Effects of Nontoxic Heat Shock Protein 90 Inhibitor Peptide Derivatives on Reversal of MDR of Tumor Cells

JOSEPH MOLNÁR1, HELGA ENGI1, YVETTE MÁNDI1, CSABA SOMLAI2, BOTOND PENKE2, ANDREA SZABÓ3 and ANTAL OROSZ3

1Department of Medical Microbiology and Immunobiology, Albert Szent-Györgyi Medical Center, Faculty of Medicine, University of Szeged, Dóm tér 10, H-6720 Szeged;
2Department of Medical Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged;
3Tumor Cell Biology Laboratory, Foundation for Cancer Prevention and Effective Treatment, Eötvös Loránd University, Pázmány sétány 1/C, H-1117 Budapest, Hungary

Abstract. Novel heat shock protein 90 inhibitor peptide derivatives \{D-Trp-Phe-D-Trp-Leu-AMB (1), p-HOPA-D-Trp-Phe-D-Trp-Leu-ψ(CH2NH)-Leu-NH2 (2), D-Trp-Phe-D-Trp-OH (3), Suc-D-Trp-Phe-D-Trp-Leu-AMB (4), D-Tyr-Phe-D-Trp-Leu-AMB (5), D-Arg-D-Trp-Phe-D-Trp-Leu-Leu-NH2 (6), Leu-ψ(CH2NH)-Leu-NH2x2HCl (7), Phe-Trp-Phe-Trp-Leu-Leu-NH2 (8), Tyr-Trp-Phe-Trp-Leu-Leu-NH2 (9) and Tyr-D-Trp-Phe-D-Trp-Leu-Leu-NH2(10)\} were synthetized, and their ability to reverse multidrug resistance (MDR) was studied. Peptide derivatives 1, 4 and 5, with D-Trp or D-Tyr residues in the N-terminal position caused a marked inhibition of MDR in cancer cells. These MDR inhibitor compounds and epirubicin were demonstrated to have additive and synergistic antiproliferative effects in checkerboard experiments on human MDR1 gene-transfected mouse lymphoma cells in vitro. It is suggested that the MDR reversal effects of these anticancer peptide derivatives, together with their antiproliferative effects on lung cancer cells, may open up new horizons in cancer chemotherapy.

Heat shock protein 90 (Hsp90) is responsible for the refolding of denatured proteins and for the conformational activation of nascent polypeptides. Numerous oncogenic functions are Hsp90-dependent, because this chaperone protein stabilizes different growth factor receptors and signaling molecules in cancer cells (1). Hsp90 molecules undergo weak interactions with their client proteins, and in this way may affect the function of the overall cell signaling network (2). This type of molecule is active via conformational cycles between the nucleotide free state and the ‘closed’ state brought about by binding of ATP production a molecular clamp, that depend on its ATPase activity (3), where the N-terminal domain contains the same binding site for ATP and Hsp90 inhibitors (4).

The treatment of tumor cells with Hsp90 inhibitors (benzoquinone ansamycins and their derivatives, radicicols, etc.) causes selective degradation of the Hsp90 client proteins and arrests the growth of cancer cells, although no clear explanation has been presented for the selective action on cancer cells. However, all these derivatives have high hepatic toxicity and other toxic side-effects (5-7). Besides, there are publications or patents that describe various pyrazole or purine-based and other compounds, which exhibit in vitro modest antiproliferative activity, but their in vivo effects have not been reported (8).

Recently, a novel family of short peptide derivatives, which strongly inhibit the proliferation of lung cancer cells in vitro, but have no effect on normal lung cells, were first reported by Orosz et al. (9, 11). In in vivo experiments these peptide derivatives restrained the growth of xenografted lung cancers in nude mice while no signs of toxic side-effects were detected. It was found that the 10 tested peptide derivatives exerted noteworthy antiproliferative effects on various human small cell lung cancer, colorectal and mammary adenocarcinoma cells. There were differences in the antiproliferative effects of the peptide derivatives studied, the effect being dependent on the fine chemical structures of the compounds. It was also found in the in vivo model experiments that growth inhibitors inhibited tumor growth in nude mice bearing the xenografts without general toxicity (11).

In view of the earlier results and the similarity between ABC transporter drug efflux proteins and other ATP binding proteins as substrates, it appeared promising to investigate...
the multidrug resistance (MDR) reversal effects of the novel peptide derivatives. In this paper the inhibition of MDR by novel anticancer peptide derivatives is reported, with special consideration of the active core at the C-terminal end and the residue at the N-terminal end of these molecules.

