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Synthesis and Multidrug Resistance Reversal Activity of 1,2-Disubstituted Tetrahydroisoquinoline Derivatives

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Abstract. *Background: Cancer treatment often fails due to multidrug resistance (MDR) of the tumor cells. One of the major causes is overexpression of P-glycoprotein (P-gp). Materials and Methods: By N-substitution reactions of diamine, amino acid and amino alcohol derivatives with 1-substituted tetrahydroisoquinoline skeleton, structurally diverse 1,2-disubstituted 1,2,3,4-tetrahydroisoquinolines were synthesized. The compounds were assayed as P-gp inhibitors using a standard functional assay with rhodamine (6G) on MCF-7/Adr cells. Cytotoxicity was investigated on HeLa cells using an antiproliferative assay. Results: Five of the 24 compounds showed greater P-gp inhibition than the control compound verapamil with AC_{50} values (concentration of the compound eliciting 50% of the maximal rhodamine 6G accumulation) significantly lower than that of verapamil. Conclusion: Novel compounds were synthesized that showed MDR-reversal effect. One of them, (1'R*,2R*)-2-{2'-[2"-hydroxy-3"-(α-naphthyloxy)propyl]-6',7'-dimethoxy-1',2',3',4'-tetrahydro-1'-isoquinolyl}propan-1-ol hydrochloride, showed two times higher efficacy than verapamil at 10 times lower concentrations. The outcome makes this molecule an attractive subject for further investigation and development.*

The successful treatment of metastatic cancer often fails due to the development of multidrug resistance (MDR) of the tumor cells. Multidrug-resistant tumors show cross-resistance to many classes of chemotherapeutic agents that were never part of their treatment. One of the major mechanisms underlying MDR consists of the overexpression of a plasma membrane-bound protein (P-gp) (1,2). This mdr1 gene product extrudes antitumor agents out of the cells, thereby reducing their intracellular accumulation and therefore their efficacy. Studies have demonstrated that a wide range of structurally and functionally unrelated compounds (*e.g.* calcium channel blockers, calmodulin inhibitors, coronary vasodilators, antimalarial agents, cyclosporines), are able to reverse MDR by modulation of P-gp or MRP1 (multidrug resistance protein). However, since these first generation MDR inhibitors are active only at high concentrations, they proved to be of limited relevance for clinical practice. Due to the great therapeutic importance, in recent years enormous efforts have been made to find new natural compounds or synthetic compounds with MDR reversal activity (3-11). Some second (*e.g.* valspodar, biricodar) and third generation compounds (*e.g.* tariquidar, zosuquidar) have been developed which are in different phases of clinical trials (12-14).

The structurally diverse compounds known to show MDR reversal activity are hydrophobic and contain aromatic or heteroaromatic rings (15-18). To find new compounds with MDR reversal activity, *ca.* 150 compounds containing a carbo- and/or heterocyclic skeleton and various functional groups, which were prepared in recent years in the Institute of Pharmaceutical Chemistry, University of Szeged, Hungary, were tested for MDR reversal effect. Some derivatives of 1,2-disubstituted tetrahydroisoquinoline containing *N*-thiocarbamoyl or *N*-hydroxyalkyl moieties showed a remarkable activity. Our present aim was to synthesize further analogues for more detailed studies. To provide a wide structural diversity in the new derivatives, both substituents at positions 1 and 2 of the tetrahydro-isoquinoline ring were varied to a great extent.

Materials and Methods

Chemistry. Synthesis of the target compounds was performed starting from 1-substituted-1,2,3,4-tetrahydroisoquinoline difunctional compounds (diamine, amino acid and amino alcohol derivatives) by using *N*-substitution reactions.

Tetrahydroisoquinoline 1,2- and 1,3-diamines were prepared from homoveratryl amine and the corresponding *N*-protected α- or β-

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Key Words: Tetrahydroisoquinolines, multidrug resistance, P-glycoprotein.

