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Biocatalytic production of a monoamine oxidase B/ catechol-O-methyltransferase inhibitor from piperine by engineered P450 BM3.

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ABSTRACT

The single-step biotransformation of the natural compound piperine into a known dual inhibitor of monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT), was achieved by cytochrome P450 BM3 wild-type and the D251G/Q307H double mutant. This compound is used for research in neurodegenerative disorders, such as Parkinson's disease, and its value in the market is ~14,000 €/g. Currently, it is produced by chemical synthesis requiring incubation of piperine with boron tribromide (BBr_3) in dichloromethane with yield of product not exceeding 55 % and using tedious and long procedure for its production and isolation. The P450 D251G/Q307H double mutant exhibited a 3-fold increase in catalytic efficiency compared to the wild-type enzyme, achieving high conversion (51.6 % of conversion in 15 minutes) under mild, environmentally friendly conditions. The yield of production was 0.01 mg of the inhibitor in 1 mL of reaction in 15 minutes at 28°C using the purified enzyme. Moreover, biological assays demonstrated that the resulting compound has a novel and stronger antioxidant and antimicrobial activities, respectively, when compared to piperine. The data further demonstrates the broader potential of engineered enzymes as versatile and sustainable tools in industrial biotechnology, offering an efficient platform for the modification of natural compounds to produce bioactive molecules.

1. Introduction

The replacement of synthetic methods with biocatalytic processes is an important challenge nowadays required for the green transition towards more sustainable processes. Biocatalytic processes are currently used in many areas, including the production of pharmaceuticals, taking advantage of the variety of naturally occurring enzymes and metabolic pathways that produce biologically active compounds. However, since the direct use of natural products is often limited by poor bioavailability or biological activity, low quantities, and undesired side effects, they have been widely used as molecular scaffolds to generate derivatives through chemical or biological catalysis (Lobiuc et al., 2023; Howes et al., 2020; Chaachouay and Zidane, 2024).

Chemical synthesis often has limitations, such as requirement of harsh conditions, lacking selectivity of reaction, and high amounts of byproducts (Chakrabarty et al., 2021). These issues can be overcome by implementation of biocatalytical methods, offering an alternative that is green, and can be optimized to give almost complete selectivity of introduced modifications (Heath and Turner, 2022).

Cytochromes P450 (P450s) are attractive enzymes for biocatalytic purposes due to many reasons that include high regio- and stereospecificity, ability to catalyse a broad variety of modifications also on poorly reactive atoms, mild conditions of reaction and high plasticity for protein engineering (O'Reilly et al., 2011; Isin and Guengerich, 2007).

A particularly interesting candidate of P450s for biocatalysis is CYP102A1 (P450 BM3) discovered in the *Prestria megaterium* (Miura and Fulco, 1974). It is a self-sufficient enzyme with a catalytic domain linked to a reductase domain by a flexible linker. In 40 years of extensive studies, it has shown remarkable flexibility in substrate recognition, and high potential for engineering approach (Whitehouse et al., 2012). Random and directed mutagenesis research resulted in thousands of variants with diverse activity, specificity and stability (Fansher et al., 2024).

Our laboratory has previously generated through directed evolution

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a variant of cytochrome P450 BM3 carrying two mutations (Asp251Gly/Gln301His) and named further P450 BM3 A2 (Tsotsou et al., 2012; Di Nardo et al., 2016). This mutant shows a broader substrate scope and/or increased activity compared to the wild-type (WT) enzyme and, interestingly, it is able to produce hydroxy, epoxy and dealkylated metabolites starting from different pharmaceuticals and natural compounds (Tsotsou et al., 2012; Di Nardo et al., 2016; Correddu et al., 2022). In this work, we used this mutant to introduce modifications on the natural compound piperine, one of the main alkaloids present in plants from Piperaceae family (Chopra et al., 2016) and the main compound responsible for the pungent taste of black pepper, that is one of the most commonly used spices worldwide. Apart from the exceptional value for the food industry, black pepper was widely used in traditional therapies, traditionally associated with pain management, inflammation, and fever treatment. Indeed, piperine was proven to be one responsible for these effects. From the structural point of view piperine consists of an aromatic benzodioxy moiety linked with an amide moiety through an aliphatic chain (Fig. 1) (Tiwari et al., 2020).

In recent years, piperine has been found to exhibit a range of beneficial activities, including antimicrobial, insecticidal, antidepressant, appetite-stimulating, and blood circulation-enhancing properties, and bioenhancing activity (Tiwari et al., 2020; Ajazuddin et al., 2014). Among others, piperine has been shown to express inhibitory effects on P-glycoprotein (P-gp), an ATP-dependent efflux pump involved in removal of drugs from cancer cells. The overexpression of P-gp has been reported in many cancer cell lines and is responsible for multidrug resistance (MDR) (Kovalev et al., 2013). Therefore, inhibition of P-gp can reverse MDR in cancer even if most of the currently tested P-gp inhibitors are not specific, and affect proper function of other proteins, leading to unwanted side effects (Callaghan et al., 2014).

Thanks to this broad biological activity, piperine was used as a starting compound to produce derivatives with improved properties (Wang et al., 2020; Zhong et al., 2023; Syed et al., 2017). Piperine derivatives can have several beneficial effects as they were shown to modulate the activity of several targets related to neurological disorders, including epilepsy, Parkinson's disease, depression and pain related disorders (Al-Baghdadi et al., 2012; Muhammad et al., 2024). Here, we present the production of a biologically active piperine derivative through the biocatalytic conversion using P450 BM3 and its mutant A2. We also show how the enzymatically introduced chemical modification affects the biological properties of the starting natural compound.

2. Methods

2.1. Protein expression, purification and enzymatic reaction

Protein expression and purification were carried out as described before (Tsotsou et al., 2012; Sideri et al., 2013). The activity of P450 BM3 WT and P450 BM3 A2 was initially assayed with the same conditions for both proteins. The reaction mixture was prepared by mixing 6 μM of enzyme, 100 μM piperine and 1 mM NADPH in a 30 mM phosphate buffer pH 7.4 and incubated for 5 – 60 min at 30°C. After incubation, the reaction was stopped by adding phosphoric acid (20 % v/v). As a control, samples were prepared in the same way, but phosphoric acid was added before the NADPH to ensure inactivation of the enzyme and lack of activity.