Materials and Methods

Synthetic methods. Solid-phase syntheses of the peptide derivatives [D-Trp-Phe-D-Trp-Leu-AMB (1), p-HOPA-D-Trp-Phe-D-Trp-Leu-ψ(CH₂NH)-Leu-NH₂ (2), D-Trp-Phe-D-Trp-OH (3), Suc-D-Trp-Phe-D-Trp-Leu-AMB (4), D-Tyr-Phe-D-Trp-Leu-AMB (5), D-Arg-D-Trp-Phe-D-Trp-Leu-Leu-NH₂ (6), Leu-ψ(CH₂NH)-Leu-NH₂x2HCl (7), Phe-Trp-Phe-Trp-Leu-Leu-NH₂ (8), Tyr-Trp-Phe-Trp-Leu-Leu-NH₂ (9) and Tyr-D-Trp-Phe-D-Trp-Leu-NH₂ (10)] were carried out manually on a p-methylbenzhydrylamine (MBHA) resin (1 g, 0.39 mmol/g) and Merrifield resin (1 g, 1%, 200-400 mesh), with standard methodology, using the Boc strategy. The side-chain protecting groups were tosyl for arginin and 2-bromo-benzyl for tyrosine (Arg(Tos) and Tyr(2Br-Z)). All protected amino acids were coupled in dichloromethane (CH₂Cl₂; 5 ml) using N,N'-Dicyclohexylcarbodiimidie (DCC; 2.5 equiv.) and 1-Hydroxybenzotriazole (HOBt; 2.5 equiv.) until completion (3 h) as judged with the Kaiser ninhydrin test. After coupling of the appropriate amino acid, Boc deprotection was effected by the use of trifluoroacetic acid/ dichloromethane (TFA/CH₂Cl₂; 1:1, 5 ml) for 5 min, which was then repeated for 25 min. Following neutralization with 10% triethylamine/dichloromethane (TEA/CH₂Cl₂) three times (5 ml each), the synthetic cycle was repeated to assemble the resin-bound protected peptide. The peptide derivatives were cleaved from the resin with simultaneous side-chain deprotection by aciolyis at 5 °C for 45 min with anhydrous hydrogen fluoride (5 ml) containing 2% anisole, 8% dimethyl sulfdde and indole. The crude peptide derivatives were dissolved in aqueous acetic acid and lyophilized. Preparative and analytical high performance liquid chromatography (HPLC) of the crude and the purified peptides were performed on an LKB Bromma apparatus-Bromma Sweden (for preparative HPLC; column: Lichrosorb RP C18, 7 μm, 250x16 mm; gradient elution: 30-100%, 70 min; mobile phase: 80% acetonitrile, 0.1% TFA; flow rate: 4 ml/min, 220 nm, for analytical HPLC; column: indicated below; mobile phase: 80% acetonitrile, 0.1% TFA; flow rate: 1 ml/min, 220 nm). Electron Spray Ionization-Mass Spectrometer (ESI-MS): Finnigan TSQ 7000 (San Jose, USA). HPLC data on the peptide-derivatives are listed in Table I.

Leu-AMB and Leu-ψ(CH₂NH)-Leu-NH₂ were obtained by using previously published procedures (12-14).

Cell cultures. L5178 mouse T-cell lymphoma cells (obtained from Prof. Gottesmann, NCI and FDA, USA) were transfected with pHₐ MDR1/A retrovirus, as previously described (15). MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain the expression of the MDR phenotype. L5178 (parent) mouse T-cell lymphoma cells and the human MDR1-transfected subline were cultured in McCoy’s 5A medium supplemented with 10% heat-inactivated horse serum L-glutamine and antibiotics.

Materials. The following were used in cultures and assays: Epirubicin (Wako Pure Chem., Ind., Osaka, Japan); rhodamine 123 (R123) (Sigma, St. Louis, MO, USA); verapamil (EGIS, Hungarian Pharmaceutical Company, Budapest, Hungary); horse serum (Gibco, Auckland, New Zealand); PBS (phosphate-buffered saline); MT 123 (4-(4,5-dihydroxylazo)-2-yl)-2,5-dihydroxytetrazolium bromide; Sigma); SDS (sodium dodecysulfate; Sigma); DMSO (dimethyl sulfoxide). Stock solutions of R123 and verapamil were prepared in water.