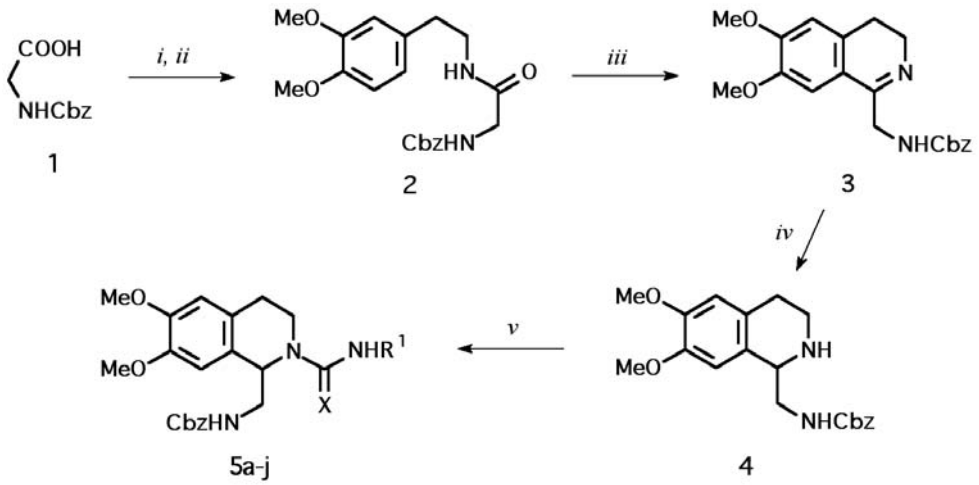


Figure 1. *i: ClCOOEt/Et_3N/toluene, ii: 2-(3,4-dimethoxyphenyl)ethylamine/CH_2Cl_2 (62%: i + ii), iii: $POCl_3$/$CHCl_3$ (71%), iv: $NaBH_4$/MeOH (83%), v: R^1NCX/toluene (64-86%)*

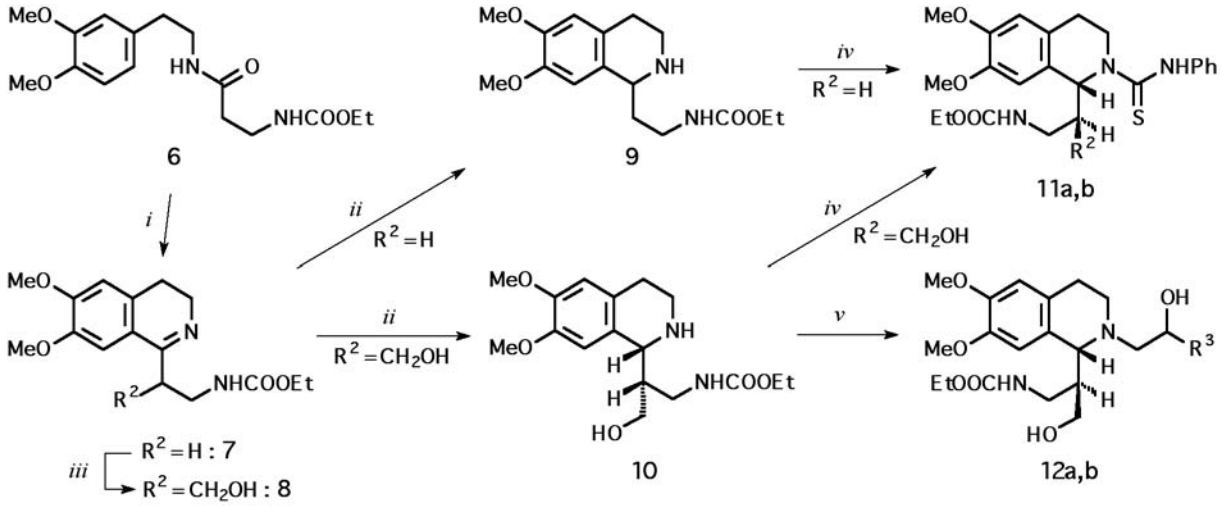


Figure 2. *i: $POCl_3$/$CHCl_3$ (74%), ii: $NaBH_4$/MeOH (78-82%), iii: $(CH_2O)_x$/NaOEt/EtOH (77%), iv: PhNCS/toluene (80-87%), v: R^3-substituted oxirane/EtOH (72-85%).*

amino acid, which were coupled by using a mixed anhydride method. Bischler-Napieralski ring-closures of carboxamides **2** and **6** resulted in dihydroisoquinolines **3** and **7**, which were reduced with sodium borohydride to yield tetrahydroisoquinolines **4** and **9** (Figures 1 and 2) (19). The activated methylene group of compound **7** was hydroxymethylated by treatment with paraformaldehyde in the presence of sodium ethylate (20,21). Sodium borohydride reduction of **8** resulted in the corresponding tetrahydro-isoquinoline **10** with a high diastereoselectivity. The relative configuration of amino-alcohol **10** was deduced from the steric structure of its azeto[2,1-a]isoquinoline derivative (Figure 2) (22).

