Synthesis and biological evaluation of tumor targeted lysosomotropic detergents

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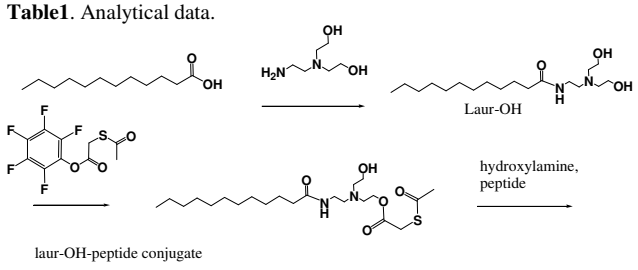
Abstract—Conjugation of a lysosomotropic detergent to tumor affine peptides (octreotatee and melanocortin analogous) resulted in receptor mediated uptake of the peptide conjugates into tumor cells expressing the peptide receptors. During intracellular trafficking the lysosomotropic detergent was cleaved from the peptide. Cytotoxicity studies showed IC_{50} values in the range of 11-72 µM caused by lysosomal rupture as proven by confocal laser scanning microscopy. For cells not expressing the receptors the conjugates were less cytotoxic.

Classical anticancer drugs lack specificity, as they enter the cells mainly by diffusion (1). Due to their DNA reactivity anticancer drugs can cause a second tumor several years after a curative treatment. Another strategy that might be exploited for cancer therapy is the controlled rupture of lysosomes (2-5). This treatment lacks the mutagenic potential of currently used chemotherapeutic agents. Tertiary amines that contain a hydrophobic chain, have been shown to accumulate in acidic cell organelles and become detergents upon protonation inside the acidic organelles resulting in organelle rupture and cell death (2). At neutral pH, they are rarely protonated and have no surface-active properties (2). It has been reported that lysosomal proteases, if released to the cytosol, may cause apoptosis directly by pro-caspase activation and/or indirectly by mitochondrial attack with ensuing discharge of pro-apoptotic factors (4). In cells with no lysosomes or small amounts of acidic cell organelles, lysosomotropic detergents were shown to be non-cytotoxic (5). The cytotoxic effect of lysosomotropic detergents is best in cells with high amounts of acidic cell organelles – such as cancer cells. The lipophilic nature of lysosomotropic detergents results in inselective cell uptake by passive diffusion through the lipid bilayer and cytotoxicity for cancer cells and normal cells. In order to increase the selectivity of lysosomotropic detergents, we propose the following strategy. By coupling lysosomotropic detergents to tumor affine peptides, they should become more water soluble. As the peptide derivatives of lysosomotropic detergents are more hydrophilic and do not diffuse passively through the lipid bilayer, involvement of the peptide receptor is essential for the cellular uptake of the conjugates. A further benefit of these conjugates should be the fact, that by exploiting the physiological transport process of the peptide-receptor complexes the peptide-conjugates are targeted to the lysosomes, the compartment where lysosomotropic detergents are activated. Receptor mediated cell uptake is supposed to be followed by intralysosomal, enzymatic liberation of the lysosomotropic detergent moiety. Employing this strategy should provide a more selective exploitation of the lysosomotropic detergents in cancer therapy. Peptides are used as targeting moieties in cancer therapy (6). Conjugates of chemotherapeutic agents and peptides can significantly increase the toxicity of anticancer drugs to tumors and reduce their adverse side effects on normal tissues (7). Somatostatin receptors (SSTR) have been found in a variety of neuroendocrine tumors, such as carcinoids and paragangliomas, as well as in most pancreatic endocrine and breast tumors (8). Structure–activity relationship studies have shown that the N-terminus of the cyclic octapeptides octreotatee (a somatostatin analogous) can tolerate N terminal substitution with minimal effect on SSTR affinity and internalization rate (9, 10). The Tyr3 modification of the peptide moiety was shown to result in a favourable pharmacokinetic behavior (9, 10). Melanoma cells display melanocortin stimulating hormone receptors. MSH analogous have been shown not to lose their receptor affinity when they contain N terminal substituents (11-13). For this reason octreotate (H-^{D}Phe-cyclo(Cys-Tyr-^{D}Trp-Lys-Thr-Cys)-Thr-OH) and a mela-nocortin analogous (His-Phe-Arg-Trp-NH_2) were chosen as tumor affine peptide carriers for the lysosomotropic detergent (11-13). We propose of coupling lysosomotropic detergents to tumor affine peptides might provide a new, more selective anticancer drugs. We synthesized and characterized the conjugates and evaluated their in vitro cytotoxicity, cell uptake characteristics and their mode of action. Scheme 1 shows the structures of the conjugates as well as the supposed intracellular cleavage. The lysosomotropic detergent Laur(OH) was obtained by reacting *N*,*N*´-bis(hydroxyethyl)ethylendiamine with dodecanoic acid using HATU (=HATU (O-(7-azobenzo-. triazol-1-yl)-1,1,3,3-tetramethyluronium) as amidation reagent. For coupling Laur(OH) to tumor affine peptides it was reacted with S-acetylmercaptoacetic acid-

