its metabolites was performed on plasma samples collected from six young healthy volunteers (Caucasians, male and female) who received a 400 mg single-dose of nebicapone while participating in a clinical trial (Almeida et al., 2004) . Blood samples were taken at the following times: pre-dose, and ½, 1, 1½, 2, 3, 4, 6, 8, 12, 18 and 24 h post-dose. Blood samples were taken into potassium EDTA and centrifuged immediately at 1500 *g* for 10 min at approximately 4ºC. The resulting plasma was stored below –20ºC until required for analysis.

Extraction of Nebicapone and its Metabolites from Plasma

A 500 µl volume of plasma specimen was added to 500 µl of phosphate buffer (0.1 M, pH 2) containing 400 ng/ml of tolcapone as internal standard. The samples were placed on an automatic liquid handler (ASPEC-XL4, Gilson) for solid phase extraction. The solid phase extraction cartridges (Oasis, HLB, 30 mg, 1ml Waters) were conditioned with 1 ml of acetonitrile and then washed twice with 1 ml of phosphate buffer (0.1 M, pH 2). Specimens (900 µl) were loaded onto the cartridges and the cartridges washed twice with 1 ml of phosphate buffer (0.1 M, pH 2.0). After the second wash the cartridges were flushed with air push of 10 ml at 6 ml/min. The cartridges were eluted twice with 250 µl of acetonitrile containing 1% formic acid with an air push of 2 ml at 6 ml/min. To the eluted sample 400 µl of water containing 1% formic acid were added and mixed twice with aspiring dispensing cycles. The eluted samples were injected (5 µl) into an HPLC-MSD.

Nebicapone Glucuronidation Screening by Recombinant UGTs

Glucuronidation activity by UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15 was measured using the following assay conditions: the incubation mixture (100 µl total volume) contained 0.4 mg/ml total protein, 10 mM $MgCl_2$, 2 mM uridine 5'-diphosphoglucuronic acid (UDPGA), 25 µg/ml alamethicin, 5 mM saccharolactone in 50 mM phosphate buffer pH 7.5 and 20 µM nebicapone. Drug was dissolved in DMSO and the final concentration of DMSO in the reaction was below 0.5% (v/v). Reactions were pre-incubated 5 min and were initiated with the drug. Reaction mixtures were incubated for up to 60 min and stopped with 100 µl 1% formic acid in acetonitrile. All incubations were performed in a water bath shaking at 37ºC. After removal of the

protein by centrifugation for 3 min at 15,000 *g*, supernatant was filtered through 0.20 µm Spin-X filters (Corning, NY) and injected on a HPLC-MS.

Kinetics of Nebicapone Glucuronidation by Selected UGTs and in Pooled HLM and HIM

Rates of glucuronidation were determined, as described above for UGT screening, with nebicapone concentrations ranging 5-1000 µM and with 15 min incubation time for all recombinant UGTs except UGT1A9. With UGT1A9, HLM and HIM incubation time was 5 min and total protein concentration was 0.1 mg/ml.

All preparations were evaluated for linearity of product formation with respect to incubation time (0-60 min). HLM, HIM and UGT1A8, 1A9 and 2B7 were also evaluated for linearity of product formation with respect to protein concentration (0.05-0.6 mg/ml). All experiments were performed with samples in duplicate.

HPLC-MS Analysis

The analysis of plasma samples extracts was performed using HPLC-MS (Agilent, AP-ESI, 1100 Series, Agilent Technologies) with negative ion detection. Separation was performed on a Zorbax SB-C_{18}, 3 µm, 30 x 4.6 mm, column (Agilent) using a mobile phase A: water containing 1 % formic acid (v:v) and B: acetonitrile containing 1% formic acid (v:v), with gradient conditions of 80 % of A and 20 % of B at 0 min and 50 % of A: 50 % of B at 20 min. Selected ion monitoring (SIM) with the detection of each compound of interest was used for quantification: namely m/z 284.3 (BIA 3-467), 352.2 (BIA 3-465), 448.4 (BIA 3-476), 272.2 (nebicapone and tolcapone) and 286.3 (BIA 3-270). A time-programmed detection was used for the following ions: group 1-BIA 3-467, BIA 3-465 and BIA 3-476; group 2- nebicapone tolcapone and

BIA 3-270. For maximal sensitivity, the fragment energy was set to 120 V and further settings were 3500 eV for the capillary voltage, 350ºC nebulizer gas temperature and 40 psi nebulizer pressure. The method was validated in accordance with FDA guidance for industry (http://www.fda.gov/cder/guidance/4252fnl.pdf). The limits of quantification (LLOQ) were defined as the lowest concentration of the range examined which has acceptable precision and accuracy (accuracy within 20% of the nominal value and the CV (%) did not deviate more than 20 %). The LLOQ were therefore 0.22 µmol/L for BIA 3-467, 0.14 µmol/L for BIA 3-476, 0.12 µmol/L for BIA 3-465, 0.15 µmol/L for nebicapone and 0.14 µmol/L for BIA 3-270. The intra and inter-batch coefficient of variation (CV) and accuracy were within 15.0 % of the actual value for all the analytes and in all concentrations checked.

For the determination of nebicapone glucuronide in *in vitro* studies the same analytical column and mobile phases were used with a gradient conditions of 70 % of A and 30 % of B at 0-5 min and 50 % of A: 50 % of B at 10 min. Selected ion monitoring (SIM) with the detection of m/z 448.4 was used for quantification of nebicapone glucuronide.

