In Vitro Cytotoxic Effect of Difluoromethylornithine Increased Nonspecifically by Peptide Coupling

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Abstract □ Difluoromethylornithine (DFMO)–peptide conjugates were synthesized as prodrugs to improve the cytotoxic efficacy of DFMO. All conjugates inhibited cell growth in different cell lines more effectively than DFMO itself. The best cytotoxic effect was achieved in all cell lines by DFMO-Glu-His-Phe-Arg-Trp-Gly-OMe, where the carrier peptide is a melanotropin hormone fragment. Although this conjugate is capable of displacing labeled melanotropin from its receptor, its cytotoxic effect on the receptor-positive human melanoma cell line has not been proven to be receptor-mediated. The differences in the cytotoxicities of the congeners seem to be influenced, at least in part, by the nature of the carrier molecule.

Introduction

DL-α-Difluoromethylornithine (DFMO) is a potent irreversible inhibitor of the ornithine decarboxylase (ODC) enzyme,[1] which plays a crucial role in polyamine biosynthesis and, subsequently, in cell proliferation. Furthermore, ODC seems to be involved in the development of tumor metastases[2] and to function as an oncogene transducer in malignant transformation.[3]

According to previous investigations, DFMO proved to be a general cytostatic agent that even exerted a cytotoxic effect in several cell lines like small cell lung carcinoma or B16 melanoma.[4] DFMO also prevents skin carcinogenesis and immunosuppression induced by ultraviolet (UV) irradiation.[5] However, in the therapy of various malignancies, DFMO alone has not proved to be efficient enough.[6] One of the main reasons for the low therapeutic efficiency is the rapid clearance of the drug from the body, which might be reduced if DFMO was applied in a prodrug form attached to a carrier molecule. It has been shown in many cases that peptide−drug conjugates have advantageous pharmacokinetic properties and can be activated more or less selectively in the extracellular space of tumoral tissues containing high levels of peptidases.[7]

Because of the high cytotoxicity of DFMO in melanoma cells, it seemed appropriate to choose biologically active fragments of α-melanotropin as peptide carriers. Melanotropins (melanocyte-stimulating hormones, α-, β-, and γ-MSH) belong to the hormone family derived from the common pro-opiomelanocortin precursor,[8] which has many different physiological functions.[9,10] The best known biological activity of MSH is skin pigmentation, not only in frogs and lizards, but in humans as well.[11] In several human melanoma cell lines, high-affinity α-MSH receptors have been found.[12,13]

According to structure−biological activity relationship studies, smaller hormone fragments also possess biological activity,[14] and the substitution of L-Phe[7] for D-Phe[7] also enhances biological activity of the hormone[15] or its active fragments.[16] Therefore, for carriers for DFMO, we chose Glu-His-Phe-Arg-Trp-Gly-OMe, the methyl ester of the central 5−10 sequence of α-melanotropin, and its Gly[5],D-Phe[7] analogue that, according to our previous experiments, specifically bind to melanotropin receptors on a human melanoma cell line.[17] For the sake of completeness, a third fragment, the *C*-terminal Lys-Pro-Val sequence that lacks any receptor-recognizing ability in the aforementioned studies, was also selected. DFMO was attached to the *N*-terminus of the peptides so that aminopeptidases could liberate the drug from the conjugate.

In this paper we report on the synthesis of the conjugates and on the growth inhibitory activity of DFMO−peptide conjugates in various cell lines. We also try to elucidate whether the DFMO−peptide conjugates possess specific melanotropin receptor binding ability, and whether they exert a targeting effect on a human melanoma cell line that expresses MSH receptors.