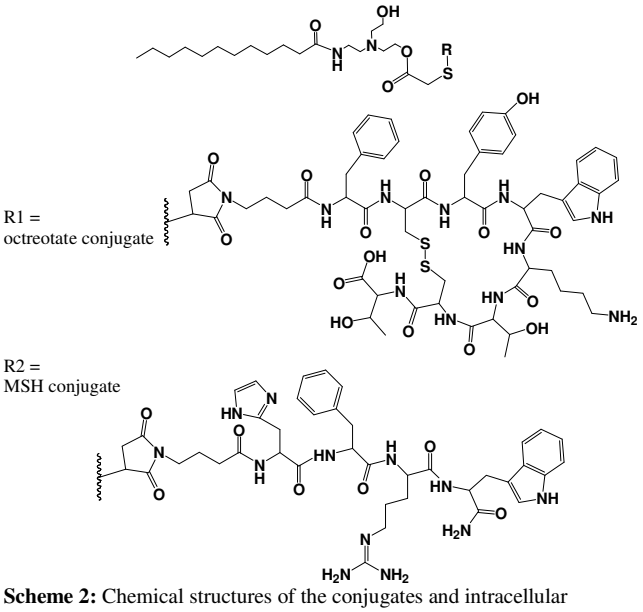
pentafluorophenylester (SAMA-OPfp) to form Laur(OH)-SAMA (14). Scheme 2 shows the synthetic route of Laur(OH)-SAMA. After deprotection of Laur(OH)-SAMA with hydroxylamine a Michael addition was performed to link the lysosomotropic detergent to the thiol reactive maleimido modified peptides (14). According to the Merrifield strategy, the peptides (octreotate and a melanocortin analogous) containing a maleimido moiety were synthesized manually. The peptides were synthesized manually, according to the Merrifield strategy. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were coupled in a stepwise manner to the corresponding resin. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) in DMF was used as coupling agent. Fmoc groups were removed using 20% piperidine/DMF. In the case of octreotatee the linear peptide H-^{D}Phe-Cys(Acm)-Tyr(tBu)-^{D}Trp(Boc)-Lys(Boc)-Thr(tBu)-Cys(Acm)-Thr(tBu)-OH was assembled by solid-phase synthesis, then the disulfide bond was formed on the solid support using thalliumtrifluoroacetate, and the N terminus was derivatized with a maleimido functionality (10). For the synthesis of the melanocortin analogous Fmoc-His(Trt), Fmoc-Phe, Fmoc-Arg(Pbf) and Fmoc-Trp(Boc) were used. The peptides were cleaved from the resin with trifluoroacetic acid (TFA/H_2O/triisopropylsilane (95:2.5:2.5). Reversed-phase HPLC purification of the crude products yielded the pure peptides which were characterized by analytical reversed-phase HPLC, and ion-spray mass spectrometry to reveal identity and purity.

