The plant alkaloid piperine as a potential inhibitor of ethidium bromide efflux in *Mycobacterium smegmatis*

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Piperine, a major plant alkaloid found in black pepper (*Piper nigrum*) and long pepper (*Piper longum*), has shown potential for inhibiting the efflux pump (EP) of *Staphylococcus aureus*. In this study, a modulation assay showed that piperine could decrease the MIC of ethidium bromide (EtBr) twofold at 32 μg ml⁻¹ and fourfold at 64 μg ml⁻¹ against *Mycobacterium smegmatis* mc² 155 ATCC 700084. A real-time, 96-well plate fluorometric method was employed to evaluate the EP inhibition ability of piperine in *M. smegmatis*. Reserpine, chlorpromazine, verapamil and carbonyl cyanide m-chlorophenylhydrazone were used as positive controls. Piperine significantly enhanced accumulation and decreased the efflux of EtBr in *M. smegmatis*, which suggests that it has the ability to inhibit mycobacterial EPs.

INTRODUCTION

Members of the family *Mycobacteriaceae* include pathogens that can cause significant morbidity and mortality, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, in addition to other opportunistic pathogens such as *Mycobacterium avium*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis* (Flores et al., 2005). It is estimated that approximately 8 million new tuberculosis (TB) cases appear annually, causing an estimated 2 million deaths (Bloom & Murray, 1992). The 13th annual TB report from the World Health Organization, published on World TB Day, 24 March 2009, showed that there were an estimated 9.27 million new cases of TB worldwide in 2007 (WHO, 2009). The resurgence of TB caused by *M. tuberculosis* is related to the emergence of the human immunodeficiency virus and the rapid spread of multidrug-resistant (MDR) strains and drug-resistant TB (WHO, 2000). Intrinsic or acquired multidrug resistance has been important for the resurgence of TB (Victor et al., 2002). Reports have shown that the efflux pump (EP) is a major fundamental factor in intrinsic resistance (Viveiros et al., 2003; De Rossi et al., 2006). Mycobacterial strains can show an MDR phenotype by increasing the activity of EPs, which prevents the compounds from reaching their intended targets (Gupta et al., 2006). Inhibition of EPs is increasingly becoming a way to fight MDR micro-organisms (Werle, 2008). Effective bacterial EP inhibitors (EPIs) should reduce the intrinsic resistance of bacteria to antibiotics, reverse any acquired resistance and decrease the frequency of emergence of resistant mutant strains (Marquez, 2005). Publications have focused predominantly on the identification of EPI molecules with activity against the Gram-positive bacterium *Staphylococcus aureus* and Gram-negative species of *Pseudomonas*, *Escherichia* and *Acinetobacter* (Stavri et al., 2007). According to Lechner et al. (2008), only one new natural EPI, isoflavone biochanin A, has been identified for mycobacteria; however, several experimental compounds, such as reserpine, chlorpromazine, verapamil and carbonyl cyanide m-chlorophenylhydrazone (CCCP), have been shown to have EPI activity.

†These authors contributed equally to this paper.

**Abbreviations:** CCCP, carbonyl cyanide m-chlorophenylhydrazone; EP, efflux pump; EPI, efflux pump inhibitor; EtBr, ethidium bromide; EUCAST–ESCMID, European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases; FICI, fractional inhibitory concentration index; MDR, multidrug resistant; MF, modulation factor; TB, tuberculosis.
against mycobacteria both in vitro and ex vivo (Rodrigues et al., 2008). Therefore, the identification of EPIs for mycobacteria is of some importance.

Piperine is a trans–trans isomer of 1-piperonyl-piperidine found within the family Piperaceae and occurs in black pepper (Piper nigrum) and long pepper (Piper longum). It is believed to have bioavailability-enhancing activity for some nutritional substances and for some drugs (Atal et al., 1985; Badmaev et al., 1999). Piperine has previously been shown to inhibit several cytochrome P450-mediated pathways and phase II reactions in animal models (Atal et al., 1981; Singh et al., 1986). It also reportedly inhibits human P-glycoprotein and CYP3A4 (Bhardwaj et al., 2002). In addition, Khan et al. (2006) described the potentiating effect of piperine with ciprofloxacin in in vitro combination studies against S. aureus and suggested its role as an EPI in S. aureus. These results motivated the current study of piperine for use as an EPI in mycobacteria.