Experimental Section

Merck Kieselgel precoated sheets (Art no. 5553) were used for thin-layer chromatography (TLC) and Merck Kieselgel 60 (Art no. 10832) was used for column chromatography. High-performance liquid chromatography (HPLC) was performed on a Knauer instrument (gradient system with variable UV detector and integrated PC-based gradient controller). The following solvent systems (v/v) were used: A, butanol:pyridine:acetic acid:water (4:1:1:1); B, ethyl acetate: pyridine:acetic acid:water (60:20:6:11); C, 2-propanol:acetic acid:water (6:1:1); D, ethyl acetate; E, ethanol:triethylamine:formic acid:water (39:18:6:15); and F, ethylacetate:pyridine:acetic acid:water (120:20:6:11). Mass spectra were taken by a plasma desorption mass spectrometer (Bioion 2000). Amino acid analyses were performed after hydrolysis [acidic: 6 N HCl, 105 °C, 24 h; or enzymatic: aminopeptidase M (Röhm), pH 7.4, 37 °C, 18 h] on Beckmann 6300 equipment. Nuclear magnetic resonance (NMR) spectra were measured on Bruker WM-250 FT-spectrometer. Infrared (IR) spectra were taken on Specord IR75 (Karl Zeiss, Jena). DFMO was prepared in our laboratory according to the procedure of Bey et al.[18]

DFMO(Boc)-OH·HCl (I)—DFMO·H_2O·HCl (1.42 g, 6 mmol) was dissolved in the 1:1 mixture of dioxane and water (18 mL), and then 12 mL of 1.0 N NaOH solution (12 mmol) and 2.65 g of di-*tert*-butyl dicarbonate (12 mmol) was added and stirred. After several hours (TLC monitoring), when the reaction was finished, the dioxane was distilled off under reduced pressure, and the remaining solution was cooled to 0 °C, and the pH was adjusted to 4 with 1 N HCl solution. The acidic solution was extracted with ether and then lyophilized. The lyophilized powder was dissolved in 20 mL of absolute ethanol, the insoluble NaCl was filtered off, the solvent was evaporated under reduced pressure, and the residue was dried in a desiccator over concentrated H_2SO_4: yield, 1.53 g (68%); TLC R_f 0.75 (A), 0.42 (B); IR (KBr): ν 3365 (NH), 3230−2300 (OH), 1690 (CO, Boc), 1062 (CF), δ 1525 (NH) cm^{-1}; ^1H NMR (D_2O, $\delta_{TMS} = 0$ ppm): CH_3(*t*-Bu): 1.42[1] (*s*, 9H); $CH_2(\gamma)$: 1.4* (where * indicates overlapping signals) and 1.60 (*dt*, 2 × 1H); $CH_2(\beta)$: 1.80 and 1.95 (2 × *dt*, 2 × 1H); $CH_2(\delta)$: 3.11 (*t*, 2H); CHF_2: 6.29 (*t*, 1H), ^2J(F,H): 53.5 Hz; NH: 6.81 (*t*, 1H), $^3J(CH_2$, NH): 5.5 Hz[2] (in DMSO-d_6 solution); ^{13}C NMR (D_2O, $\delta_{TMS} = 0$ ppm): $CH_2(\gamma)$: 25.8; CH_3(*t*-Bu): 30.5; $CH_2(\beta)$: 31.0; $CH_2(\delta)$: 42.2; C(α): 68.3 (*t*), ^2J(F,C): 18.7 Hz; CHF_2: 118.2 (*t*), ^1J(C,F): 246.8 Hz; CO (urethane): 160.9; CO (carboxylic): 172.3 (*d*), ^3J (F, C): 6.0 Hz.

DFMO-Glu-His-Phe-Arg-Trp-Gly-OMe Acetate (II)—To the solution of 320 mg (1 mmol) of **I** in 5 mL of dimethylformamide (DMF), 0.16 mL (1.5 mmol) of *N*-methylmorpholine (NMM), 135 mg (1 mmol)

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.