The synthesis of tetrahydroisoquinoline β-amino acid derivatives **14** and **15** was achieved by condensation of 6,7-dimethoxy-3,4-dihydroisoquinoline either with cyanoacetic acid or with monoethyl malonate, respectively (23). Lithium aluminium hydride reduction of **15** led to the corresponding amino alcohol (homocalycotomine: 16) (Figure 3) (24). 1-Methyl-substituted homocalycotomine (18) was prepared according to the known procedure (20,21).

On reacting tetrahydroisoquinolines **4**, **9**, **10** and **18** with isocyanates or isothiocyanates, the corresponding urea or thiourea derivatives (**5a-j, 11a,b** and **19a,b**) were formed in good yields.

Hereby the synthesis of **5h** is given as a typical procedure for the preparation of urea or thiourea derivatives. A mixture of 1.07 g (3 mmol) **4** and 0.49 g (3.3 mmol) *p*-tolyl isothiocyanate was stirred and refluxed in 25 ml toluene for 1 hour. The cooled mixture was allowed to stand at ambient temperature after adding of 25 ml Et_2O. The separated crystals were filtered off, washed with Et_2O and recrystallized. The yield was 1.03 g (68%).

On refluxing tetrahydroisoquinolines **10**, **14-16** and **18** with substituted epoxides, the corresponding (2-hydroxy-alkyl)-substituted derivatives (**12a-c, 17a-f** and **20**) were formed by ring-opening reactions (Figures 1-4).

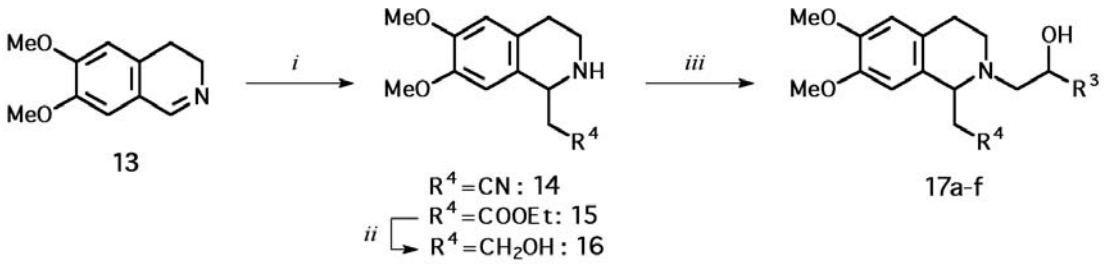


Figure 3. *i: R^4CH_2COOH (ref. 14), ii: $LiAlH_4$/THF (ref. 15), iii: R^3-substituted oxirane/EtOH (65-92%).*