Data Analysis

The pharmacokinetic parameters of nebicapone and its metabolites were calculated from plasma concentration-time profiles using GraphPad Prism v4 (CA, USA).

Kinetic parameters of nebicapone glucuronidation were obtained by fitting velocity data to the following models (equations 1 to 3) with GraphPad Prism (CA, USA): Michaelis-Menten equation,

$$v = \frac{V_{max} \cdot S}{K_m + S} \qquad (1)$$

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Where v is the rate of the reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant and S is the substrate concentration. Hill equation, which describes sigmoidal kinetics (Houston and Kenworthy, 2000),

$$v = \frac{V_{\max} . S^n}{S_{50}^n + S^n} \quad (2)$$

where S_{50} is analogous to K_m and *n* is the Hill coefficient. Substrate inhibition model (Houston and Kenworthy, 2000),

$$v = \frac{V_{\max} . S}{K_m + S + \left(\frac{S^2}{K_{si}} \right)} \quad (3)$$

Where K_{si} is the constant describing the substrate inhibition interaction.

All data is reported as mean±sem.

Results

Metabolism and Elimination of Nebicapone in Human Plasma

Nebicapone and its major metabolites where quantified for an administration dose of 400 mg. The concentration-time profiles are presented in Figure 2 and the pharmacokinetic parameters derived from this curves are summarized in Table 1. Nebicapone, was rapidly absorbed, reaching a maximum plasma concentration (C_{max}) of 28.3±1.4 µmol/L within 1.3 h before falling back to low levels over the next 8 h and returning to baseline at 12 h. Nebicapone presented an AUC_{0-24} of 99.1±17.2 h.µmol/L and was mainly conjugated to the 3-*O*-β-glucuronic acid derivative (BIA 3-476)

representing ~70 % of the total nebicapone and metabolites AUC. BIA 3-476 reached C_{max} of 60.8 ± 7.8 µmol/L within 2.5 h post administration and remained the major circulating metabolite of nebicapone at 8 h after administration. The mean AUC_{0-24} of the fraction of nebicapone glucuronide was 303.1±96.3 h.µmol/L. The lower limit of quantification for BIA 3-476 was approached by 12 h. After this time, the 3-*O*-methyl derivative of nebicapone, BIA 3-270, became the predominant metabolite in plasma with a t_{max} of 12 h and with an AUC_{0-24} of 30.6±9.1 h.µmol/L and C_{max} of 1.7±0.1 µmol/L, representing ~7% of the total nebicapone and metabolites AUC. The 3-*O*-sulfate and acetamino derivatives of nebicapone (BIA 3-465 and BIA 3-467, respectively) were very minor metabolites (representing less than ~1% of the total nebicapone and metabolites AUC) and were not quantified.

Nebicapone Glucuronidation by Human Microsomes

Kinetic analysis of nebicapone was performed in human liver and intestine pools of microsomes. As shown in Figure 3, both preparations displayed typical hyperbolic kinetics, though the Eadie-Hofstee plots of the data were clearly biphasic (Hutzler and Tracy, 2002), which is indicative of the involvement of more than one enzyme in the reaction. The apparent kinetic parameters derived from these curves fitted to the Michaelis-Menten equation, (1) are listed in Table 2. Both apparent K_m and V_{max} values were higher for liver than for intestine microsomes (about 2 and 4 fold higher, respectively). The intrinsic clearance ($Cl_{int} = V_{max}/K_m$) calculated for intestine was 222 µl mg $prot^{-1}$ min^{-1} and the one calculated for liver was 475 µl mg $prot^{-1}$ min^{-1}, 2.1 fold higher than the one for intestine.

Nebicapone Glucuronidation by Recombinant UGTs

Nine commercially available UGT enzymes were used to evaluate their ability to conjugate nebicapone to BIA 3-476 (Figure 4). From the tested UGTs only UGT 1A9 produced significant amounts of BIA 3-476 (24.3±1.3 nmol mg $prot^{-1}$ min^{-1}). UGT 1A6, 1A7, 1A8, 2B7 and 2B15 produced little amounts of BIA 3-476 (between 0.1-1.1 nmol mg $prot^{-1}$ min^{-1}). UGT1A1 and 1A3 also conjugated nebicapone, however, only at longer incubation times (above 30 min) and at extremely low levels, respectively 0.028±0.002 and 0.016±0.002 nmol mg $prot^{-1}$ min^{-1}. No metabolite formation was detected with control baculosomes/supersomes over an incubation period of 60 min.