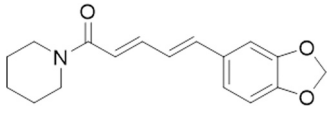

Fig. 1. Structure of piperine.

2.2. HPLC method for the separation of piperine and derivatives

The separation and detection of piperine and its derivatives was achieved through HPLC equipped with a diode array detector (Agilent 1200 Binary DAD HPLC System) using a ZORBAX Eclipse Plus C18 column (4.6 ×250 mm 5-micron) for compounds separation.

The mobile phase consisted of methanol (A) and water containing 0.1 % phosphoric acid (B). The samples were analysed with the following steps: 1) 25 % A; 2) 5–15 min, linear gradient up to 75 % A; 3) 15–21 min, 75 % A. The UV–vis spectra between 200 and 600 nm were recorded and the chromatographic traces at 343 nm were considered for the detection of piperine ($\varepsilon_{343\ nm}$=31,000 $M^{-1}cm^{-1}$) and the derivatives (Upadhyay et al., 2013).

2.3. Mass spectrometry

Electro Spray Ionization (ESI) and Tandem ESI MS/MS analysis experiments were conducted using a Thermo Finnigan Advantage Max Ion trap spectrometer in positive ion acquiring mode; sheath gas flow rate was set at 25 (arbitrary unit), auxiliary gas flow rate at 5 (arbitrary unit), spray voltage at 3.25 (KV), capillary temperature at 270 °C, capillary voltage at −7 (V), and tube lens offset at −60.00 (V). Nitrogen was used as sheath and auxiliary gases.

Tandem ESI MS/MS analysis of piperine and its metabolite was performed with a CE of 32 %, and 33 % respectively to enhance the characterization of piperine and its metabolite.

2.4. Analysis of reaction kinetic parameters

To investigate the kinetic parameters of the enzymatic reaction and evaluate possible differences between P450 BM3 WT and P450 BM3 A2, the conversion over time was first monitored to establish the linear range of product formation. Samples were prepared as described in the previous section and the reaction was stopped at various times ranging from 2 and 60 min.

Then, different concentrations of substrate were applied for 5 minutes (based on the previous experiment), and all reactions were prepared in at least four independent replicates. Piperine was tested at 8 different concentrations ranging from 10 μM to 150 μM. Higher substrate concentration could not be used because of poor piperine solubility in aqueous solutions. Protein (2 μM) and NADPH (1 mM) concentrations were constant for each experiment. The reaction products were separated and quantified using HPLC, as described before. The major reaction product was quantified using a calibration curve obtained from integrated area of peaks obtained after injection of known concentration of analytical standard of hMAO-B/MB-COMT-IN-1. The reaction rates that were plotted as a function of substrate concentration and the kinetic parameters were calculated based by fitting the data to the Michaelis-Menten model.

2.5. Products recovery and quantification

The reaction product was directly collected from HPLC. Once collected, a solid phase extraction (SPE) was performed, using strata-X 33 μm Polymeric Reversed Phase 200 mg/ 6 mL tubes (Phenomenex) to concentrate the product. Each tube matrix was previously prepared with 6 mL of pure methanol (MeOH), followed by 6 mL of ddH_2O; then, the sample was loaded into the matrix, let dry for 30 minutes and washed with 6 mL of 5 % MeOH. The elution was performed with 3 mL of pure MeOH, to concentrate the product.

The quantification of the major piperine metabolite (identified by MS analysis) was carried out using a calibration curve obtained from integrated area of peaks obtained after injection of known concentration of analytical standard of hMAO-B/MB-COMT-IN-1.

2.6. Antioxidant activity – ABTS assay

The antioxidant activity of piperine, and its metabolite was measured with ABTS assay. ABTS radical was prepared by mixing 7 mM ABTS solution (solution A), prepared by dissolving ABTS in acetic acid buffer pH 4.5, with 2.45 mM $K_2S_2O_8$ (solution B), prepared by dissolving $K_2S_2O_8$ in deionized water. Solutions A and B were mixed in a 1:1 ratio and further diluted with acetic acid buffer pH 4.5 to obtain the ABTS radical working solution. To assess the antioxidant activity, 190 µL of working solution was transferred to 96 well microtiter plate and 10 µL of solution containing the molecule of interest was added. The samples were left in the dark for 7 minutes and then the absorbance at 734 nm was measured. The results were then compared to the standard curve obtained by using ascorbic acid as a positive control (Rumpf et al., 2023)

2.7. Antibacterial activity

The effects of piperine and its metabolite on the proliferation of *E. coli* ATCC 8739 strain, used as representative Gram-negative model (U.S. Pharmacopeia General Chapters:51 Antimicrobial effectiveness testing. Rockville, MD:U.S. Pharmacopeia; USP USP34-NF29, 2011), was tested with microdilution method. Antibacterial activity was measured using a turbidimetric broth method based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI M07). Cells were plated on LB agar plate and grown at 37°C for 16 hours. A single colony was taken from the plate and used to inoculate 15 mL of LB broth. The liquid culture was incubated at 37°C until OD_{600} reached 0.5 and then adjusted with MH2 medium to $OD_{600} = 0.1$ and then further 100 times with the same medium obtain a concentration of $1.0 * 10^6$ CFUs/mL. The diluted culture was mixed in a 1:1 ratio with the solution containing the compound to be tested and incubated at 37°C for 16 hours. Then, the culture was diluted with 0.9 % NaCl in a $1{:}10^6$ v/v ratio and 100 µL of diluted culture was plated on the LB agar plate. Plates were incubated for 16 h before the colonies count. The counted colonies number represents viable CFUs/mL remaining after incubation with the tested compound.