Table I. HPLC data on the synthesized peptide derivatives.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Retention factor (min)</th>
<th>Gradient elution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-Trp-Phe-D-Trp-Leu-AMB</td>
<td>7.173²</td>
</tr>
<tr>
<td>2</td>
<td>p-HOPA-D-Trp-Phe-D-Trp-Leu-ψ(CH₂NH)-Leu-NH₂</td>
<td>16.480²</td>
</tr>
<tr>
<td>3</td>
<td>D-Trp-Phe-D-Trp-OH</td>
<td>9.184²</td>
</tr>
<tr>
<td>4</td>
<td>Suc-D-Trp-Phe-D-Trp-Leu-AMB</td>
<td>12.976²</td>
</tr>
<tr>
<td>5</td>
<td>D-Tyr-Phe-D-Trp-Leu-AMB</td>
<td>4.500²</td>
</tr>
<tr>
<td>6</td>
<td>D-Arg-D-Trp-Phe-D-Trp-Leu-Leu-NH₂</td>
<td>11.380²</td>
</tr>
<tr>
<td>7</td>
<td>Leu-ψ(CH₂NH)-Leu-NH₂</td>
<td>Identified by TLC*, mp** and OR***</td>
</tr>
<tr>
<td>8</td>
<td>Phe-Trp-Phe-Trp-Leu-Leu-NH₂</td>
<td>14.600³</td>
</tr>
<tr>
<td>9</td>
<td>Tyr-Trp-Phe-Trp-Leu-Leu-NH</td>
<td>14.410⁴</td>
</tr>
<tr>
<td>10</td>
<td>Tyr-D-Trp-Phe-D-Trp-Leu-NH₂</td>
<td>21.380⁵</td>
</tr>
</tbody>
</table>

¹TLC: thin layer chromatography; **mp: melting point; ***OR: optical rotation; AMB: 1-amin0-3-methyl-butane; p-HOPA: para-hydroxyphenyl acetyl; Suc: succinyl.

²Nucleosil 5C 18, 300Å, 10 μm 250x4.6 mm
³Luna C18, 5 μm 250x4.6 mm
⁴Vydac Protein&Peptide C18 250x4.6 mm
⁵Bondapac C18 255x4.6 mm
⁶Eurosil Bioselect 5C 18, 300Å 250x4.6 mm
⁷Phenomenex Jupiter, 5C 18, 300Å 250x4.6 mm
⁸Fennigian TSQ 7000 (San Jose, USA).

[430 in vivo 21: 429-434 (2007)]
**Assay for antiproliferative effect.** The effects on cell growth of increasing concentrations of the drugs alone and then with potential MDR resistance modifiers were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted to a volume of 50 μl. Then, 1x10⁴ cells in 0.1 ml of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C for 72 h; at the end of the incubation period, 20 μl of MTT solution (from a 5 mg/ml stock) was added to each well. After incubation at 37°C for 4 h, 100 μl of SDS solution (10%) was measured into each well and the plates were further incubated at 37°C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX (Guernsey, GB) vertical beam ELISA reader (16). Inhibition of cell growth (as a percentage) was determined according to the formula:

\[
\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}} \times 100 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}}
\]

A checkerboard microplate method was applied to study the effects of drug interactions between potential MDR modifiers and the cytotoxic compound on cancer cells as an *in vitro* model of combination chemotherapy. The effects of the anticancer agent epirubicin and the potential MDR modifiers in various combinations were studied. The dilutions of epirubicin (A) were made in a horizontal direction, and the dilutions of potential MDR modifiers (B) vertically in the microtiter plate in a volume of 100 μl. The cell suspension in the tissue culture medium was distributed into each well in 100 μl containing 5x10⁴ cells. The plates were incubated for 72 h at 37°C in a CO₂ incubator. The cell growth rate was determined after MTT staining and the intensity of the blue color was measured on a micro ELISA reader (17). Drug interactions were evaluated according to the following system:

\[
\text{FIC}_A = \frac{\text{ID}_{50A} \text{ in combination}}{\text{ID}_{50A} \text{ alone}} \quad \text{and} \quad \text{FIC}_B = \frac{\text{ID}_{50B} \text{ in combination}}{\text{ID}_{50B} \text{ alone}}
\]

where ID= inhibitory dose and FIC= fractional inhibitory concentration. FIX= FICₐₐ / FICₐ₉ where FIX= fractional inhibitory index. FIX<0.5 indicated synergism, FIX>1 indicated antagonism between the anticancer drug and the potential MDR modifier.