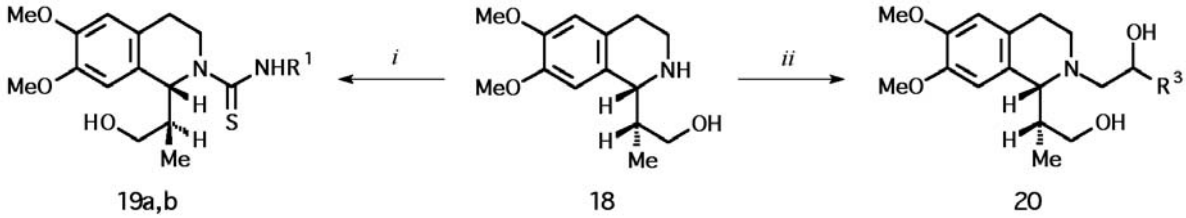


Figure 4. *i: R^1NCS/toluene (85-91%), ii: R^3-substituted oxirane/EtOH (78%)*

Furthermore, the synthesis of **17a** is given as a typical procedure for the preparation of (2-hydroxy-alkyl)-substituted derivatives. A solution of 0.70 g (3 mmol) **14** and 0.99 g (6 mmol) 3-(*m*-tolyloxy)-1,2-epoxypropane in 25 ml EtOH was refluxed for 10 hours. The solvent was evaporated off and the oily residue was converted to the crystalline HCl salt of **17a** by treatment with 20% ethanolic HCl and Et_2O. The separated crystals were filtered off, washed with a mixture of MeOH and Et_2O and recrystallized. According to the 1H NMR spectrum, the crystalline product proved to be a *ca.* 1:1 mixture of diastereomers. Ring-openings occurred on the sterically less hindered carbon to give only regioisomers bearing substituents R at position 2. The yield was 0.87 g (67%). According to the ^{1}H NMR spectrum, the crystalline product proved to be a *ca.* 1:1 mixture of diastereomers.

Diastereomers of compounds **12a,b, 17a-f** and **20** were not separated before pharmacological investigations.

Table I and Table II summarize the structures of the urea/thiourea derivatives (**5a-j, 11a,b** and **19a,b**) and the (2-hydroxy-alkyl)-substituted derivatives (**12a-c, 17a-f** and **20**), respectively. All compounds were characterized by IR, elementary analysis and NMR (400 Mhz). Data are available on request.

Bioassays

Cell culture. The parent MCF-7 human breast adenocarcinoma as well as the MCF-7/Adr cell line overexpressing P-gp were a gift from Dr. J. Carmichael (University of Nottingham, UK) and were originally derived from the laboratory of Dr. K. Cowan (NCI, Bethesda, MD, USA) (25,26). The MCF-7/Adr cells are >100-fold resistant to adriamycin, vincristine and vinblastin as compared to the parent cell line (27,28). This difference in sensitivity was investigated and confirmed on receipt of the cells. Cells were maintained in Eagle's minimum essential medium with Earle's salts containing 2 mM L-glutamine under 5% CO_2 at 37˚C. The MCF-7/Adr were cultured in the presence of 1 μg/ml adriamycin. Prior to the use in experiments, the resistant cells were grown for at least two passages in the absence of adriamycin.

Plasma membrane glycoprotein inhibition assay. The individual test compounds were dissolved in DMSO (40 mM) and stored at -20˚C. The compounds were assayed in a fluorescence microtiter plate assay using a standard functional assay with rhodamine (6G) as a fluorescent probe to assess MDR activity (10,29,30). MCF7/Adr cells were seeded in 96-well microplates (40000 cell/well). Twelve to eighteen hours later, the test compounds were added to the cells in the presence of rhodamine 6G (0.3 μM) (Sigma, St. Louis, USA) and incubated for 3.5 hours. Verapamil (Sigma), a well-known MDR modulating agent (28,31), was used as a positive control. After washing three times with ice-cold phosphate-buffered saline (PBS), the cells were dislodged with 100 μl phenol red-free trypsin (Gibco BRL, UK), transferred to new 96-well microplates and solubilized in 100 μl 4% sodium dodecylsulphate (SDS) solution. Fluorescense due to released rhodamine 6G was assayed at 530/590 nm (excitation/emission) using a Biotek FL600 microplate fluorescence reader. The amount of rhodamine 6G accumulation observed in the presence of the individual test compounds (40 μM) was expressed relative to the accumulation observed in untreated MCF7/Adr cells. This accumulation factor (AF) was calculated as the mean of 3 independent experiments. For the compounds that displayed an AF greater than 3.0 (approximately the AF of verapamil), concentration-response curves were constructed and the AC_{50} values (concentration of the test compound eliciting 50% of its maximal effect on rhodamine 6G accumulation) were determined using Prism 2.01 (GraphPad Software Inc. CA, USA). ANOVA was performed to determine whether the observed differences in the AF and the AC_{50} values, respectively, were statistically significant.