Compounds	formula/ $(M+H)^+$ calc.	$(M+H)^+$ found	t_R (min)
Lau-OH	331.29	331.3	3.488
Laur-Sama	447.28	447.3	3.842
octreotatee conjugate	1618.73	1618.9	3,514
maleimido-octreotatee	1215.37	1215.4	2.803
MSH conjugate	1213.65	1212.8	2.779
maleimido-MSH	809.38	809.5	2.477

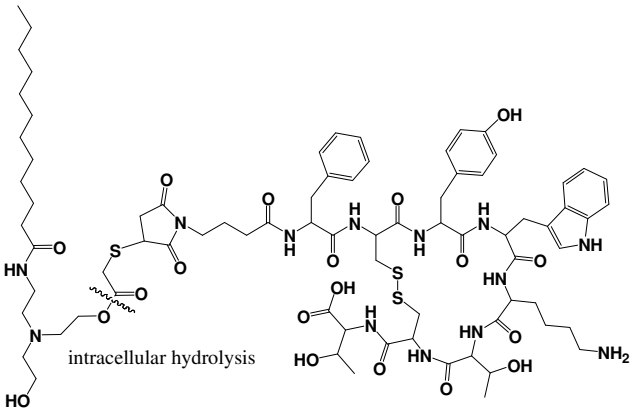
Table1. Analytical data.



Scheme 1: Synthesis of Laur(OH)-SAMA



Scheme 2: Chemical structures of the conjugates and intracellular hydrolysis.



Scheme 3: Intracellular hydrolysis of the conjugate.

Viability measurements using the MTS non radioactive proliferation assay revealed that Laur-OH (unconjugated lysosomotropic detergent) showed toxicity against AR42J, MCF-7, ZR-75, 3T3NIH, B16, SkMel3 cells with IC_{50} values in the range of 19- 52 µM. The octreotatee conjugates were cytotoxic for AR42J and MCF-7 cells (both overexpressing somatostatine receptors) compared to ZR-75 and 3T3NIH cells (both expressing no somatostatine receptors). The MSH-conjugate was more cytotoxic for the MSH expressing cells B16 and SkMel3 compared to the other cells lines, all lacking MSH receptors. The IC_{50} values for each cell line and compound are given in Table 2. By coupling the lysosomotropic detergent to tumor targeting peptides their cytotoxicity for cells expressing the receptor is greater (lower IC_{50} value) and shows a preferential cytotoxicity for these cell lines. However, the compounds were cytotoxic for the cells not expressing the receptor. This can be explained by partial hydrolysis of the compounds. The hydrolysed proportion is taken up by passive diffusion and is cytotoxic. HPLC studies revealed a hydrolysis of about

40% at the ester bond after one hour incubations in cell culture medium at 37°C. The IC_{50} values for lysosomotropic detergents are in the µM range (2). Even the conjugates show a cytotoxicity in the µM range. This can be explained by the mode of action. The detergents are incorperated into the lysosomal membrane (2). Only a µM concentration can destroy the lysosomal membrane. The conjugates are more effective compared to the unconjugated lysosomotropic detergent as allow more effective transport to the lysosomes.

Table 2. Results of the cytotoxicity assay for compounds **1–6**.

Compd	cell line	Cytotoxicity IC_{50}, µM[a]	Compd	cell line	Cytotoxicity IC_{50}, µM[a]
Laur-OH	AR42J	25 (±8)	**OC**	AR42J	11 (±8)
Laur-OH	MCF-7	34 (±5)	**OC**	MCF-7	20 (±8)
Laur-OH	ZR-75	37 (±8)	**OC**	ZR-75	55 (±8)
Laur-OH	3T3NIH	52 (±14)	**OC**	3T3NIH	64 (±8)
Laur-OH	B16	19 (±8)	**MSH**	SkMel3	15 (±8)
Laur-OH	SkMel3	28 (±8)	**MSH**	B16	25 (±8)
			MSH	3T3NIH	72 (±8)

[a]Values are means of three experiments, standard deviation is given in parentheses. OC = octreotatee conjugate, MSH = MSH conjugate.