Ethidium bromide (EtBr) is an efflux substrate for many efflux systems in various MDR micro-organisms, including S. aureus and mycobacteria (Gibbons et al., 2003; Khan et al., 2006; Stavri et al., 2007). M. smegmatis has been used extensively as a model system for M. tuberculosis and other pathogenic mycobacteria. M. smegmatis is non-pathogenic, requiring less stringent containment facilities, and grows at a relatively high rate in a variety of defined and nutrient-restricted media (Chacon et al., 2002). EtBr and M. smegmatis have been used collaboratively to study EPI activity (Lechner et al., 2008; Rodrigues et al., 2008). In this paper, we utilized a real-time, 96-well plate fluorometric method that was modified from methods described in recent reports (Rodrigues et al., 2008; Coldham et al., 2010) to evaluate the inhibiting effects of piperine on the EtBr efflux system in M. smegmatis strain mc² 155.

**METHODS**

**Materials.** Reserpine, chlorpromazine, verapamil, CCCP and piperine (all from Sigma-Aldrich) were dissolved in DMSO (Sigma-Aldrich). EtBr (Sigma-Aldrich) was dissolved in water. The chemical structure of piperine is shown in Fig. 1.

**Bacterial strains.** M. smegmatis mc² 155 ATCC 700084 (LGC Promochem) was used throughout the study. Bacterial cells were grown at 37 °C in Middlebrook 7H9 broth or Middlebrook 7H11 agar medium (Difco Laboratories). Both media were supplemented with 10% oleic acid–albumin–glucose complex (OADC). Cultures of these strains served as the source of bacteria for the preparation of a standard inoculum in PBS and were used to determine the MIC. The number of c.f.u. corresponding to aliquots of the inoculum was routinely calculated to ensure a constant number of bacterial cells in experiments (Rodrigues et al., 2008).

**MIC determination.** The MICs of EtBr, reserpine, chlorpromazine, verapamil, CCCP and piperine were determined by the broth microdilution method adapted from other studies and in accordance with Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) guidelines (NCCLS, 2003; Rodrigues et al., 2008). Briefly, M. smegmatis mc² 155 was grown in 7H9/OADC-supplemented medium at 37 °C until an OD₆₀₀ of 0.8 was reached. The bacterial cultures were diluted in PBS and the suspension adjusted to equal a McFarland no. 0.5 turbidity standard. The final inoculum was prepared by diluting the adjusted bacterial suspension 1:100. Aliquots of 0.1 ml were transferred to each well of a 96-well plate containing 0.1 ml of each agent at concentrations prepared from twofold serial dilutions in 7H9/OADC-supplemented medium. The inoculated plates were incubated at 37 °C and the MIC results were noted after a period of time at which growth in the agent-free control well was evident. The MIC was defined as the lowest concentration of compound that inhibited visible growth.

**Modulation assay.** The modulator compounds were further screened for their synergistic effects with EtBr prior to the efflux assays, according to modification of a method described by Lechner et al. (2008). Compounds were dissolved in DMSO and diluted in 7H9/OADC-supplemented medium at subinhibitory concentrations. The concentration of the compounds remained the same throughout the experiment, whereas the EtBr was serially diluted for MIC determination with and without modulators. A modulation factor (MF) was used to express the modulating effects of compounds on the MIC. (EtBr), where MF=MIC (EtBr)/MIC (EtBr + modulator). The fractional inhibitory concentration index (FICI) for M. smegmatis mc² 155 was calculated by the following equation (EUCAST–ESCMID, 2000): FICI= FIC(A) + FIC(B) = (C₅₀(A) / MIC₅₀(A) alone) + (C₅₀(B) / MIC₅₀(B) alone), where MIC₅₀(A) alone and MIC₅₀(B) alone were the MIC values of drugs A and B when acting alone, and C₅₀(A) and C₅₀(B) were the concentrations of drugs A and B at the isoeffectivc combinations, respectively. Interpretation of the FICI was as follows: an FICI value ≤0.5 represented synergy, whilst an FICI value of between 1 and 4 represented indifference and an FICI value >4 represented antagonism (Odds, 2003).