Colonies from each experiment were counted separately and compared with respective positive control before the average was calculated. The number of colonies on the positive control plate was taken as 100 %. Lack of colonies means that tested concentration is higher than MIC (minimum inhibitory concentration), and lower colonies count than in the control means partial growth inhibition.

2.8. HT29 and HT29-dx cell lines

Human colon adenocarcinoma HT29 cells (a kind gift of Prof. Chiara Riganti, Department of Oncology, University of Torino, Italy) were grown on the cell culture plates at 37°C with 95 % humidity and 5 % CO_2 in RPMI-1640 medium with stable glutamine (VWR International), supplemented with 10 % bovine fetal serum, and 1 % penicillin-streptomycin mix. HT29-dx doxorubicin resistant cell line was selected by cultivation on medium containing 70 nM of doxorubicin for 20–25 passages, as previously described (Riganti et al., 2005). After this period resistance was verified by measurement of intracellular level of drug. For further passages 50 nM of doxorubicin was always present in the growing medium to maintain the resistance.

2.9. Cell viability - WST-1 assay

HT29-dx were seeded in 96-well plates (3000 cells/well, 100 µL/well), and, after 24 h, exposed to different doses of piperine and hMAO-B/MB-COMT-IN-1 for 48 hours. The concentration range of the tested compounds was between 5 µM and 25 µM. Cells exposed to 10 % of DMSO were used as a positive control, while untreated cells were used as a negative control. After incubation with the compounds to be tested, cell viability was measured by performing WST-1 assay (Roche) as per manufacturer's instructions.

WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was added at 10 % (v/v) of cell medium and the absorbance was read at 440 nm after 30 min, using a SPECTROstar nano-microplate reader (BMG labtech, Aylesbury, UK). The absorbance value at the reference wavelength (630 nm) was subtracted.

2.10. Doxorubicin accumulation

For the detection of doxorubicin accumulation, HT29 and HT29-dx cells were seeded in 2 mL of RPMI-1640 medium supplemented with fetal bovine serum in 6-well plates. A 5 mM doxorubicin stock solution was prepared by dissolving in deionized water and diluted in culture medium to reach 5 µM final concentration in the wells. Cells were incubated for 24 h at 37°C with 5 % CO_2. After incubation, the medium was removed, and the cells were washed with phosphate buffered saline (PBS). Adherent cells were treated with 0.1 % trypsin, shaken for 3 minutes, and resuspended in 1 mL of PBS. After this treatment cells were centrifuged for 30 seconds at 13k RPM. Supernatant was discarded and the pellet was resuspended in 0.6 mL of a solution containing equal volume of ethanol and 0.3 M HCl. Samples were sonicated for on ice with two 10-second bursts. 50 µL were used for the measurement of cellular proteins with the bicinchoninic acid kit from Sigma-Aldrich; the remaining part was checked for doxorubicin content by measuring fluorescence at $\lambda_{abs} = 475$ nm and $\lambda_{emis} = 553$ nm with a Perkin-Elmer LS-5 spectrofluorimeter (Perkin-Elmer). Cells not incubated with doxorubicin were used as a reference to subtract natural fluorescence from cellular metabolites.

2.11. Statistical analysis

All data in text and figures are provided as means $\pm$ SD. The results were analyzed by a one-way ANOVA and Tukey's test. $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Metabolites detection

The P450 enzymes were overexpressed in *Escherichia coli* and purified by affinity chromatography to reach a final yield of 20 mg of pure enzyme/L of liquid culture (Tsotsou et al., 2012; Sideri et al., 2013). The purified enzymes were used in the reaction mixtures containing piperine as substrate and the possible products of bioconversion were analysed by HPLC. The chromatographic traces of the reaction mixture with piperine compared to the control reactions with pre-inactivated enzymes, revealed the presence of 4 new peaks when the compound was incubated with P450 BM3 WT and 6 new peaks in the presence of P450 BM3 A2 (Fig. 2). The product ratio for the wild type enzyme was 76:9:8:7 for products numbered as 3:4:5:6. The product ratio for the mutant was 1:3:80:7:3:6 for products numbered as 1:2:3:4:5:6 in Fig. 2. In both reactions, the peak with retention time of 15.6 min (named MetP3) was predominant (~80 % of the total products) and this compound was selected for identification through LC-MS analysis (Fig. 3).

Fig. 3A shows the MS spectrum of an analytical standard of piperine in positive ionization mode, that resulted with detection of the isotopic pattern at *m/z* 286 (100 %), *m/z* 287 (19 %), and *m/z* 288 (2.5 %) attributed to protonated piperine ($[M + H]^+$).

The assignment of *m/z* 286 signal to piperine was confirmed by tandem ESI MS/MS analysis that resulted in the detection of fragments with *m/z* 84, *m/z* 112, *m/z* 135, *m/z* 201, and *m/z* 202 (Fig. 3B) in agreement with previously reported fragmentation pattern of piperine (Praneetha et al., 2019). The MS analysis of MetP3 sample resulted in the detection of a new peak with *m/z* 274 (Fig. 3C). The difference of 12 au was associated with the opening of benzodioxol ring and the loss of a carbon atom and formation of a catechol moiety. Its tandem ESI MS/MS