Antiproliferation assay. HeLa (human cervix carcinoma) cells were seeded in 96-well microplates (5000/well). After 12-18 hours, the test compounds were added to the cells, in concentrations ranging from 0.25 μM to 80 μM. After 72 hours the surviving fraction was determined with a MTT antiproliferation assay, which is based on the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)

Table I. *Structure of the tetrahydroisoquinoline urea and thiourea derivatives.*

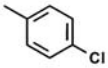
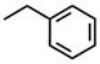
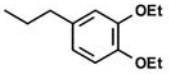
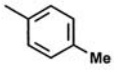
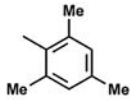
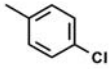
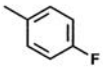
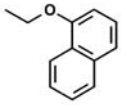
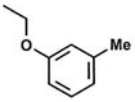
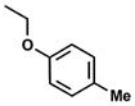
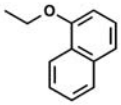
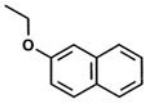
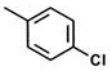
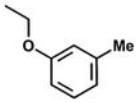
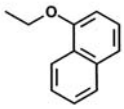
Comp.	R^1	R^2	X
5a		–	O
5b		–	O
5c		–	S
5d		–	S
5e		–	S
5f		–	S
5g		–	S
5h		–	S
5i		–	S
5j		–	S
5k		–	S
11a		H	S
11b		$-CH_2OH$	S
19a		–	S
19b		–	S

Table II. *Structure of the N-(2-hydroxyalkyl)tetrahydroisoquinoline derivatives.*

Comp.*	R^3	R^4
12a		–
12b		–
17a		CN
17b		CN
17c		CN
17d		CN
17e		COOEt
17f		CH2OH
20		–

*Compounds 12, 17 and 20 were purified and tested as hydrochloride salts.

(Sigma, Steinheim, Germany) into purple formazan crystals. For this purpose, the medium was replaced by a 1 mg/ml MTT solution in fresh medium, followed by incubation at 37˚C for 3 hours. The MTT solution was then removed and replaced with 200 μl DMSO. The concentration of formazan per well was determined by measuring its absorbance at 550 nm using a microplate reader (FL600, Biotek, Winooski, VT, USA). The surviving fractions of the cells were calculated from 3 replicates. Concentration-response curves were constructed and the IC_{50} values (concentration of the test compound inhibiting 50% of cell proliferation) were determined using Prism 2.01 (GraphPad Software Inc.)

Results

Increase in rhodamine 6G accumulation. In case of MCF7/Adr treated with verapamil (20 μM) (positive control), the AF (rhodamine 6G accumulation observed in treated relative to the accumulation observed in untreated MCF7/Adr cells) amounted to 3.11±0.10, while the accumulation of rhodamine 6G in the non-resistant parent MCF7 cells was 12.5-fold higher when compared to the resistant subline. Therefore the results obtained with rhodamine 6G, as a compound mimicking chemotherapeutics that are pumped out by MDR tumor cells, are consistent with previous work where it was shown that verapamil (10 μM) was able to increase the intracellular concentration of adriamycin and vinblastine in MCF7/Adr cells with a factor 4 and 7.5, respectively (28), and that the net accumulation of vinblastine by sensitive MCF7 cells was approximately 12-fold greater than MCF7-Adr cells (27).

The influence of the synthesized molecules on the rhodamine 6G accumulation was determined at a concentration of 40 μM. The calculated AF values for all 24 compounds and verapamil are given in Table III.

Nine compounds (**5a-b, 5d, 5g, 5i, 5j, 12b, 17c, 20**) were found to have an AF greater than 3.0, which indicates that they are at least as effective inhibitors of the P-gp as verapamil. Consequently, their AC_{50} values (concentration of the compound eliciting 50% of the maximal rhodamine 6G accumulation) were determined from their concentration-response curves (see Table IV). With the exception of 17c, all compounds were found to have an AC_{50} significantly lower than verapamil ($p<0.001$).

Ratio IC_{50}/AC_{50}. To better understand their potential as safe MDR inhibitors, the cytotoxic character of the selected compounds was assessed by means of an antiproliferative assay. The IC_{50} values (concentration of the compound inhibiting 50% of cell proliferation) are given in Table IV. Furthermore the respective IC_{50}/AC_{50} ratios were calculated (see Table IV). The larger this ratio, the less the compound induces a cytotoxic effect at concentrations that are effective in inhibiting the P-gp, reverting the MDR-phenotype of the treated malignant cells.