**EtBr accumulation assay by the fluorometric method.** This assay was performed according to a modification of published methods (Rodrigues et al., 2008; Coldham et al., 2010). Organisms were grown in 10 ml 7H9/OADC-supplemented medium at 37 °C to an OD₆₀₀ of 0.8. The culture was centrifuged at 13 000 r.p.m. in a microfuge for 3 min. The supernatant was discarded, and the pellet washed once and resuspended in PBS. After adjusting the OD₆₀₀ to 0.4, glucose and EtBr (to yield final concentrations of 0.4% and 1 μg ml⁻¹, respectively) were added to one set of microtubes containing 1 ml bacterial suspension. Aliquots of 95 μl were distributed to replica sets of 0.2 ml microtubes, and 5 μl each EPI was added. Replica tubes that did not receive any EPI served as a control. The fluorescence was measured in real time using a Victor3 1420 multi-label counter (Wallac Oy) equipped with a plate heater set at 25 °C. The fluorescence at excitation and emission wavelengths of 530 nm (bandwidth 5 nm) and 600 nm (bandwidth 10 nm), respectively, was measured at 3 min intervals for 45 min. Each experiment was repeated three times.

**EtBr efflux assay by the fluorometric method.** The effect of the agents on EtBr efflux activity was measured according to a modification of recently reported fluorescence techniques (Rodrigues et al., 2008; Coldham et al., 2010). The conditions that caused the maximum

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**Fig. 1.** Chemical structure of piperine.
accumulation of EtBr without causing any significant inhibition of growth, as confirmed by c.f.u. counting, were used for the loading of *M. smegmatis* mc² 155 cells with this fluorochrome. The following conditions were used: accumulation at 25 °C in the absence of glucose, use of an EtBr concentration that caused a higher accumulation without compromising the cellular viability (4 μg ml⁻¹, corresponding to half the MIC) and in the presence of verapamil at half its MIC (150 μg ml⁻¹, to ensure that all of the *M. smegmatis* mc² 155 cells could load up to the maximum EtBr). The EtBr-loaded cells were centrifuged at 13 000 r.p.m. in a microfuge for 3 min and resuspended in EtBr-free PBS containing 0.4 % glucose. After adjusting the OD₆₀₀ to 0.4, aliquots of 95 μl were transferred to replicate 0.2 ml microtubes, and 5 μl each EPI was added. Replica tubes that did not receive any EPI served as a control. The EtBr efflux from the cells was monitored with a Victor3 1420 multi-label counter equipped with a plate heater set at 25 °C. The fluorescence at excitation and emission wavelengths of 530 nm (bandwidth 5 nm) and 600 nm (bandwidth 10 nm), respectively, was measured at 3 min intervals for 45 min. Raw fluorescence values were analysed using Excel (Microsoft) and included the subtraction of appropriate control blanks. Each experiment was repeated three times.

**RESULTS**

**MIC results**

According to the agent susceptibility tests, the MIC values for EtBr, reserpine, chlorpromazine, verapamil, CCCP and piperine in *M. smegmatis* mc² 155 were 8, 256, 32, 300, 25 and 128 μg ml⁻¹, respectively (Table 1). These results showed that piperine had a moderate anti-mycobacterial activity compared with the other positive-control EPIs.