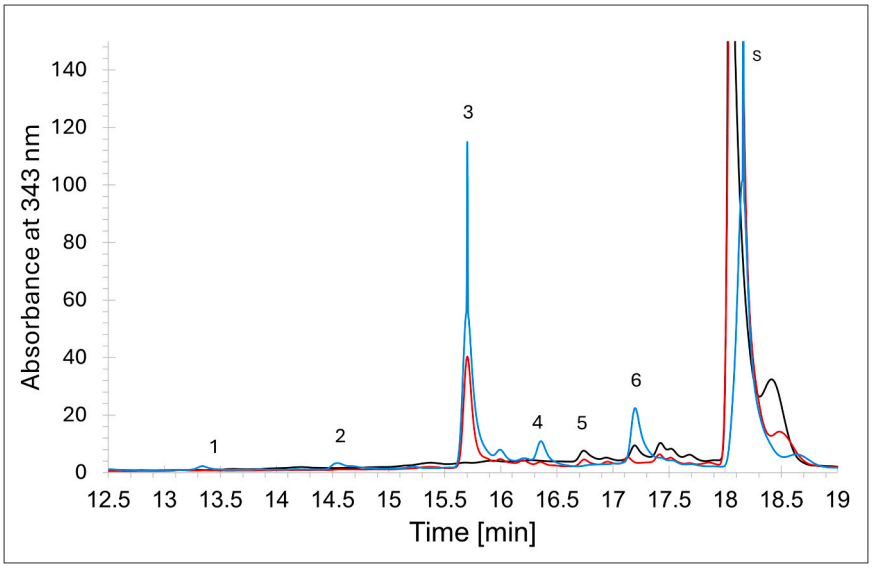

Fig. 2. Chromatographic traces obtained from samples of 100 μM piperine incubated with 6 μM P450 BM3 WT (red), 6 μM P450 BM3 A2 (blue), and negative control (pre-inactivated enzymes, black). The numbers indicate the products of bioconversion: the peak number 3 is referred to as MetP3.

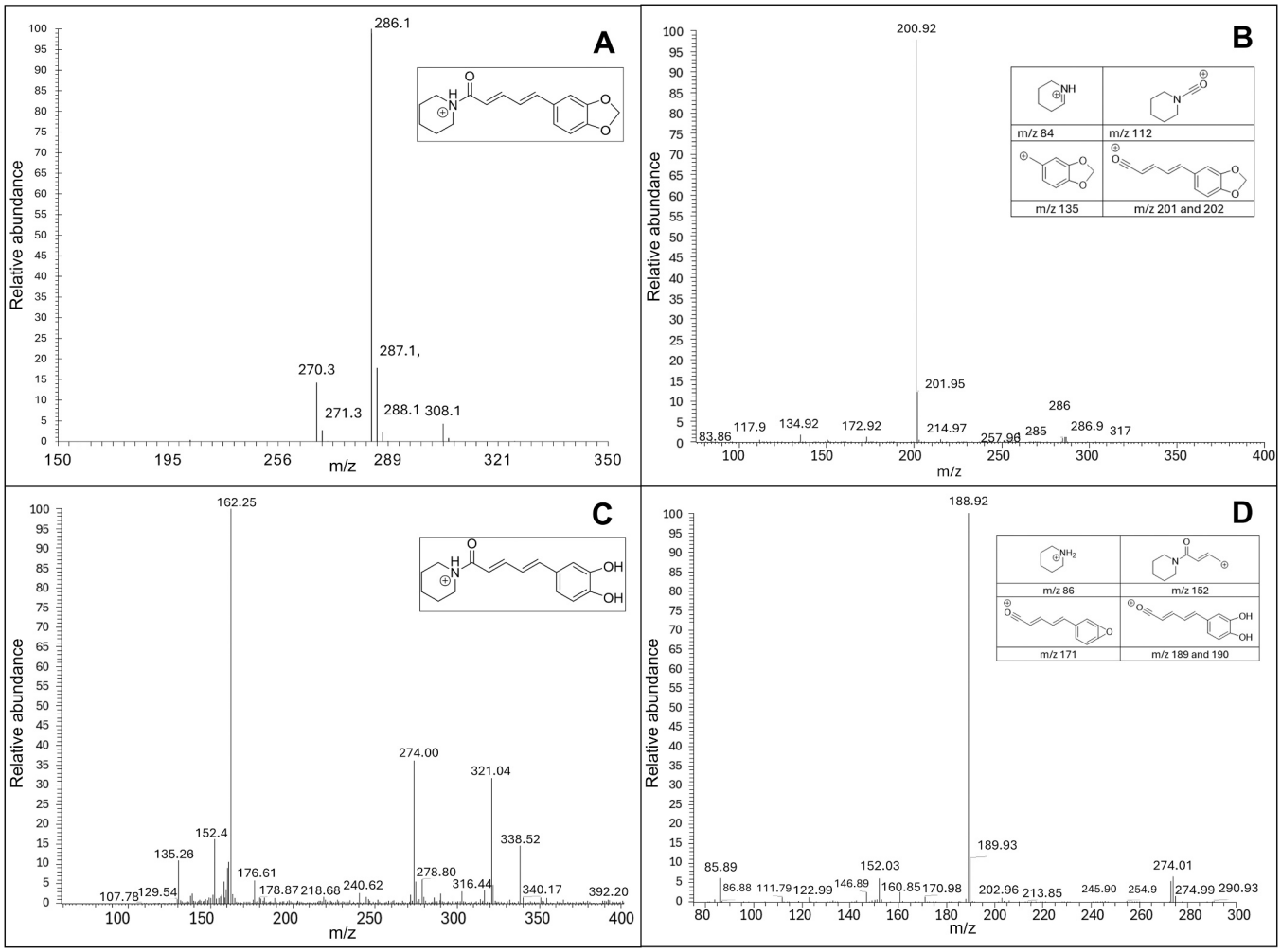

Fig. 3. LC-MS results of samples containing A) piperine, B) isolated MetP3. C) Fragmentation pattern of piperine and D) MetP3. Insets A) protonated piperine $m/z = 286$, B) prevalent ions obtained from piperine fragmentation, C) protonated catechol derivative $m/z = 274$, and D) prevalent ions obtained from catechol derivative fragmentation.

analysis showed fragments at *m/z* 86, *m/z* 152, *m/z* 171, *m/z* 189, and *m/z* 190 (Fig. 3D), consistent with the protonated catechol derivative of piperine described in literature (Fig. 4) (Gao et al., 2017; Praneetha et al., 2019).