In order to prove that disrupture of lysosomal membranes is mediated by the lysosomotropic detergent and the peptide conjugates, the localization of lysosomal entrapped FITC(fluorescein isothiocyanate)-dextran in B16, 3T3 NIH, AR42J, MCF-7 and ZR-75 cells was studied in the presence and in the absence of the lysosomotropic detergent and the peptide conjugates. FITC-dextran is a macromolecular drug that localises to acidic organelles by endocytosis and that is unable to escape from these organelles (15). Viable cells incubated with FITC-dextran alone served as controls. In the control cells FITC-dextran was located in lysosomes and endosomes, but not in other subcellular structure. An additional treatment of the cells with a peptide conjugate or a lysosomotropic detergent led to a change in the subcellular distribution of FITC-dextran which could now be imaged in the cytoplasm. There was a concentration dependent influence on the subcellular distribution of FITC-dextran. Incubation concentrations near the IC_{50} values resulted in a release of FITC-dextran into the cytoplasm. The micrographs of MCF-7 cells in Figure 1 show the subcellular distribution of FITC-dextran in the absence and in the presence of Laur-OH. FITC-dextran is located in the cytoplasm of cells treated with Laur-OH. Similar results were obtained in the with the octreotatee conjugate (not shown). The same changes in the subcellular distribution of FITC-dextran were obtained by Prasmickaite et al. who disrupted the endosomal membranes by photochemical delivery [15].

The data presented in this paper support the further evaluation of conjugates as novel targeted anticancer drugs.

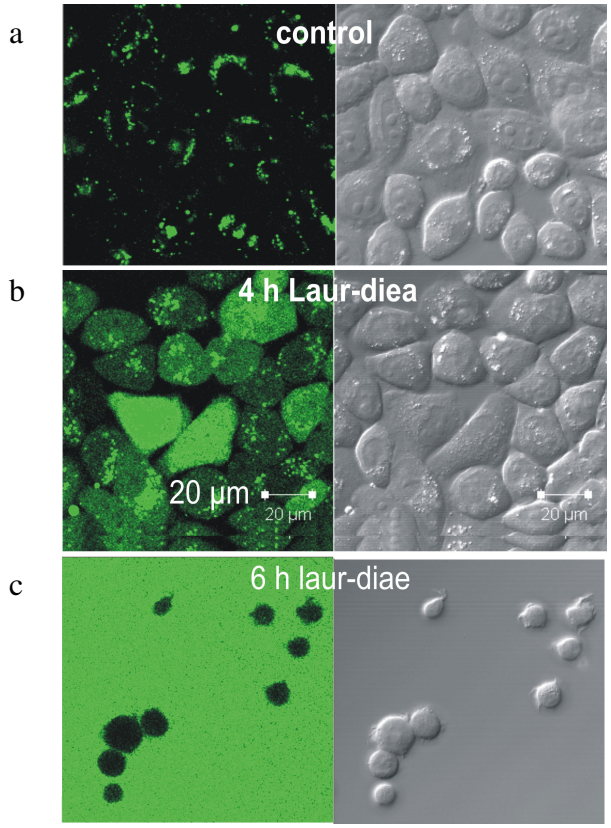


Figure 1: Subcellular distribution of fluoresceinisothiocyanate-dextran in the absence (a and b) and presence of a lysosomotropic detergent or a peptide conjugate in MCF-7 cells.