**Effect of EPIs on EtBr MICs**

Reserpine, chlorpromazine, verapamil, CCCP and piperine were further tested for their modulating activities of EtBr at subinhibitory concentrations against *M. smegmatis* mc² 155 ATCC 700084. Piperine decreased the MIC of EtBr twofold at 32 μg ml⁻¹ and fourfold at 64 μg ml⁻¹. The FICI between piperine and EtBr demonstrated indifference (FICI=0.75). Reserpine decreased the MIC of EtBr twofold at 32 μg ml⁻¹ and fourfold at 64 μg ml⁻¹, chlorpromazine decreased the MIC of EtBr fourfold at 16 μg ml⁻¹, verapamil decreased the MIC of EtBr twofold at 64 μg ml⁻¹ and CCCP decreased the MIC of EtBr twofold at 16 μg ml⁻¹. The FICIs between chlorpromazine, verapamil, CCCP and EtBr all showed indifferent interactions (FICI = >0.5 to ~2), whereas a combination of reserpine and EtBr demonstrated weak synergy (FICI =0.5). Compared with the positive-control EPIs, the reduction caused by piperine indicated the role of an EP system, which suggests that piperine can potentially inhibit EtBr efflux in *M. smegmatis*.

**EtBr efflux inhibition experiments**

Figs 2 and 3 show the fluorescence intensity of the four positive-control EPIs and piperine within 45 min obtained from the EtBr accumulation and efflux assays. In the EtBr accumulation assay (Fig. 2), the fluorescence intensity of the no-EPI group was lower than the other groups containing these agents. The verapamil group had the highest fluorescence intensity level among all the groups, and CCCP showed a higher fluorescence intensity than reserpine, with chlorpromazine being the weakest. The accumulated fluorescence intensity of piperine was between that of chlorpromazine and reserpine for the first 15 min; however, it was lower than CCCP and higher than reserpine for the final 30 min.

In the EtBr efflux assay (Fig. 3), verapamil displayed a noticeable capability to prevent a decrease in the fluorescence intensity in *M. smegmatis* cells. In addition, CCCP had a stronger ability to prevent this decrease

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (μg ml⁻¹)</th>
<th>Concns used in modulatory activity test (μg ml⁻¹)</th>
<th>MF of EtBr*</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>256</td>
<td>32</td>
<td>2</td>
<td>0.625</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>32</td>
<td>8</td>
<td>1</td>
<td>1.25</td>
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<tr>
<td></td>
<td></td>
<td>16</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>Verapamil</td>
<td>300</td>
<td>32</td>
<td>1</td>
<td>1.107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>2</td>
<td>0.713</td>
</tr>
<tr>
<td>CCCP</td>
<td>25</td>
<td>16</td>
<td>2</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>Piperine</td>
<td>128</td>
<td>32</td>
<td>2</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
<td>4</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*MIC of EtBr=8 μg ml⁻¹; MF=MIC (EtBr)/MIC (EtBr + modulator).
compared with reserpine, whilst chlorpromazine demonstrated the weakest ability to prevent the reduction in fluorescence intensity. The fluorescence intensity was highest at the beginning of testing when the \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155 cells were loaded with EtBr. Gradually over 45 min, the fluorescence intensity decreased because the EP decreased the concentration of EtBr in the cells. From the results of the EtBr efflux inhibition experiments, the four positive-control EPs tested exhibited efflux inhibition in \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155 cells, with concentrations at half their MICs compared with the no-EPI group. Piperine also achieved EP inhibition levels comparable to the reserpine standard EPI control, and the EtBr efflux inhibition ability of piperine was greater than chlorpromazine but lower than CCCP. These results suggest that piperine is a potential inhibitor of EtBr efflux in \( M. \text{smegmatis} \).

Fig. 4 shows that higher concentrations of piperine exhibited a stronger EtBr efflux inhibitory effect in \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155 cells. This result demonstrated that piperine inhibited this efflux in a concentration-dependent manner during the assay period.