The ESI-MS/MS fragmentation pattern observed for both piperine and its metabolite MetP3 is consistent with their respective molecular structures and typical ionization behavior under positive-mode electrospray conditions. For piperine, key fragment ions at *m/z* 84, 112, 135, 201, and 202 reflect characteristic cleavages involving the piperidine moiety and the methylenedioxybenzene ring. The fragment at *m/z* 135 likely arises from the loss of the side chain and stabilization of the benzodioxole cation. In the case of MetP3, the fragment ions at *m/z* 86, 152, 171, 189, and 190 suggest a shift in the fragmentation pathway, consistent with the transformation of the methylenedioxy group into a catechol. The presence of these ions supports the structural assignment of MetP3 as the catechol derivative of piperine, with fragmentation occurring preferentially around the newly formed hydroxylated aromatic ring. This pattern reinforces the chemical identity deduced from the enzymatic mechanism and confirms that the observed mass spectral features align with established fragmentation behavior of similar catecholic and amide-bearing structures.

The P450 BM3-catalyzed transformation of piperine proceeds via hydrolytic ring opening, releasing methylene glycol and yielding the catechol derivative of piperine. The enzymatic activity reflects a demethylenation reaction typical of mammalian detoxification pathways, but here achieved under mild, aqueous conditions. This supports the structural assignment of MetP3 as a catechol derivative, in agreement with mass spectrometry and UV–visible data. The reaction scheme (Fig. 4) illustrates the enzymatic selectivity towards the benzodioxole moiety and the feasibility of this biocatalytic conversion.

Interestingly, this catechol derivative is commercially available under the name of hMAO-B/MB-COMT-IN-1 as it was reported as a highly specific inhibitor of monoamine oxidase B (MAO-B, IC_{50} = 2.5 ± 0.22 μM) and membrane-bound catechol-*O*-methyltransferase (MB-COMT, IC_{50} = 3.84 ± 0.35 μM) (Chavarria et al., 2022). It showed antioxidant activity and a probable ability to cross blood-brain barrier (Chavarria et al., 2022). Thanks to these characteristics, it is used in neurodegenerative disorders research and can be potentially used in the treatment of Parkinson and Alzheimer's diseases (Carradori and Petzer, 2015; Silva et al., 2020).

To the best of our knowledge, there is no reported green, enzymatic way of producing this compound. Instead, the most widely reported method of piperine demethylation requires incubation of piperine with BBr_3 for 24 h in dichloromethane. After this long time, dichloromethane is removed under vacuo and the yellow solid is washed, dried and the pure product is obtained by chromatography (Muthuraman et al., 2019).

Monoamine oxidase B (MAO-B) is a FAD-containing flavoprotein that carries out oxidative deamination of brain monoamines such as dopamine and 5-hydroxytryptamine. Inhibition of its activity leads to the accumulation of these monoamines in brain and was proven to have a high pharmacological importance, especially in Parkinson and Alzheimer's diseases helping with motoric dysfunction (Carradori and Petzer, 2015).

Catechol-*O*-methyltransferase (COMT) is another enzyme involved in degradation of brain amines. It catalyses transfer of methylene moiety to one of two hydroxyl groups in dopamine and epinephrine. Similarly to MAO-B, COMT inhibitors are mainly used in treatment of Parkinson's disease symptoms, especially in combination with L-DOPA, a dopamine precursor (Silva et al., 2020).

3.2. Kinetic parameters

In order to evaluate the kinetic parameters of piperine metabolism, the enzymatic reaction was carried out in the presence of increasing substrate concentrations with a constant reaction time of 5 minutes. The catechol derivative of piperine was quantified using the theoretical calculated epsilon at 343 nm and the rates of enzymatic conversion of piperine to hMAO-B/MB-COMT-IN-1 were plotted as a function of substrate (piperine) concentration and fitted to the Michaelis-Menten model (Fig. 5).

Both enzymes followed a very similar behaviour but P450 BM3 A2 reached higher rates. The kinetic parameters of both enzymes are presented in Table 1 and show that there is no statistically significant difference in the K_M values between the P450 BM3 WT and the P450 BM3 A2. However, the k_{cat} value is increased by 3-folds in P450 BM3 A2 when compared to P450 BM3 WT. Overall, the data suggest that the mutant showed better substrate conversion (Fig. 5), with a catalytic efficiency increased by 4-folds (Table 1), but lower product selectivity (Fig. 2).

The use of higher concentrations was also limited due to very poor solubility of piperine in aqueous solution being 40 mg/L (140 μM) in pure water at room temperature.

Piperine conversion was then studied as a function of time with the highest substrate concentration possible (150 μM piperine) (Fig. 6). The substrate was incubated with P450 BM3 and P450 BM3 A2 in presence of NADPH, and the reaction was stopped at various time points between 2- and 60-minutes incubation.

Both enzymes convert most of the substrate in the first 15 minutes of incubation, and longer incubation time did not result in a significant increase in substrate conversion (Fig. 6A).

To check whether uncoupling reactions could limit NADPH availability, we performed the reactions following the same protocol but with refilling the NADPH after 5 and 10 minutes of incubation. However, with this experimental setup we did not observe any increase in the formation of products and thus we could exclude a possible lack of NADPH due to uncoupling. Therefore, our hypothesis is that the observed slowdown in the reaction is due to the limited substrate amount left in the reaction mixture. In fact, after 15 minutes of reaction, the concentration of substrate is close to calculated K_M that can explain the significant decrease in the reaction rate. The maximum conversion was noted in samples incubated for 60 minutes and was 32 % and 63 % for P450 BM3 WT and P450 BM3 A2, respectively (Fig. 6B). In this conditions, 0.01 mg of hMAO-B/MB-COMT-IN-1 is produced in 1 mL of reaction in 15 minutes at 28°C.