Acknowledgements

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References and notes

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Experimental

Materials and Methods. The protected amino-acids were obtained from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). The peptides were analyzed and purified by liquid chromatography (HPLC) on an Agilent 1100 system (Waldbronn, Germany) equipped with a variable UV detector. The columns used were Chromolith® RP- (5 µm, 250×4 mm; 10 µm, 250×10 mm; (VWR International GmbH, Darmstadt, Germany). All analytical runs were performed with at a flow rate of 4 mL/min. Unless otherwise stated, all preparative runs were performed with a linear gradient over 10 min of 0–100% acetonitrile in water (both solvents containing 0.1% TFA) and a flow rate of 8 mL/min. The peptides were synthesized manually. Low-resolution mass spectrometric analysis (ca. ±2 m.u.) of the peptides and conjugates was performed on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (MALDI-1, Kratos Instruments, UK). Sample preparation was performed with a solution of 2′-(4-hydroxyphenylazo)benzoic acid in acetonitrile/water (1:1) containing 0.1% TFA. The matrix (0.6 µL) was placed on the target, followed by one drop (0.6 µL) of the analyte, and allowed to dry at room temperature. Analyses with higher resolution and absolute accuracy were obtained with a Reflex 3 MALDI-TOF (Bruker Daltonics) (accuracy: ca. ±0.5 m.u.) and confirmed with a Finnigan MAT TSQ-7000 triple-quadrupole mass spectrometer using electrospray ionization (ESI) in the positive-ion mode (accuracy: ca. ±0.4 m.u.).

Synthesis and analytical data

Laur(OH). 2 g (10 mmol) dodecanoic acid and 3.8 g (10 mmol) mg HATU were dissolved in 3 mL of acetonitrile. Then 1.48 g (10 mmol) of *N*,*N*´-bis(hydroxyethyl)ethylendiamine were added and the reaction mixture was stirred at ambient temperatur for one hour. The reaction mixture was evaporated to dryness and then dissolved in dichlormethane. The organic phase was extracted several times with 1 N NaOH. The organic phase was dried over $MgS0_4$ and then purified by column chromatography (silica gel/ solvent: $CHCl_3$: MeOH : Et_3N = 80:18:2). Evaporation to dryness resulted in a white powder.

Laur-OH-Sama. To a stirred solution of Laur(OH) in dichlormethane was added a solution of SAMA at ambient temperature at a dropwise manner over 2 hours. Then the reaction mixture was washed with 1 N NaOH. The organic phase was dried over $MgSO_4$ evaporated to dryness. Column chromatography (silica gel/ solvent: $CHCl_3$: MeOH : Et_3N = 95:4:1) and evaporation to dryness resulted in a yellowish oil.

Maleimido-MSH was assembled by Fmoc chemistry on Rink amide resin (0.61 mmol/g). *N* α -Fmoc amino acids with the following side chain protecting groups were employed: Trp(Boc), Arg(Pbf), Phe and His(Trt). *N*-maleimido-3-propionic acid was coupled as described above, the resin was washed and dried under vacuum overnight. Cleavage was performed with 5 mL of 37:1:1:1 TFA/H_2O/phenol/TIS for 2 h at room temperature. The resin was filtered and washed. The peptide was precipitated by the gradual addition of tert-butyl-methylether at 4 °C. Purification was accomplished by reversed-phase HPLC.

Maleimido-octreotatee was assembled by Fmoc chemistry on Fmoc-Thr(tBu)-Wang resin (0.61 mmol/g). *N* α -Fmoc amino acids with the following side chain protecting groups were employed: Cys(Acm), Thr(tBu), Lys(Boc), Trp(Boc), D-Phe, D-Trp(Boc), and Tyr(tBu). All couplings were performed in DMF. The peptide chain was constructed manu-ally according to a modified in situ neutralisation cycle. Briefly, this cycle consisted of a twofold decoupling (1 min and 5 min) with 50 % piperidine in DMF and 30 min coupling with 4 eq of the Fmoc-amino acid (0.4 M in DMF, incubated for 5 min with 3.9 eq of HBTU and 6 eq DIPEA). After completion, the resin (1.75 g dry weight) was treated with piperidine in DMF to deprotect the terminal α -aminogroup of the peptide. The resin-bound peptide was cyclized at room temperature with a 4-fold molar excess of thallium(III)trifluoroacetate in dichlormethane for one45 minutes. After thorough washing, *N*-maleimido-3-propionic acid was coupled as described above, the resin was washed and dried under vacuum overnight. Cleavage was performed with 5 mL of 37:1:1:1 TFA/H_2O/phenol/TIS for 2 h at room temperature. The resin was filtered and washed. The peptide was precipitated by the gradual addition of tert-butyl-methylether at 4 °C. Purification was accomplished by reversed-phase HPLC.