S0022-3549(97)00080-4 CCC: $14.00

of 1-hydroxybenzotriazole (HOBt), and 444 mg (1 mmol) of (benzotriazolyloxy)*tris*(dimethylamino)phosphonium hexafluorophosphate (BOP) were added. After 10 min of stirring, the solution of 1.0 g (1 mmol) of *H*-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OMe·3HCl[19] and 0.22 mL (2 mmol) of NMM in 10 mL of DMF was added, and the reaction mixture was stirred overnight. The DMF was distilled off under reduced pressure, the residue was triturated with sodium bicarbonate solution, and the precipitate was filtered and washed with water on the filter. The crude product was purified on silica gel column in solvent system B to give 720 mg (57%) of DFMO(Boc)-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OMe (**IIa**). TLC R_f 0.77, 0.82 (A), 0.34, 0.37 (B) for the epimeric components, respectively. Then, 720 mg of **IIa** was dissolved in 7 mL of trifluoroacetic acid (TFA) containing 0.5 mL of anisole and stirred for 1 h. The solution was then poured into 70 mL of ether, and the precipitate was filtered off, washed on the filter with ether, and dried in a desiccator over P_2O_5. The solution of the trifluoroacetate salt was applied onto a QAE Sephadex column in the acetate cycle and then lyophilized to yield 638 mg (83%) of **II**; TLC R_f 0.25, 0.35 (A) for the epimeric components, respectively; amino acid analysis: Glu 1.00, His 1.02, Phe 0.99, Arg 0.98, Gly 0.99, DFMO 0.95; MS: calc M for $C_{46}H_{62}N_{14}O_{10}$: 1009, found $(MH)^+$: 1010.

Separation and Hydrolysis of the DFMO-Glu-His-Phe-Arg-Trp-Gly-OMe Epimeric Peptides—Crude **II** was separated by HPLC on a silica gel SI60 (10 μm) column (4 × 200 mm; Labor MIM, Budapest, Hungary) using D:E (6:4) as eluent. Amino acid analyses of the two peaks gave the same results after both acidic (see previous section) and enzymatic [aminopeptidase M (10% of the amount of the peptide) in 0.1 M tris buffer solution, pH 7.4, 37 °C, 18 h] hydrolyses.

DFMO-Gly-His-*D*-Phe-Arg-Trp-Gly-OMe acetate (III)—**I** was coupled to *H*-Gly-His-*D*-Phe-Arg-Trp-Gly-OMe·3HCl[20] in the same way as just described, yielding DFMO(Boc)-Gly-His-*D*-Phe-Arg-Trp-Gly-OMe (**IIIa)** with 60% yield; TLC R_f 0.25 (B), 0.59 (C). The Boc group from **IIIa** was removed as in the case of **IIa** to give **III** with 82% yield; TLC R_f 0.46 (A), 0.53 (B); amino acid analysis: Gly 2.10, His 0.95, Phe 1.03, Arg 0.92, DFMO 0.93. For MS measurements, a sample was further purified by HPLC [BST-SI100 C18 (10 μm, 4 × 200 mm, acetonitrile gradient (30 to 50%, 30 min)] in 0.1% TFA; MS: calc M for $C_{43}H_{58}F_2N_4O_8$: 937, found $(MH)^+$: 938.

DFMO-Lys-Pro-Val-NH$_2$·3HCl (IV)—To the ice-cooled solution of 160 mg (0.5 mmol) of **I** in 5 mL of DMF, 0.09 mL of diisopropylethylamine (DIEA) and 200 mg (0.5 mmol) of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were added and the mixture was stirred for 10 min. Then the solution of 240 mg (0.5 mmol) of *H*-Lys(Boc)-Pro-Val-NH$_2$·HCl[21] and 0.09 mL of DIEA in 2 mL DMF was added. The reaction mixture was stirred with ice cooling for 1 h and at room temperature for 2 h. After the evaporation of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, extracted with saturated sodium bicarbonate solution and then with water, dried on $MgSO_4$, and evaporated under reduced pressure to give 260 mg of DFMO(Boc)-Lys(Boc)-Pro-Val-NH$_2$ (**IVa**): TLC R_f 0.89 (A), 0.82 (F). Then, 200 mg of **IVa** was stirred as a suspension in 4 N HCl containing ethylacetate for 30 min, ether was added next, and the precipitate was filtered off and washed with ether on the filter to give **IV** in quantitative yield: TLC R_f 0.40 (A); amino acid analysis: Lys 0.95, Pro 1.04, Val 1.01, DFMO 0.96; MS: calc M for $C_{22}H_{41}N_7O_4F_2$: 506, found $(MH)^+$: 507.