**Assay for reversal of MDR in mouse lymphoma cells (drug accumulation).** The L5178 MDR and L5178Y parental cell lines were grown in McCoy’s 5A medium containing 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a density of 2x10⁶/ml, resuspended in serum-free McCoy’s 5A medium and distributed in 0.5-ml aliquots into Eppendorf centrifuge tubes. The tested compounds were added at various concentrations in different volumes (2.0-20.0 μl) of the 1.0-10.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature. Next, 10 μl (5.2 μM final concentration) of rhodamine 123 was added to the samples and the cells were incubated for a further 20 min at 37°C, washed twice and resuspended in 0.5 ml PBS for analysis. The fluorescence of the cell population was measured with a Beckton Dickinson FACScan flow cytometer (cell sorter, Oxford, U.K). Verapamil was used as a positive control in the rhodamine 123 exclusion experiments. The percentage mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated controls (17-19). An activity ratio R was calculated via the following equation on the basis of the measured fluorescence values:

\[
R = \frac{\text{MDR}_{\text{treated/MDR}_{\text{control}}}}{\text{parental treated/parental control}}
\]

**Results**

On the basis of the antiproliferative activities of the non toxic heat shock protein peptide derivatives the drug accumulation in human MDR1 gene-transfected mouse lymphoma cells was investigated in the presence of the Hsp90 inhibitor peptide derivatives.

The peptide derivatives 1 and 4 caused a 10-fold increase of the drug accumulation in the MDR cancer cells relative
to the untreated cells (Table II). Compound 5 proved ineffective in inhibiting the ABC transporter, but was selected as a negative control in the experiments because of its somewhat similar chemical structure to that of peptide derivative 4. The majority of antiproliferative peptide practical ineffective in MDR reversal. Apparently derivatives having D-Trp or D-Tyr residue in the N-terminal position of the peptide were the effective compound, that mean some sequence specificity of peptide-Pgp interaction.

In further experiments, the possible practical importance of the reversal of MDR was analyzed in combination experiments, when the antiproliferative effect of the three representative peptide derivatives (1, 4 and 5) were studied in combination with epirubicin on the human MDR gene transfected mouse lymphoma cells. Here we had direct evidents for the synergistic interaction, when the most effective MDR reversal compound, 1, synergized the antiproliferative effect of epirubicin (FIX=0.37). The other MDR reversal peptides, the peptide 4 (FIX=0.63) had shown only additive effect as peptide 5 (FIX=0.52).

Discussion

Di-, tri-, tetra-, penta- and hexapeptide derivatives of Hsp90 inhibitor with different structural features were investigated for their ability to reverse the MDR of tumor cells in order to obtain additional evidence concerning the relationship between chemical structure and biological activity. A structure-activity relationship was observed in the MDR reversal effect since two of the three strongly hydrophobic compounds (1 and 4) were the most potent. The correlation between the inhibition of the ABC transporter and the chemical properties of the active compounds is possibly similar to that for a certain group of carotenoids in which the total polar surface areas of the compounds is possibly similar to that for a certain group of ABC transporter and the chemical properties of the active compounds is possibly similar to that for a certain group of MDR reversal peptides, the peptide 4 (FIX=0.63) had shown only additive effect as peptide 5 (FIX=0.52).

It is well known that the majority of anticancer chemotherapeutics have lost their antiproliferative effects on multidrug-resistant cells. Accordingly, it was useful to investigate the antiproliferative effects of the combinations of epirubicin as a known substrate of the MDR efflux pump, and our MDR inhibitor compounds.

The interactions between three of the MDR inhibitor peptide derivatives and the anticancer chemotherapeutic epirubicin were studied on multidrug-resistant cancer cells in checkerboard assays. These MDR inhibitors increased the antiproliferative effect of epirubicin on human MDR1 gene-transfected mouse lymphoma cells resulting from synergism for compound 1 and additive antiproliferative effects for compounds 4 and 5. The combination of these MDR inhibitor peptide derivatives and traditional cancer chemotherapy might possibly improve the efficacy of cancer treatment in clinical practice.

Acknowledgements

This study was supported by the Szeged Foundation for Cancer Research. The technical assistance of Mrs Aniko Várdai Vigyikán was highly appreciated.

References

10 Orosz A, Szabo A, Szeman G, Somlai Cs, Janaky T and Penke B: New anticancer peptide analog compounds targeting the tumor Hsp90 protein have no toxic effect on normal cells. 11th Word Conference on Lung Cancer, Barcelona, Spain, 3-6 July, 2005.

Received October 12, 2006
Revised December 19, 2006
Accepted January 3, 2007