Kinetics of Nebicapone Glucuronidation by Recombinant UGTs

The characterization of nebicapone glucuronidation kinetics was performed for all UGTs with activities higher than 0.1 nmol mg $prot^{-1}$ min^{-1}, namely UGTs 1A6, 1A7, 1A8, 1A9, 1A19, 2B7 and 2B15. Each enzyme was incubated with different concentrations of nebicapone (1-1000 µM) and the initial rates determined. Rates were fitted to the most suitable model using Eadie-Hofstee plots (not shown) as diagnostic tool. Accordingly, the experimental data from 1A9, 2B7 and 2 B15 was fitted with Michaelis-Menten equation (1), data from 1A6 and 1A8 was fitted with the Hill model (equation 2) and data from 1A7 and 1A10, with an obvious substrate inhibition profile, was fitted with equation 3. The resulting curves are represented in Figure 5 and the apparent kinetic parameters K_m and V_{max} derived from these curves, are shown in Table 1. There is a rather considerable range of affinities for the conjugation of nebicapone as shown by the apparent K_m values determined. The enzyme with the highest affinity was UGT 2B15, with a K_m of 9 µM, followed by UGTs 1A9 and 1A7

with K_m values of, respectively, 50 and 75 µM. UGTs 1A6 and 1A10 had apparent affinities close to 100 µM and UGTs 1A8 and 2B7 were the ones with the lower affinities, K_m of respectively 149 and 206 µM.

When comparing the K_m values for the HLM (87 µM) and the liver expressed UGTs, specifically UGT 1A6, 1A9, 2B7 and 2B15, it is not possible to establish which is the most important enzyme/s involved in the conjugation of nebicapone in the liver, since two of the enzymes (UGTs 2B15 and 1A9) have higher affinities than the HLM and the other two (UGT 1A6 and 2B7) have lower affinities.

The comparison of the affinities of the intestinal expressed enzymes (namely 1A6, 1A8, 1A9, 1A10, 2B7 and 2B15) with the affinity of HIM (Km = 42 µM) suggests an important contribution of UGT 1A9 in the intestinal conjugation of nebicapone, since it is the enzyme with similar affinity to the one of HIM, however involvement of the high affinity UGT 2B15 together with one or more of the lower affinity enzymes (UGT 1A6, 1A8, 1A10 and 2B7) cannot be excluded.

Discussion

In the work here presented, the major metabolites of nebicapone were quantified in human plasma (Figure 6) and the kinetic parameters from UGT enzymes able to metabolize nebicapone where determined. The UGT kinetic parameters for human liver and intestinal microsomes were compared in an attempt to elucidate which enzymes contribute to the nebicapone glucuronidation *in vivo*.

Nebicapone 3-*O*-glucuronide was rapidly formed and accounted for most of the nebicapone-related material in human plasma, whereas the 3-O-methylated derivative, was responsible for low but sustained nebicapone related material in plasma over 24 h. This profile is similar to the one observed for tolcapone upon human administration (Jorga et al., 1999). The 4-*O*-glucuronide of nebicapone was not found in human plasma, similarly to what was observed for other nitrocatechols, such as tolcapone (Jorga et al., 1999), and thus providing additional evidence for the hypothesis that nitrocatechol glucuronidation in humans is highly regioselective (Wikberg et al., 1993).

Glucuronidation and sulfation are often competing reactions for phenolic substrates, however in the case of nebicapone glucuronidation is preferred over sulfation which is the case of tolcapone (Jorga et al., 1999) and entacapone (Wikberg et al., 1993).

N-acetylation of nebicapone resulting from the 5-nitro group reduction was detected in some samples, however, the levels were below the limit of quantification. Limited amounts of N-acetylamino derivative of tolcapone were also reported in human plasma (Jorga et al., 1999), on the other hand no nitro reduction was observed for entacapone (Wikberg et al., 1993).

The small amount of the nebicapone *O*-methylated derivative observed in this study is in agreement with the fact that nitrocatechols are poor substrates for COMT because of the strong electronegative nitro substituent (Ovaska and Yliniemela, 1998). The 3-*O*-methylation of nebicapone reduces the polarity of the molecule and yields the long-lived metabolite, BIA 3-270, with slower elimination that peaks 12 h after administration.

The results obtained herein indicate that the major metabolic pathway of nebicapone is glucuronidation in meta position to the nitro group of the catechol. The extensive glucuronidation of nebicapone was evaluated *in vitro* using human microsomes and recombinant UGTs. It is clear that correlations of the activities determined with *in vitro* systems and the whole organism are highly limited, not only because of the differential expression of UGTs in the various tissues, but also because it is not possible to quantify the levels of the individual enzymes in the microsomal preparations. The use of recombinant systems further adds limitations due to the variability of recombinant enzyme expression levels. In addition, increasing evidence suggests that UGTs can form dimers *in vivo* (Fisher et al., 2001; Miners et al., 2004), possibly heterodimers whose role is not evaluated with the recombinant enzymes.

The kinetic analysis of nebicapone glucuronidation in liver and intestine microsomes revealed an affinity for the conjugation within the same order of magnitude for the two organs, with liver having a 2-fold higher K_m than intestine (87±4 µM *versus* 42±3 µM). The intrinsic clearance of intestine, on the other hand, was about half the one of the liver. These results suggest that first-pass glucuronidation of nebicapone may represent a significant contribution to BIA 3-476 in plasma, which is further corroborated with the rapid increase of BIA 3-476 in circulation after oral

administration of nebicapone to humans. The efficient glucuronidation of some catechols by human stomach and intestine microsomes, similar to the one obtained in liver microsomes has already been described (Antonio et al., 2003). There is no information available concerning glucuronidation of tolcapone or entacapone in intestine microsomes; however, both compounds are glucuronidated at high rates by human liver and kidney microsomes, with entacapone having an affinity for HLM glucuronidation (K_m=47±5μM) within the same order of magnitude of the one of nebicapone. Tolcapone was reported to have a lower affinity (K_m=201±102μM) however the error is too large to draw any conclusion (Lautala et al., 2000b).