Cells—HBL and LND1 human melanoma cell lines and F-NBB2 newborn human fibroblasts (used between passages 15 and 20) were established in our laboratory. HBL was the only cell line expressing detectable MSH receptors (MSHR$^+$). Me-180 epidermoid carcinoma cell line was purchased from ATCC (no: HTB33). All cells were grown as monolayer cultures in Ham-F10 nutrient mixture containing 5% fetal calf serum, 5% newborn calf serum, 2 mM glutamine, 100 U/mL penicillin-streptomycin, and 100 μg/mL kanamycin (all from Gibco). All cell lines were routinely checked for the absence of mycoplasma contamination (Mycoplasma T. C., Gen-Probe). Twenty-four hours prior to the assays, cells were harvested, counted, washed, resuspended in a fresh medium at the adequate density, and seeded in 96-well microtiter plates. The doubling times of Me-180, HBL, LND1, and F-NBB2 cells were 40, 51, 80, and 100 h, respectively.

Receptor Binding Assay—All the drug conjugates were checked as to their ability to bind specifically to α-MSH receptors on the HBL cell line by a modification of a method reported elsewhere.[12] Briefly, 1.2 × 10^6 Bq of [^{125}I-Tyr^2,Nle^4,*D*-Phe^7]α-MSH$_{1-13}$ (NDP-MSH) analogue were added to 10^6 cells in 200 μL of 0.067 M phosphate buffered saline (pH 7.4) containing 1% BSA. Serial dilutions of each compound were prepared, and 100 μL of each solution were added to the incubation medium. After mixing, the tubes were let stand for 45 min at 37 °C before cell separation by repeated centrifugations and washings. The pellet-associated radioactivity was then measured in a γ-counter. Incubations were run in triplicate and the results are expressed as percent of the control value (without effector).

Cell Exposure to Drugs—Cell incubation was carried out at 37 °C for 48 h in 96-well microtiter plates at a density of 1.2 × 10^4 cells/well 24 h before performing the assay described next. Assays were performed (n = 6) in flat-bottomed wells at 37 °C in humidified air containing 5% CO_2 .

^{3}HTdR Uptake Assay—Thymidine uptake was performed as previously described.[22] Briefly, 3.7 × 10^{10} Bq of tritiated thymidine ([methyl-^{3}H]thymidine; TRA120, 1.85 × 10^{11} Bq/mmol, Amersham) was added to each well 24-h after the begining of the assay. Microplates were incubated at 37 °C for an additionnal 24 h period. Cells were then harvested onto glass fiber filters (Whatman) with a PHD-cell harvester (Cambridge Technology, Inc.). The filters were allowed to dry at room temperature overnight before 4 mL of scintillation liquid (Ready Safe, Beckman) were added to each vial. The radioactivity was measured in a β-counter. Incubations were run in quadruplicate, and cell growth is expressed as percent of the untreated cells after deduction of the background. Compound concentrations are expressed in μg/mL DFMO equivalent, which represents the amount of drug contained in each of the conjugates.

Results

Chemistry—To prepare an N^{α},N^{δ}-*bis*-protected DFMO derivative suitable for the acylation of a peptide *N*-terminus, we tried to synthesise the *bis*(*tert*-butyloxycarbonyl)-DFMO (Boc$_2$DFMO). However, with the aid of di-*tert*-butyl dicarbonate we succeeded only in the preparation of a mono-Boc derivative [DFMO(Boc), **I**], and the *bis*-Boc derivative was not formed either after longer reaction time or at higher temperatures.