Discussion

As shown by the results, 9 out of the 24 synthesized and investigated tetrahydroisoquinoline derivatives were found to inhibit the P-gp transport pump at least as effectively as verapamil. In the case of 6 of them, the observed increase in rhodamine 6G accumulation was even significantly greater. In parallel, all selected compounds (with the exception of compound **17c**) exhibited an AC_{50} significantly lower than that of verapamil. These findings suggest that, in the case of 5 compounds that displayed AFs higher than twice the AF value of verapamil (**5a-b, 5i, 12b, 20**), the maximal inhibitory effect of verapamil could be reached by administering a significantly lower drug concentration.

However, this increased potency is only advantageous when at effective concentrations the compounds are free of side-effects such as cytotoxicity. Fortunately, from the selected tetrahydroisoquinoline derivatives, it was compound **20** that was found to combine the highest AF (8.43±0.07) and the lowest AC_{50} value (2.4±0.22 μM) with the highest IC_{50}/AC_{50} ratio (22) recorded. As a matter of fact, the maximal P-gp inhibition caused by verapamil could be reached by using 2.4 μM of compound **20,** which is 22 times lower than its IC_{50}. Alternatively, 24 μM of verapamil only produced half of the inhibition reached by 2.4 μM of compound **20**, implying that approximately a two-fold greater P-gp inhibition could be reached at a ten-fold lower concentration without obvious cellular side-effects.

Further experiments will need to be carried out to verify the P-gp inhibitory and cytotoxic effects on other resistant cell lines of this compound. Besides acute and chronic toxicity data, the *in vivo* efficacy of reverting MDR of resistant cells should complement the present findings for appreciation of its potential as a P-gp inhibitor.

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Table III. *Accumulation factor (AF) (rhodamine 6G accumulation observed in treated cells relative to the accumulation observed in untreated MCF7/Adr cells) calculated for the investigated tetrahydroisoquinoline derivatives and verapamil.*

Comp.	AF	Comp.	AF	Comp.	AF	Comp.	AF
5a	8.69±0.94***	**5g**	3.73±0.28	**11b**	1.35±0.2	**17b**	0.98±0.01
5b	7.04±0.21***	**5h**	1.52±0.08	**19a**	1.28±0.01	**17c**	3.08±0.09
5c	2.49±0.11	**5i**	8.22±0.52***	**19b**	1.11±0.12	**17d**	1.79±0.09
5d	3.67±0.16	**5j**	5.69±0.57**	**12a**	1.45±0.08	**17e**	1.44±0.05
5e	2.72±0.06	**5k**	1.39±0.04	**12b**	8.21±0.5***	**17f**	1.68±0.02
5f	1.73±0.05	**11a**	1.44±0.03	**17a**	1.22±0.06	**20**	8.43±0.07***
Verapamil	3.11±0.11						

Data are given as mean±s.e.m. (n=3). (**$p<0.01$, ***$p<0.001$ significantly higher as compared to verapamil).

Table IV. *The IC_{50} values (concentration inhibiting 50% of cell proliferation), AC_{50} values (concentration eliciting 50% of the maximal rhodamine 6G accumulation) of the selected tetrahydroisoquinoline derivatives and verapamil, and the corresponding calculated IC_{50}/AC_{50} ratios. IC_{50} and AC_{50} values are given as mean±s.e.m. (*=at the highest concentration used (80 μM), still more than 50% cell proliferation was observed)*

Comp.	IC_{50} (μM)	AC_{50} (μM)	Ratio	Comp.	IC_{50} (μM)	AC_{50} (μM)	Ratio
20	52.4±7.1	2.4±0.22	22	**5d**	> 80*	7.8±0.2	>10
5i	7.5±0.48	3.9±0.08	1.9	**5j**	13.3±2.8	3.8±0.4	3.5
5a	14.0±3.9	7.3±0.9	1.9	**5g**	7.7±0.12	8.1±0.5	0.9
12b	23.2±1.53	9.9±0.6	2.3	**17c**	41.8±5.5	25.0±0.7	1.7
5b	> 80*	8.0±0.18	>10	verapamil	> 80*	24.0±4.3	>3.3

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