The kinetics of nebicapone glucuronidation in human liver and intestinal microsomes suggest the involvement of more than one UGT in glucuronidation. All recombinant human UGTs tested, namely UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15, were capable of conjugating nebicapone albeit at different levels. Nebicapone was shown to be a particularly good substrate for UGT1A9, suggesting a role for this enzyme in *in vivo* metabolism. UGT1A9 is suggested to be the major enzyme responsible for tolcapone and entacapone glucuronidation in vivo (Lautala et al., 2000b). Although different recombinant enzyme sources were used for the determination of glucuronidation affinities (Lautala et al., 2000b), the UGT1A9 K_m for tolcapone (66±12.0μM) is similar to the K_m of nebicapone obtained in this study, which is in accordance with structure similarity of these two compounds.

The comparison of the apparent K_m values obtained for the recombinant enzymes evaluated with those for human microsomes, did not clarify which enzymes were involved in microsomal glucuronidation. Among the hepatic expressed UGTs, UGT 1A9 had the highest formation rate of BIA 3-476, however its affinity was superior to

the one of liver microsomes (50±2 *versus* 87±4µM, respectively) clearly indicating the involvement of one or more of the lower affinity enzymes in this sample. Regarding the intestinal expressed UGTs, UGT1A9 is the enzyme with the same binding affinity in the glucuronidation of nebicapone as intestine microsomes (50±2 *versus* 42±3µM, respectively) suggesting that it could be the major enzyme responsible for nebicapone conjugation in the intestinal microsomal sample. With the available data it is not possible to discriminate the enzymes involved in *in vivo* glucuronidation of nebicapone. Besides there are other UGTs that were not evaluated in the present study and might be involved. Furthermore the enzymes involved will be dependent not only on the kinetics of the reaction but also on the amount of compound that reaches the respective tissue and most significantly on the enzyme levels present in the tissues.

In conclusion, glucuronidation is an important feature of nebicapone pharmacokinetics contributing undoubtedly to nebicapone elimination and several UGTs are involved in this reaction.

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Legends

Figure 1. Structures of A) entacapone, B) tolcapone and C) nebicapone.

Figure 2. Mean plasma nebicapone (BIA 3-202) and its metabolites, 3-*O*-glucuronide (BIA 3-476) and 3-O-methylated (BIA 3-270) concentration-time profile following *p.o.* administration of 400 mg nebicapone to humans. Symbols are means ± S.E.M. of n=6 humans.

Figure 3. Kinetics of nebicapone (BIA 3-202) glucuronidation in human liver (A) and intestine (B) microsomal pools. Nebicapone concentrations ranged 1-1000 µM. Each inset shows the Eadie-Hofstee representation of the experimental data with computer generated best fit. Values represent means ± sem of duplicates.

Figure 4. Apparent glucuronidation rates catalysed by recombinant human UGT enzymes. Rates were determined at 20 µM nebicapone. Values represent means ± sem of 3-4 determinations.

Figure 5. Kinetics of nebicapone glucuronidation by recombinant human UGT enzymes (1A6, 1A7, 1A8, 1A9, 1A10, 2B7 and 2B15). nebicapone concentrations ranged 1-1000 µM. Values represent means ± sem of duplicates. Lines represent the fitting curves to either Michaelis-Menten equation (1A9, 2B7, 2B15), Hill equation (1A6, 1A8) or substrate inhibition equation (1A7, 1A10) as described under Materials and Methods.

Figure 6. Nebicapone and metabolites detected.

TABLE 1

Mean (S.D.) pharmacokinetic parameters of nebicapone and its metabolites BIA 3-476 and BIA 3-270 in human after p.o. administration of nebicapone (n=6).

	C_{max} (µml/L)	T_{max} (hr)	AUC_{0-24} (hr. µml/L)
Nebicapone	28.3 (1.4)	1.3 (0.5-3)	99.1 (17.2)
BIA 3-476	60.8 (7.8)	2.5 (1.5-4)	303.1 (96.3)
BIA 3-270	1.7 (0.1)	12 (8-18)	30.6 (9.1)

t_{max} values are the median with range values in parentheses

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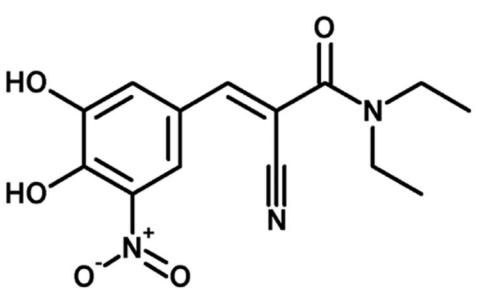
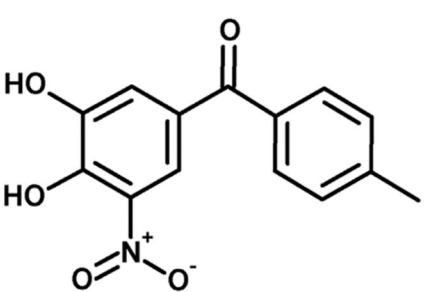
TABLE 2

Apparent kinetic parameters of nebicapone glucuronidation in human liver (HLM) and intestine microsomes (HIM) and recombinant UGT enzymes