Having obtained the mono-Boc-derivative it had to be decided whether the α or the δ amino group was substituted with the Boc group. This decision was made possible with the aid of the NH signals of the ^{1}H NMR spectrum in DMSO-d_6 solution. In our case the N^+H_3 signal was approx. 4.8 ppm (broad maximum), and the NH signal of the carbamoyl group is at 6.81 ppm with 1H intensity. The latter signal was a triplet [^3J(CH_2, NH) = 5.5 Hz] with the coupling of the neighboring hydrogens unambigously proving the presence of the -CH_2-NH(Boc) group. Furthermore, we performed a differential nuclear Overhauser effect (DNOE) measurement. During the saturation of the *t*-Bu signal (1.42 ppm), the intensity of the NCH_2 triplet increased, indicating its spatial proximity to the *t*-Bu group. Because there was no similar intensity increase on the ^{1}H NMR signal of the CHF_2 group, the DNOE measurement supports the N^{δ}-Boc-DFMO structure.

On the basis of our experiences in the synthetic work, we concluded that the α-amino group of DFMO, being so unreactive, would not be acylated during the peptide coupling procedure, which requires even milder reaction conditions than those used during the amino group protection. Indeed, no detectable *bis*-acylation was observed. After deprotection and purification, the structure of the DFMO—peptides was proved by mass spectrometry and amino acid analysis.

Enzymatic Hydrolysis—Because DFMO is a racemate, all the peptides formed are epimers. In the case of DFMO-Glu-His-Phe-Arg-Trp-Gly-OMe (**II**), we were able to separate the epimeric peptides by HPLC on a silica gel column. The separated peptides were hydrolyzed with amino peptidase M and, according to the amino acid analyses, both of the hydrolysates had the same amino acid composition indicating that both L- and D-DFMO had been split from the carrier peptide by the enzyme. DFMO was detected as a distinct peak between the peaks of Phe and His.

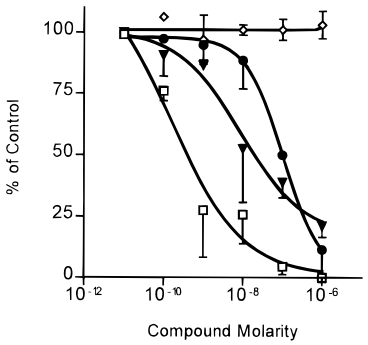

Figure 1—MSH receptor binding assay performed on HBL human melanoma cells (MSHR$^+$) using (Nle^4, D-Phe^7)α-MSH (□), **II** (●), **III** (▼), and **IV** (◇).

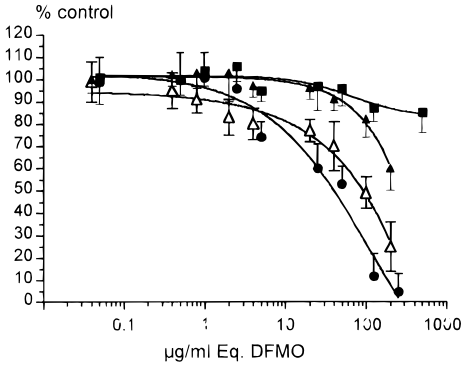

Figure 2—Cytotoxic effect of DFMO alone (■) and DFMO conjugated to MSH-related peptides [**II** (●), **III** (△), **IV** (▲)] on HBL human melanoma cells (MSHR$^+$)(^{3}HTdR assay).

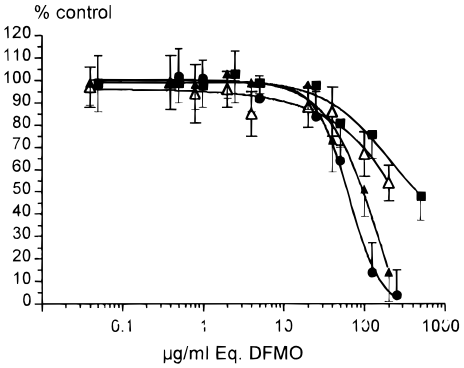

Figure 3—Cytotoxic effect of DFMO alone (■) and DFMO conjugated to MSH-related peptides [**II** (●), **III** (△), **IV** (▲)] on LND1 human melanoma cells (MSHR$^-$)(^{3}HTdR assay).