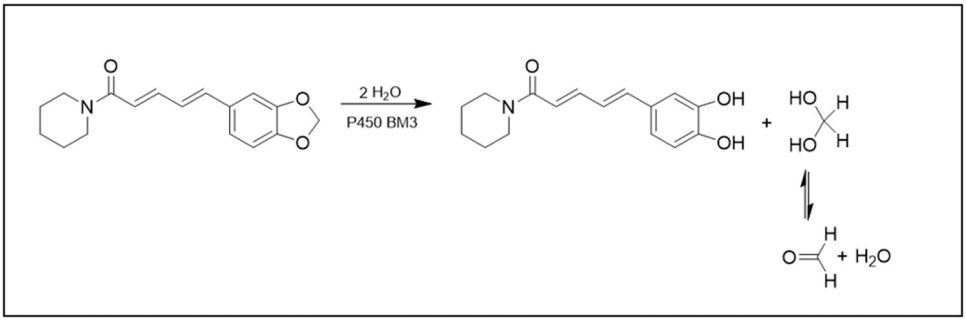

Fig. 4. P450 BM3-catalyzed reaction scheme and structure of catechol derivative of piperine (hMAO-B/MB-COMT-IN-1).

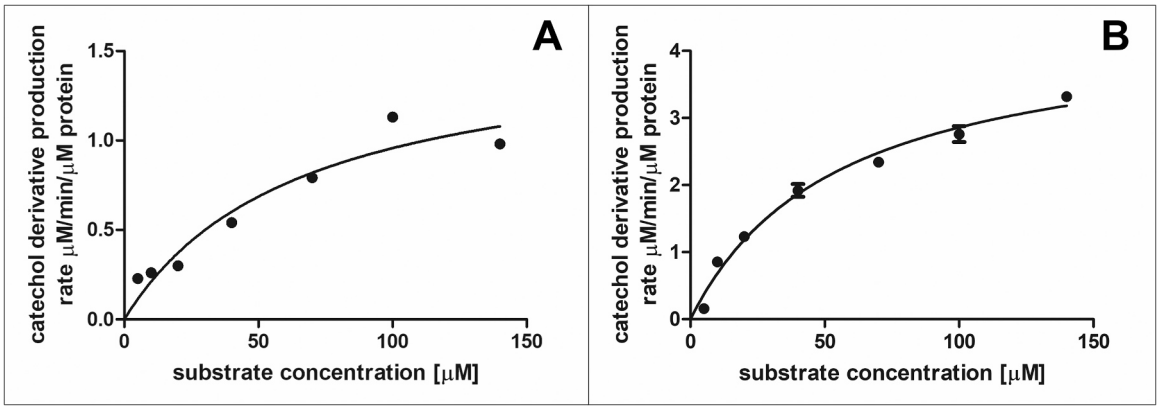

Fig. 5. Kinetic of hMAO-B/MB-COMT-IN-1 production observed for A) P450 BM3 and B) P450 BM3 A2.

Table 1
Kinetic parameters for piperine metabolism by P450 BM3 WT and P450 BM3 A2.

Kinetic parameters of hMAO-B/MB-COMT-IN-1 formation			
	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
P450 BM3 WT	1.58 ± 0.13	65.1 ± 12.1	0.02
P450 BM3 A2	4.44 ± 0.17	55.2 ± 5.0	0.08

3.3. Antioxidant and antimicrobial activity

The biological properties of piperine and hMAO-B/MB-COMT-IN-1 were tested in terms of their antioxidant and antimicrobial activity. The antioxidant activity of piperine and its derivative were tested by ABTS assay, in the range of concentration chosen on the basis of previous studies and of the limitations due to the solubility of the compounds (Zarai et al., 2013). The antimicrobial activity was tested on

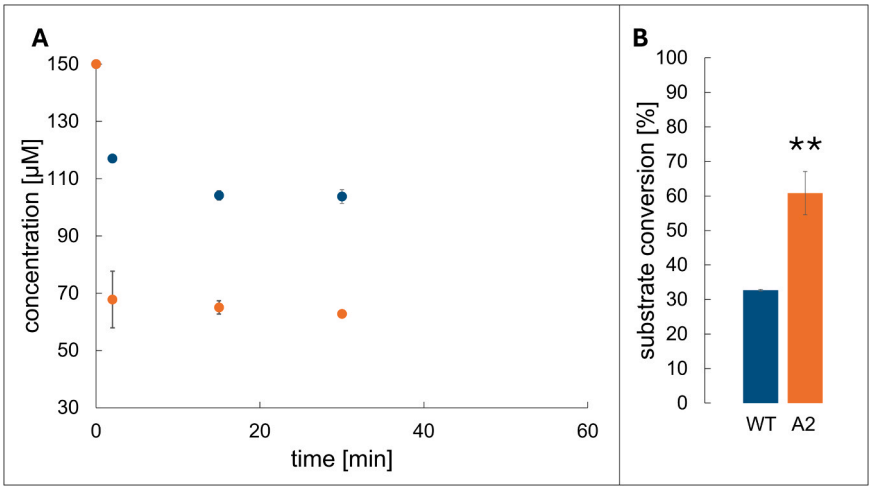

Fig. 6. Piperine conversion over time and yield of conversion. A) Changes in piperine concentration as a function of time from the incubations with P450 BM3 WT (blue) and P450 BM3 A2 (orange). B) Comparison of the maximal conversion obtained for piperine by P450 BM3 WT (blue) and P450 BM3 A2 (orange). Measurements were performed in triplicate and data are presented as means ± SD (n = 3); versus ctrl *p < 0.05; * *p < 0.01.