	K_m	V_{max},	
	µM	*nmol mg prot^{-1} min^{-1}*	
HLM	87 ± 4	41.29 ± 0.62	
HIM	42 ± 3	9.32 ± 0.14	
UGT 1A6[a]	111 ± 5	0.76 ± 0.02	*n* = 1.4 ± 0.1
UGT 1A7[b]	75 ± 5	0.75 ± 0.03	K_{si} = 472 ± 69µM
UGT 1A8[a]	149 ± 9	4.58 ± 0.12	*n* = 1.4 ± 0.1
UGT 1A9	50 ± 2	33.05 ± 0.30	
UGT 1A10[b]	105 ± 10	1.88 ± 0.11	K_{si} = 532 ± 47µM
UGT 2B7	206 ± 21	0.59 ± 0.02	
UGT 2B15	9 ± 1	0.14 ± 0.00	

Rates were fitted to Michaelis-Menten equation except a) Hill equation and b) Substrate inhibition. *n* is the Hill coefficient and *Ksi* is the substrate inhibition constant. Values represent best fit values ± sem.





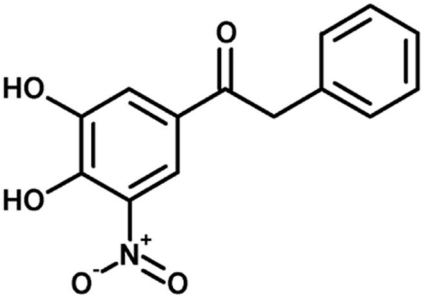
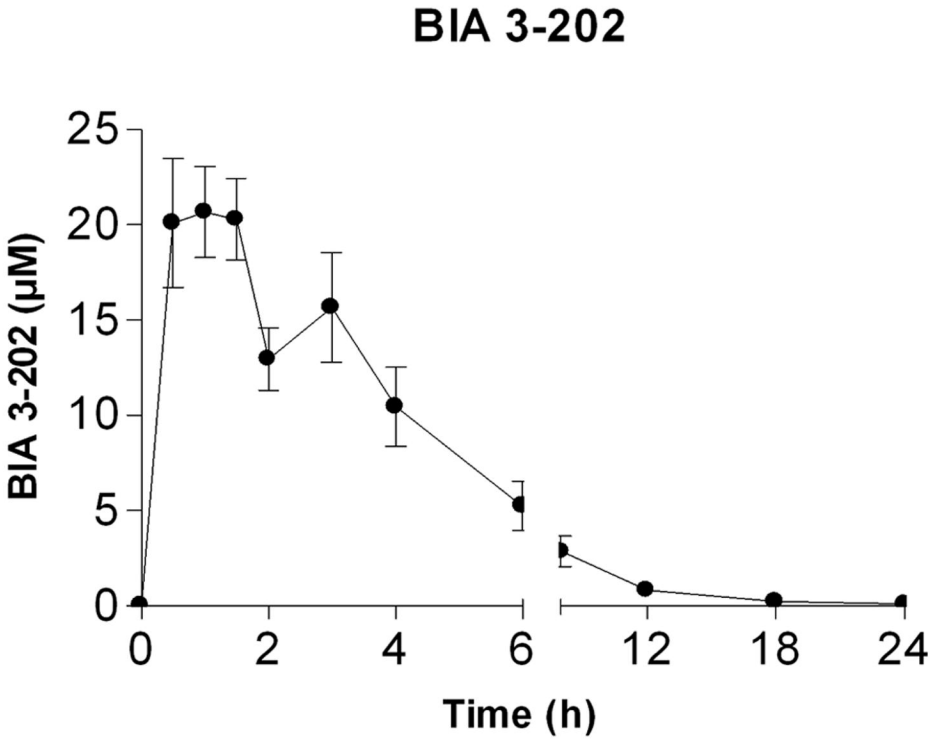
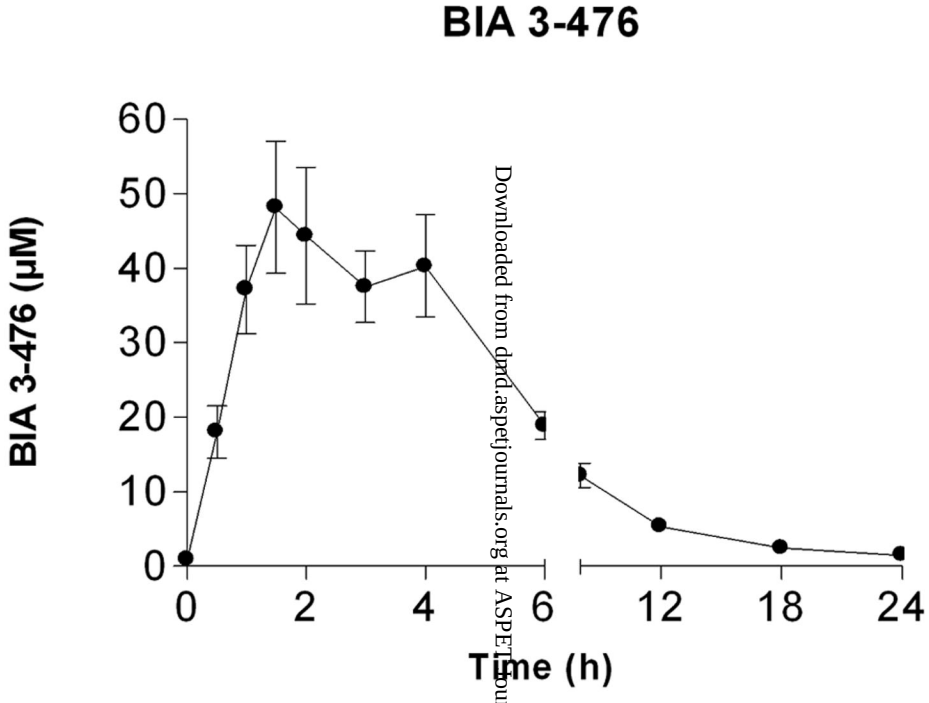