Receptor Binding Assay—The DFMO derivatives of the two central α-MSH fragments, **II** and **III,** displace labeled NDP-MSH from its receptors in a human melanoma cell line (Figure 1). Their affinity for the melanotropin receptors is different; that is, **III** is capable of displacing 50% of the labeled hormone in about one order of magnitude lower concentration than **II**. The DFMO derivative of the *C*-terminal α-MSH fragment **IV** cannot specifically displace the hormone from its binding site.

Cytotoxicity—For the cytotoxicity studies, two human melanoma cell lines (HBL containing and LND1 not containing melanotropin receptors), a carcinoma cell line (Me180), and normal human fibroblasts were used. The cytotoxicity of the DFMO–peptide conjugates was measured by the thymidine incorporation into DNA (Figures 2, 3, and 4) after long (48 h) incubation with DFMO as control. The conjugates proved to be more efficient in the inhibition of cell growth than DFMO itself, the highest effect being shown by **II** (Table 1).

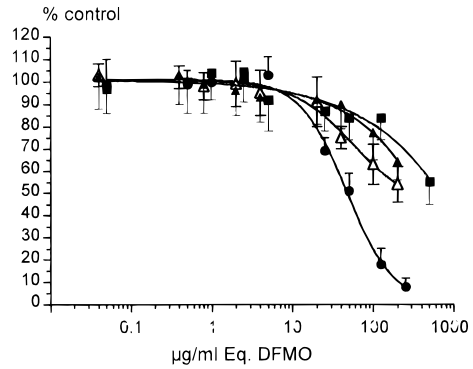

Figure 4—Cytotoxic effect of DFMO alone (■) and DFMO conjugated to MSH-related peptides [**II** (●), **III** (△), **IV** (▲)] on Me-180 carcinoma cells (MSHR$^-$) (^{3}HTdR assay).

Table 1—Comparison of IC_{50} Values of the Conjugates and Free Drug in the ^{3}HTdR Uptake Assay[a]

Compound	IC_{50} (DFMO eq., μg/mL)			
	HBL	LND1	Me180	FNBB2
DFMO	>500	462 ± 45	500 ± 20	>500
II	40 ± 4	63 ± 8	49 ± 5	>250
III	93 ± 7	200 ± 15	198 ± 16	>200
IV	103 ± 10	100 ± 10	>200	>200

[a] HBL are the only cells expressing MSH receptor (results are shown as mean ± SD from two independant experiments, with $n = 6$ and $n = 4$, respectively).

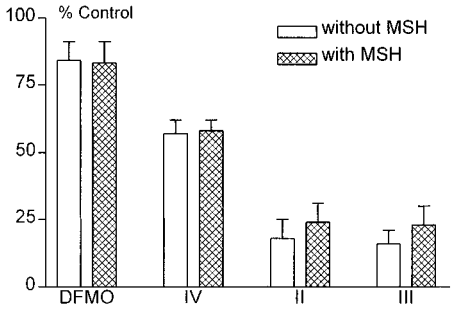

Figure 5—Effect of MSH on HBL cells (MSHR$^+$) growth inhibition with DFMO and DFMO–peptides: DFMO (250 μg/mL); **II** and **III** (125 μg/mL); and **IV** (200 μg/mL).

The possible role of the melanotropin receptor in the cytotoxicity of the conjugates was checked by measuring the cytotoxic effect of DFMO and DFMO–peptides either in the presence or in the absence of α-MSH on the receptor-positive HBL cell line,[17] and no difference was found (Figure 5).