**DISCUSSION**

In this study, we first analysed the MICs of EtBr, reserpine, chlorpromazine, verapamil and CCCP against \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155. The MIC values of these compounds were similar to the values reported by others for the same strain (Lechner et al., 2008; Rodrigues et al., 2008). Furthermore, the reversal of the MICs of EtBr by piperine was studied using well-known EPIs (reserpine, chlorpromazine, verapamil and CCCP) as controls using a modulation assay. In this pre-screen experiment, piperine showed comparable synergistic effects with EtBr to those of the positive-control EPIs of chlorpromazine, verapamil and CCCP. Piperine also decreased the MIC of EtBr (MIC = 8 \( \mu \text{g ml}^{-1} \)) by an MF of 4 at 64 \( \mu \text{g ml}^{-1} \) in \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155. These results led us to conduct the efflux assay. Many compounds that are competitors of other EP substrates could also cause the increasing concentrations of other substrates in the accumulation assay. However, they could not inhibit the efflux of other substrates in the efflux assay. For example, Phe-Arg \( \beta \)-naphthylamide, which causes accumulation of EtBr but has no effect on the extrusion of EtBr, is not an EPI but rather a competitor of EtBr (Lomovskaya et al., 2007; Martins et al., 2009). Therefore, as well as the EtBr accumulation assay, the EtBr efflux assay was also employed to further confirm that piperine is indeed an EPI. The concentrations of EPIs (including piperine) used to achieve efflux inhibition were high and may be the reason that the \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155 strain used here is not an overexpressing strain (Lechner et al., 2008). Thota et al. (2008) indicated that the effect of EPIs was more prominent in NorA-overexpressing S. aureus strain 1199B.

It has been suggested that amphipathic cations are existing natural substrates of EPs (Lewis, 1999) and these molecules have frequently been used to study EP-mediated efflux (Tegos et al., 2008). EtBr is one of most popular molecules used (Gibbons et al., 2003; Oluwatuyi et al., 2004). EtBr can bind to DNA (Jennings & Ridler, 1983) and increase fluorescence. Consequently, we took advantage of this property to directly examine the action of EPIs on EtBr uptake using fluorescence techniques. Additionally, the combinations of EtBr/reserpine, EtBr/chlorpromazine, EtBr/verapamil and EtBr/CCCP have been used internationally as standard positive controls for efflux activity in S. aureus and \( M. \text{smegmatis} \) (Oluwatuyi et al., 2004; Lechner et al., 2008). Reserpine is an inhibitor of ATP-dependent pumps, verapamil inhibits P-glycoprotein and bacterial EPs in general (De Rossi et al., 2006), and chlorpromazine affects potassium flux across the membrane in S. aureus and the yeast Saccharomyces cerevisiae (Eilam, 1983; Kaatz et al., 2003), whilst CCCP is a proton motive force uncoupler and its addition increases isoniazid accumulation in \( M. \text{smegmatis} \) (De Rossi et al., 2006). In this study, we integrated a recently reported real-time method (Rodrigues et al., 2008) and a 96-well plate fluorometric method (Goldham et al., 2010). This allowed us to quantify EP activity on a real-time basis and separate the accumulation of substrate from its extrusion and vice versa, which allowed the identification of specific EPIs with a high throughput.

An EtBr concentration of 1 \( \mu \text{g ml}^{-1} \) was used for the EtBr accumulation assay. This preliminary experiment, along with the results of other reports, showed that the lowest concentration of EtBr that resulted in minimal accumulation by \( M. \text{smegmatis} \) \( \text{mc}^2 \) 155 was 1 \( \mu \text{g ml}^{-1} \) at 25 °C for
100 min (Rodrigues et al., 2008). The minimal concentration of EtBr needed was similar to that of the intrinsic efflux systems of *M. smegmatis* (Rodrigues et al., 2008). An EtBr concentration of 4 μg ml⁻¹ (half the MIC) was used in the efflux assay to ensure that its accumulation was lower than the levels that cause EtBr to reach and intercalate into DNA (which could obviate its removal), whilst promoting the optimum conditions for efflux measurements (Sharples & Brown, 1976; Rodrigues et al., 2008). Moreover, our results showed that piperine inhibited the efflux of EtBr in a concentration-dependent manner. Previous reports have shown that chlorpromazine, verapamil, CCCP and reserpine also inhibited the efflux of EtBr in a concentration-dependent manner during the assay period (Couto et al., 2008; Rodrigues et al., 2008).