Figure 1





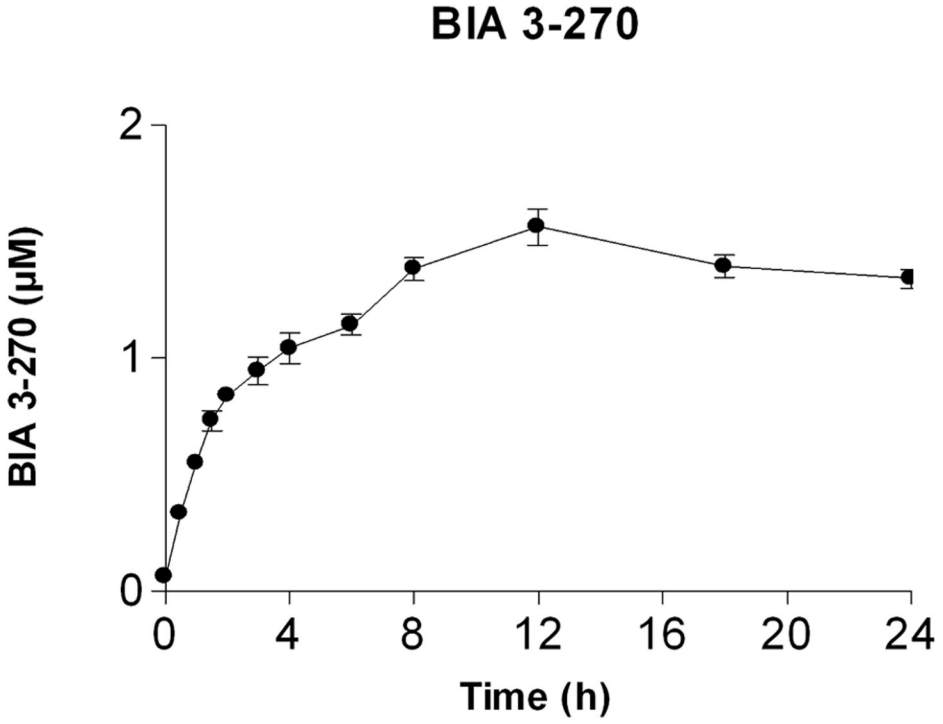


Figure 2

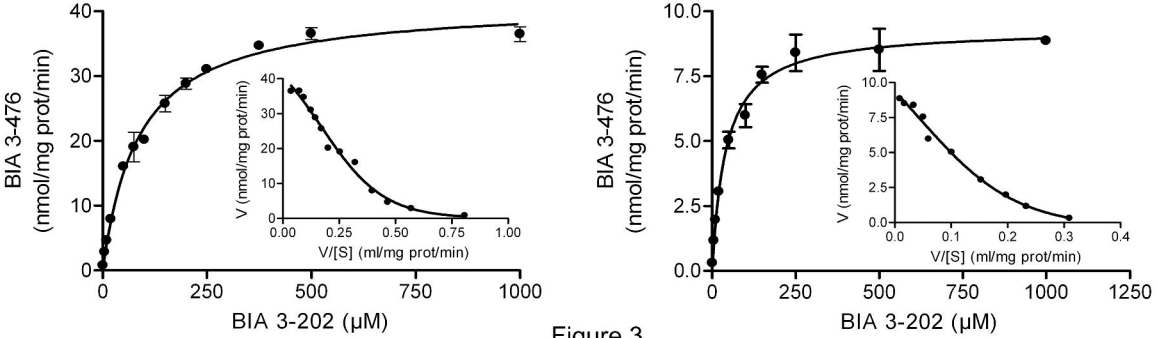
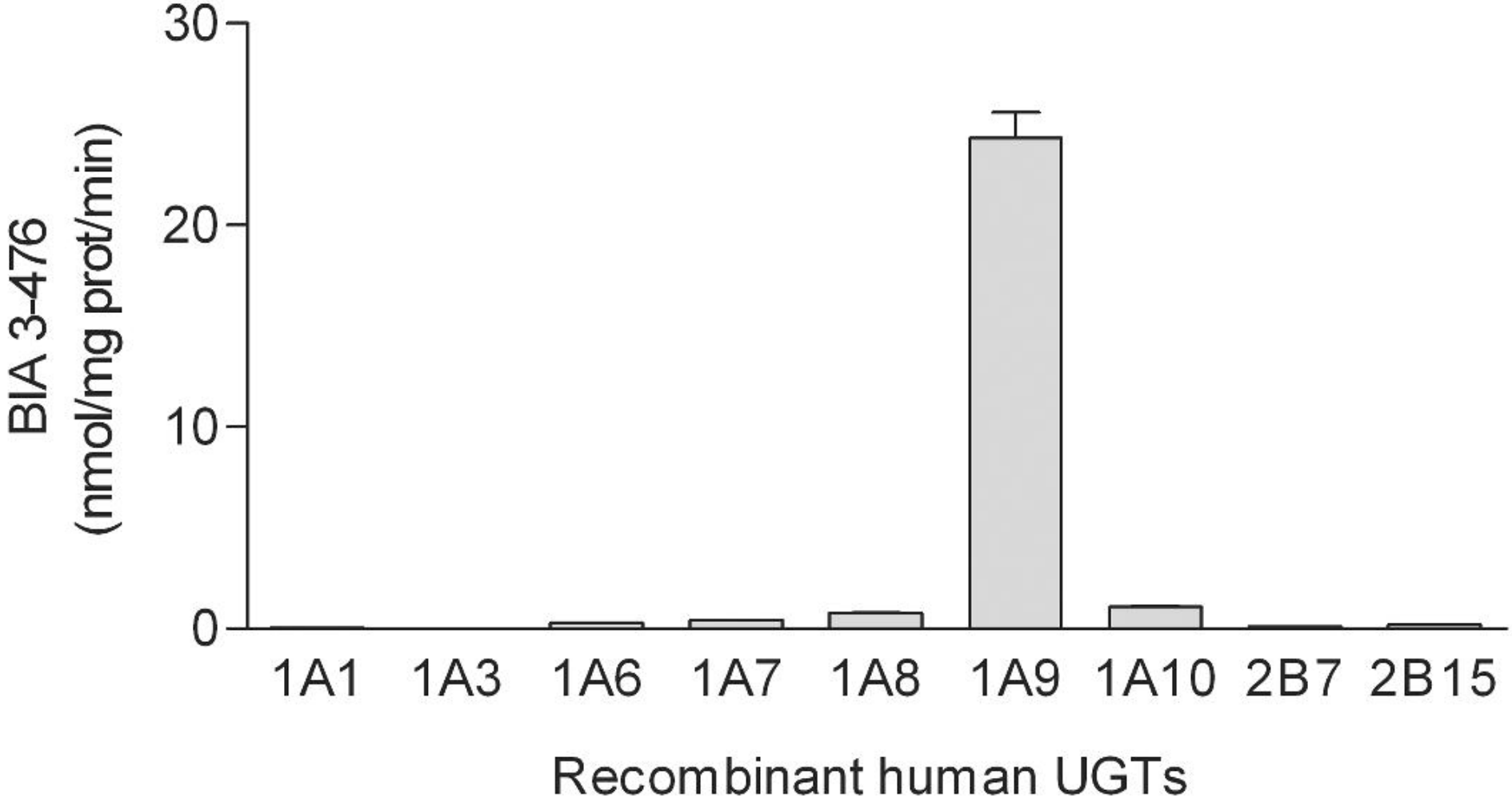
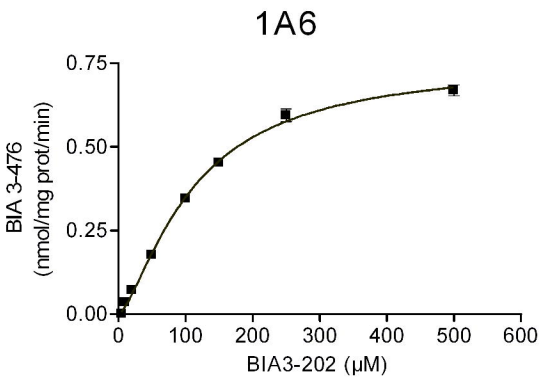

BIA 3-476
(nmol/mg prot/min)
BIA 3-202 (μM)
V (nmol/mg prot/min)
V/[S] (ml/mg prot/min)
BIA 3-476
(nmol/mg prot/min)
BIA 3-202 (μM)
V (nmol/mg prot/min)
V/[S] (ml/mg prot/min)

Figure 3

Figure 4


30
20
10
0
BIA 3-476
(nmol/mg prot/min)
1A1
1A3
1A6
1A7
1A8
1A9
1A10
2B7
2B15
Recombinant human UGTs