Discussion

DFMO, as an amino acid derivative, is ideal for coupling to a peptide carrier, thereby generating a prodrug. Therefore, it may well be expected that an elevated peptidase activity on the surface of the tumor cells will liberate the drug from the conjugate. Attaching the racemic DFMO to the *N*-terminus of an optically pure peptide carrier results in epimeric DFMO–peptide congeners. To check whether an aminopeptidase can split both enantiomers of DFMO from the DFMO–peptide conjugate, we separated one of our epimer DFMO–peptide derivatives. The separated peptides were hydrolyzed by aminopeptidase M and, according to the amino acid analysis of the hydrolysates, both of them were digested by the enzyme. As it has been shown previously, both enantiomers of DFMO are potent ODC inhibitors,[23] which is

the cause of the cytostatic effect. Thus, for therapeutic purposes, there is no need to separate the epimeric DFMO−peptide conjugates.

All the DFMO−peptide derivatives exerted inhibition of cell proliferation. The cell growth inhibition is obviously due to the enzymatic release of DFMO from the carrier peptide because, according to the experimentally proved mechanism of ODC inhibition,[24] a free α-carboxyl group is required on the inhibitor to achieve irreversible inhibition. On the other hand, according to our previous investigations MSH fragments do not inhibit cell proliferation. On the contrary, MSH has a mitogenic effect on normal melanocytes but not on melanoma cells.[25]

Comparing the cytotoxic effects of the conjugates with one another, the best results were achieved with **II,** although all conjugates are more effective in inhibiting cell proliferation than DFMO itself. A possible explanation for the increased cytotoxicity is that the carrier peptide enhances the binding of the conjugate to the tumor cell surface, where DFMO is generated enzymatically in situ by a cell membrane peptidase. In this way, a higher concentration and a higher uptake of the drug can be achieved than in cases where DFMO is applied alone. The differences in the cytotoxic effects of the conjugates on various cell lines may be the consequence of the local peptidase activity. Another explanation for the different sensitivity of the various types of human neoplastic cells to the DFMO−peptide conjugates might be, as in the case of DFMO itself, that the various types of human neoplastic cells may regulate polyamine biosynthesis differently. As a consequence, the differential sensitivity to the compounds tested may be a result of fundamental differences in the control of the polyamine biosynthetic pathway and in the variations in the specific polyamine requirements of human cancer cell types.[26] It has also been shown that normal and neoplastic cells regulate polyamine biosynthesis differently,[4] and the role of polyamines in cell differentiation seems also to vary with the model system studied.[27]

The rationale for using melanotropin fragments as carriers for DFMO was not only the favorable cytotoxic effect of DFMO on melanoma cells, but also the fact that in our previous investigations with melanotropin conjugates containing Melphalan we demonstrated a specific receptor-mediated cytotoxic effect on them.[17] In the case of the present conjugates, we also performed receptor binding investigations. Although the DFMO derivatives of the central hormone fragments are capable of specifically displacing the natural hormone from its receptor, no preferred cytotoxic action was observed on the receptor-positive cells. A possible reason for this may be that receptor-mediated transport of the conjugates into the cell decreases the potential DFMO concentration on the cell surface where peptidases are acting. Therefore, we tested the cell growth inhibition of the conjugates on melanoma cells containing melanotropin receptor either in the presence or in the absence of MSH, and found no significant difference in the cytotoxicities. This result means that either no receptors are involved in the cytotoxic action or that their mediated cytotoxic effect could not be observed under the current experimental conditions.

In conclusion it may be stated that whatever mechanism(s) might be responsible for our findings, the increased cytotoxicity caused by coupling DFMO to carrier peptides may be a successful way for increasing the therapeutic efficacy of DFMO. Although no receptor-specific targeting effect was demonstrated, it is worth mentioning that contrary to Melphalan and nitrosourea congeners of melanotropin fragments,[17,28] the DFMO−peptide conjugates have a higher cytotoxic effect than the parent drug itself, which seems to be influenced at least to some extent by the nature of the carrier peptide.

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Acknowledgments

We thank Professor P. Sohár for the interpretation of the NMR measurements. This work was supported by the Hungarian National Research Fund (OTKA T 015709).

JS970080D