A recent report demonstrated that the MDR pump inhibitor reserpine inhibits the resistance to EtBr in both wild-type *M. smegmatis* and the complemented mutant (Farrow & Rubin, 2008). The study also suggested that P55, a small-molecule transporter from the major facilitator superfamily, mediated the transport of EtBr. In *M. smegmatis*, the gene encoding the P55 EP, *Rv1410c*, forms an operon with *Rv1411c* (Bigi et al., 2000), which encodes the lipoprotein LprG. This operon is required for survival in the presence of EtBr and for maintenance of a normal cell surface composition in *M. smegmatis* (Farrow & Rubin, 2008); however, further research is needed to determine whether the efflux inhibition of EtBr by piperine in *M. smegmatis* mc² 155 ATCC 700084 involves P55. Additionally, the differential ability of the various EPIs (reserpine, chlorpromazine, verapamil, CCCP and piperine) to reduce the MICs of *M. smegmatis* suggests that intrinsic resistance to EtBr may be due to different EPs.

To our knowledge, there are few known natural EPIs that can enhance the anti-mycobacterial activities of drugs. This can partly be attributed to the fact that the characterization of mycobacterial EPs is a relatively new field compared with characterization of Gram-positive or Gram-negative

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**Fig. 4.** Effect of piperine and verapamil on the accumulation (a) and efflux (b) of EtBr from *M. smegmatis* mc² 155 cells at different concentrations. ▼, No EPI; ■, 64 μg piperine ml⁻¹; ▲, 32 μg piperine ml⁻¹; ◇, 16 μg piperine ml⁻¹; □, 150 μg verapamil ml⁻¹; △, 75 μg verapamil ml⁻¹; ○, 37.5 μg verapamil ml⁻¹.
bacteria EPs. There is an urgent need to identify new anti-TB compounds due to the high incidence levels of MDR TB cases (De Rossi et al., 2006). Piperine has been reported to increase the accumulation of ciprofloxacin by S. aureus, which suggests that piperine acts as an inhibitor of the transporter NorA (Khan et al., 2006). In this study, piperine exhibited EPI activity, although the MIC of transporter NorA (Khan et al., 2006). Mohtar et al. (2003) suggested that piperine may exert its efflux inhibitory activity via the highly conjugated double bonds (conjugated diene) connected to the aromatic ring of the molecule. The enhanced accumulation of ethidium bromide similar to the action of reserpine preloaded with EtBr by piperine suggest that piperine is involved in MDR P-glycoprotein efflux-mediated transport in rats (Sharma et al., 2005; Khan et al., 2006). Mohtar et al. (2009) suggested that piperine may exert its efflux inhibitory activity via the highly conjugated double bonds (conjugated diene) connected to the aromatic ring of the molecule. The enhanced accumulation of EtBr and blockage of its efflux from mutant cells preloaded with EtBr by piperine suggest that piperine inhibits the efflux of EtBr similar to the action of reserpine (Gibbons et al., 2003). A recent report (Sharma et al., 2010) has shown that piperine reduced the MIC and mutation prevention concentration of rifampicin for M. tuberculosis strain H37Rv, including MDR M. tuberculosis and clinical isolates. Piperine also effectively enhanced the bacterial activity of rifampicin in time-kill studies and significantly extended its post-antibiotic effect. These observations suggest that piperine is an inhibitor of Rv1258c, a putative multidrug EP of M. tuberculosis.

In summary, this study was performed on a fast-growing mycobacterial strain. Piperine was shown to be a potential inhibitor of the intrinsic EP system in mycobacteria. To date, several known bacterial EPs, such as the alkaloid reserpine, are potent inhibitors of the transporter. Piperine is a potent inhibitor of drug metabolism. J Pharmocol Exp Ther 232, 258–262.

REFERENCES


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