1A6
BIA 3-476 (nmol/mg prot/min)
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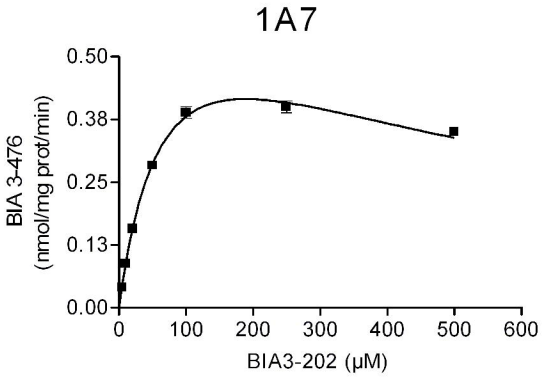
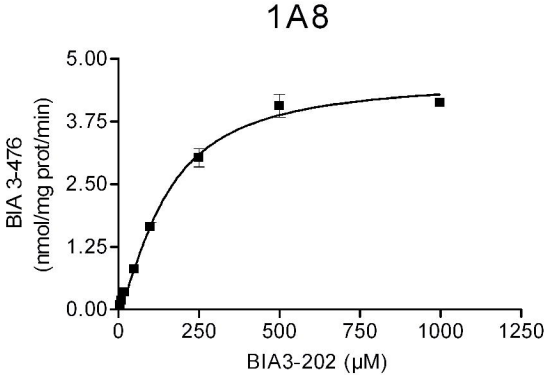
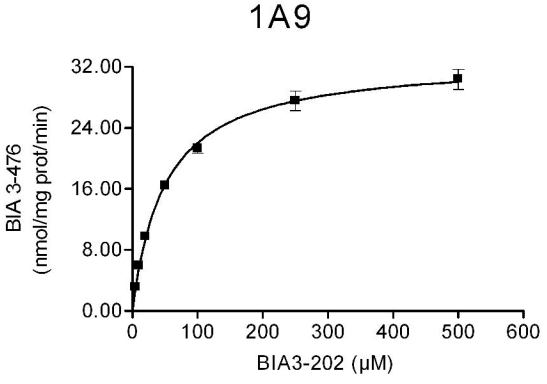
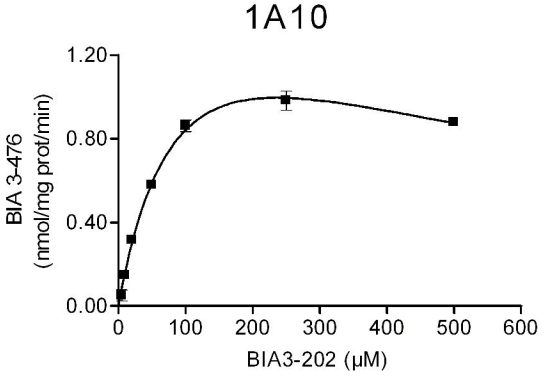
0.50
0.25
0.00
0
100
200
300
400
500
600
BIA3-202 (µM)


1A7
BIA 3-476 (nmol/mg prot/min)
0.50
0.38
0.25
0.13
0.00
0
100
200
300
400
500
600
BIA3-202 (µM)


1A8
BIA 3-476 (nmol/mg prot/min)
5.00
3.75
2.50
1.25
0.00
0
250
500
750
1000
1250
BIA3-202 (µM)


1A9
BIA 3-476 (nmol/mg prot/min)
32.00
24.00
16.00
8.00
0.00
0
100
200
300
400
500
600
BIA3-202 (µM)


1A10
BIA 3-476 (nmol/mg prot/min)
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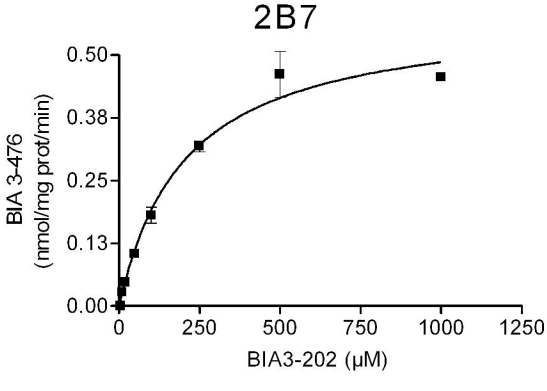
0.80
0.40
0.00
0
100
200
300
400
500
600
BIA3-202 (µM)


2B7
BIA 3-476 (nmol/mg prot/min)
0.50
0.38
0.25
0.13
0.00
0
250
500
750
1000
1250
BIA3-202 (µM)

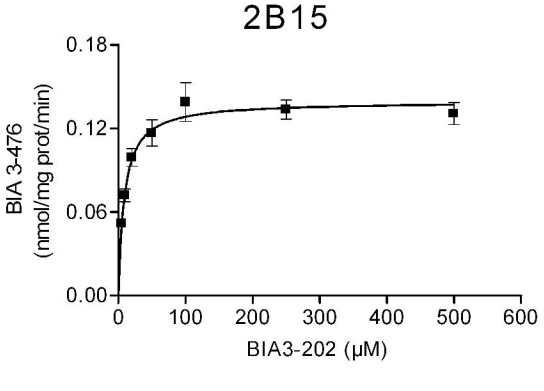

2B15
BIA 3-476 (nmol/mg prot/min)
0.18
0.12
0.06
0.00
0
100
200
300
400
500
600
BIA3-202 (µM)

Figure 5

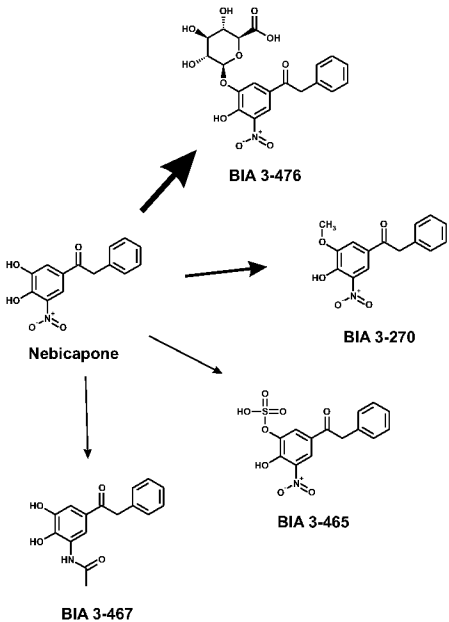


Figure 6