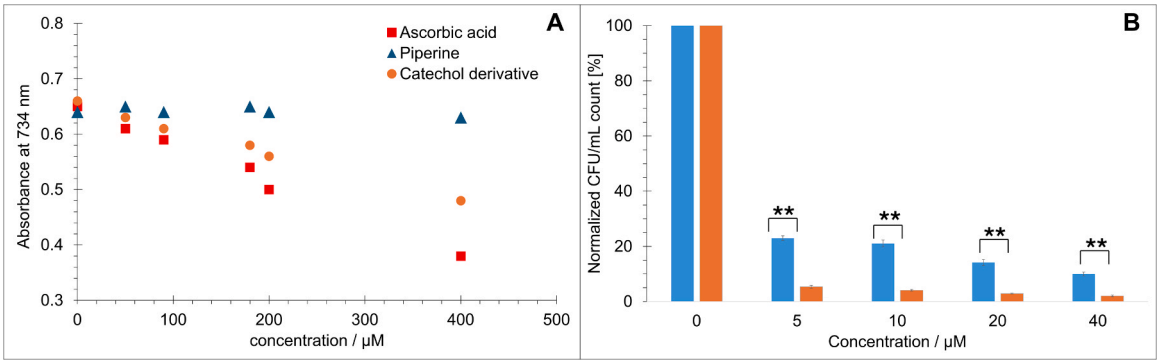

Fig. 7. Results of biological activity assays. A) ABTS assay results, representing radical scavenging force of piperine (blue triangle), hMAO-B/MB-COMT-IN-1 (orange circle), and ascorbic acid (red square). B) Proliferation of *E. coli* ATCC 8739 incubated with piperine (blue) and hMAO-B/MB-COMT-IN-1 (orange). Measurements were performed in replicates and data are presented as means ± SD (n = 4); hMAO-B/MB-COMT-IN-1 treated *versus* piperine treated *p < 0.05; * *p < 0.01.

E. coli ATCC 8739, a representative Gram-negative model. The results of both experiments are presented together in Fig. 7. Piperine did not show any noted antioxidant activity, whereas the hMAO-B/MB-COMT-IN-1 piperine derivative acquired an antioxidant activity that is 60 % of the ascorbic acid one, indicating that changes in the structure of piperine through biocatalytic demethylation caused increase of the radical scavenging power (Fig. 7A).

Both piperine and hMAO-B/MB-COMT-IN-1 inhibited *E. coli* ATCC 8739 proliferation even at the lowest of tested concentration (5 μM). We observed a dose-dependent response but none of the tested compounds inhibited completely *E. coli* growth. This effect is in line with the previous findings reporting piperine, and pepper extracts activity against various microbes including pathogenic *E. coli* strains (Manjunath et al., 2022). The effect of hMAO-B/MB-COMT-IN-1 was stronger than piperine at every tested concentration (Fig. 7B).

3.4. Anticancer activity

As previously mentioned, piperine was reported to have inhibitory effect on the ATP-dependent efflux pump P-gp, a transporter involved in drug excretion from cancer cells. Inhibition of P-gp can therefore be crucial in reverting MDR in cancer as one of the drug resistance mechanisms observed in various cancer cell types is overexpression of membrane transporters such as P-glycoprotein. Overexpression of these pumps make the transport of drug outside of the cell more efficient, resulting in lower drug activity. Due to these properties, piperine was used as a scaffold for development of various analogues. Most of these analogues were obtained by modifications on the piperidine ring, either by adding new functional groups or being completely substituted. (Syed et al., 2021) Here we tested whether the conversion of piperine to hMAO-B/MB-COMT-IN-1 affects the P-gp inhibitory activity.

To this purpose, the two compounds were tested for their ability to inhibit the viability of colon cancer doxorubicin-resistant HT29-dx cells, a subpopulation of cells, obtained from sensitive HT29 cells after a selection with doxorubicin (Riganti et al., 2005). HT29-dx accumulate less intracellular doxorubicin and are less sensitive to its cytotoxic effects, with respect to the sensitive counterpart, thanks to an increased expression of drug efflux transporters such as P-gp (Riganti et al., 2005). Doxorubicin is a common anticancer drug used in treatment of various tumours. It is a fluorescent compound, thus its concentration inside the cell can be easily monitored. In resistant cells, doxorubicin is actively eliminated by the P-gp. Therefore, a possible increase in the accumulation of doxorubicin during co-incubation with the molecule of interest can be directly correlated with P-gp inhibition.

The viability of HT29-dx cells was measured after 24 hours incubation with piperine or hMAO-B/MB-COMT-IN-1, in the absence or in the presence of 5 μM doxorubicin (Fig. 8). The two compounds tested alone (without coincubation with doxorubicin) did not show any significant toxicity on HT-29-dx cells, even at the highest tested concentration, which was 25 μM.

However, when HT-29-dx cells were co-incubated with piperine and doxorubicin, a significant reduction in viability was observed, starting from the lowest dose applied (5 μM, Fig. 8A); the incubation with the hMAO-B/MB-COMT-IN-1 was not effective in increasing doxorubicin toxicity towards resistant cells (Fig. 8B).

Indeed, when intracellular accumulation of doxorubicin was measured in HT29-dx cell line, piperine and hMAO-B/MB-COMT-IN-1 showed a significantly different effect (Fig. 9). In the absence of any compound, HT29 cells drug accumulation is 4-folds higher than in the HT29-dx cells (Fig. 9 inset). Co-incubation of HT29-dx cells with doxorubicin and piperine resulted in the increase in the drug accumulation: the addition of 10 μM of piperine results with more than two-fold increase in the doxorubicin content. On the contrary, co-incubation with hMAO-B/MB-COMT-IN-1 did not alter the doxorubicin accumulation (Fig. 9).

These findings indicate that the benzodioxole ring is crucial for the interaction between the piperine and p-glycoprotein.

4. Conclusion

In this work, we showed that piperine can be converted by P450 BM3 WT and its engineered mutant P450 BM3 A2. The most abundant piperine metabolite was successfully isolated and identified, through mass spectrometry analysis. Based on our results and previous reports, we established that the opening of methylenedioxy moiety with loss of a carbon atom leaded to formation of a metabolite that was previously described as hMAO-B/MB-COMT-IN-1. This compound is a double function inhibitor of monoaminoxidase B and catechol-*O*-methyltransferase. Both enzymes are involved in the degradation of amines in the brain and are targets in the treatment of neurodegenerative diseases.

A series of biological activity assays revealed that the changes introduced by the enzyme in piperine structure caused high differences in the activity. The obtained derivative gained antioxidant activity, and a higher antimicrobial power compared to the starting compound.

Another interesting change is that hMAO-B/MB-COMT-IN-1 did not inhibit P-glycoprotein activity in doxorubicin resistant colon cancer HT-29 cells. However, the lack of P-gp inhibition can be a positive factor for the treatment of neurodegenerative diseases. Indeed, the importance of

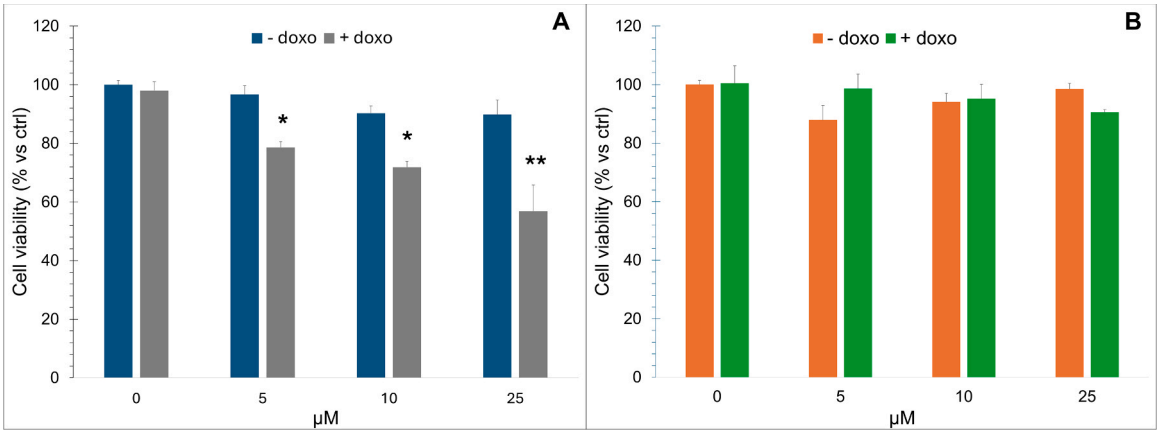

Fig. 8. HT29-dx cells viability results in the presence of piperine and hMAO-B/MB-COMT-IN-1. A) HT29-dx viability after incubation with piperine in the absence (blue bars) or presence (grey bars) of 5 μM doxorubicin (doxo); B) HT29-dx viability after incubation with hMAO-B/MB-COMT-IN-1 in the absence (orange bars) or presence (green bars) of 5 μM doxorubicin (doxo). Measurements were performed in triplicate and data are presented as means ± SD (n = 3); versus ctrl *p < 0.05; * *p < 0.01.

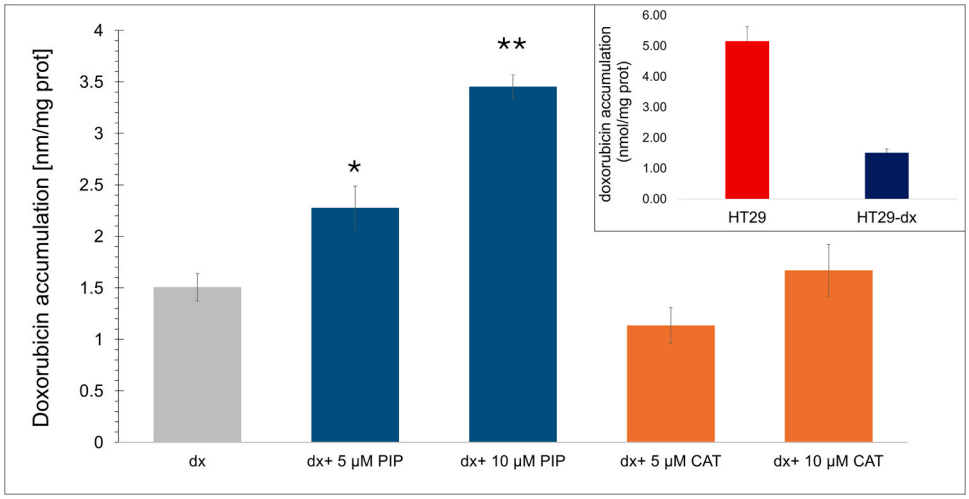

Fig. 9. Accumulation of doxorubicin in HT29-dx cells incubated with piperine (PIP, blue bars) or hMAO-B/MB-COMT-IN-1 (CAT, orange bars). Inset – comparison of doxorubicin accumulation in HT29 cells and doxorubicin resistant HT29-dx cells. Measurements were performed in triplicate and data are presented as means ± SD (n = 3); versus ctrl *p < 0.05; * *p < 0.01.

this protein in elimination of toxic metabolites in Alzheimer's disease is intensively studied and its downregulation was associated with earlier and stronger symptoms occurrence (Abuznait and Kaddoumi, 2012).

Our findings prove that P450-driven catalysis of piperine can be an interesting replacement for standard procedure. A chemical synthesis of hMAO-B/MB-COMT-IN-1 requires incubation with boron tribromide (BBr_3) in dichloromethane with yield of product not exceeding 55 % after 24 h (Muthuraman et al., 2019). Lack of alternative production methods causes the exceptionally high cost of this compound reaching up to 14,000€/g which makes it not accessible for further large-scale studies. In laboratory scale, we were able to reach up to 0.01 mg/mL of product using 0.7 mg of purified enzyme. This yield can be improved by implementing a continuous reaction setup using whole cells expressing the enzyme and a NADPH regeneration system that will further lower the biosynthesis cost.

The analysis of reaction kinetics showed that P450 BM3 A2 was more active than the P450 BM3 WT both in terms of maximum observed conversion and in terms of the reaction rate. The finding that enzymatic reactivity occurs on benzodioxyl moiety can be an interesting point for future work due to the often occurrence of this moiety in various natural and synthetic compounds with bioactive properties.

CRediT authorship contribution statement

Irudal Samuele: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Brzoski Mariusz:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Gilardi Gianfranco:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Buscaino Roberto:** Methodology, Investigation, Formal analysis, Data curation. **Gazzano Elena:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Di Nardo Giovanna:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization. **Viscardi Guido:** Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data will be made available on request. All data generated or analysed during this study are included in this manuscript.

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