Conjugation of the lysosomotropic detergent to the peptide

Preparation of the hydroxylamine solution. To 34 µL of a 50% hydroxylamine solution in water 966 µL water were added.

MSH conjugate. 43 mg (50 µmol) of the maleimidopropionic acid-His-Phe-Arg-Trp-NH_2 peptide and 22 mg (50 µmol) of the Laur(OH)sama were dissolved were dissolved in a mixture consisting of 2 mL PBS buffer (0.4 M, pH = 6.4), 600 µL DMSO and 400 µL of the aforementioned hydroxylamine solution. The resulting pH should be in the range of 7.0 to 7.5. The reaction mixture was vortexed for 2 minutes resulting in a precipitate. The precipitate was sseparated from the supernatant and dissolved in acetonitril water containing TFA. This solution was purified by preparative HPLC. The conjugate eluting at 5.714 min was collected and lyophilised. 11 mg of a white powder (68 % based on the amount of the starting maleimido-peptide) was obtained.

Octreotate conjugate. 21 mg (49 µmol) of Laur(OH)sama and 60 mg (49 µmol) of maleimido-3Tyr-octreotatee were dissolved in a mixture consisting of 2 mL PBS buffer (0.4 M, pH = 6.4), 600 µL DMSO and 400 µL of the aforementioned hydroxylamine solution. The resulting pH should be in the range of 7.0 to 7.5. The reaction mixture was vortexed for 2 minutes resulting in a precipitate. The precipitate was sseparated from the supernatant and dissolved in acetonitril water containing TFA. This solution was purified by preparative HPLC. The conjugate eluting at 5.714 min was collected and lyophilised. 7.85 mg of a white powder (68 % based on the amount of the starting maleimido-peptide) was obtained.

Stability. The conjugate was dissolved in cell culture medium and incubated for 1 hour at 37°C. Then a HPLC analysis was made using the conditions mentioned above.

Biological experiments.

FITC-dextran assay

Cells grown on coverslips were incubated with 1 mg/ml FITC-dextran (MW 70000) for 24 h. Afterwards they were incubated with or without Laur(OH) or a conjugate for 1 h at indicated concentrations (in the range of 10–200 µmol/l). Cells were washed three times with PBS buffer. The intracellular localization of FITC-dextran in unfixed cells was observed with a Zeiss Axioplan fluorescence microscope (Oberkochen, Germany) using an objective with ×100, a 450–490 nm bandpass excitation filter and a 510–540 bandpass emission filter.

Viability assay

The MTS viability assay (Cell Titer 96® Aqueous non-radioactive proliferation assay; Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. B16 (murine melanoma), SK-MEL-3 (human melanoma), AR42J (rat pancreas carcinoma), MCF-7 (human breast cancer), ZR-75 (human breast cancer) and NIH 3T3 (mouse fibroblast) cells were obtained from the tumour bank of the DKFZ (Heidelberg, Germany). Melanocytes were obtained from Promo Cell (Heidelberg, Germany). The cells were cultured for 24 h in a 96-well microtitre plate at 37°C in an atmosphere of 95% air and 5% CO_2. The culture medium consisted of RPMI-1640 and Dulbecco's phosphate-buffered saline (DPBS) (both from Pan Biotech, GmbH, Aidenbach, Germany). After 24 h, the cells were incubated with the compounds at indicated concentrations. The fraction of surviving cells was measured 48 h later, measuring the lactate dehydrogenase (LDH)-induced formazan formation at 490 nm with a microplate reader model 3350-UV (Biorad Laboratories GmbH, München, Germany). The compounds were dissolved in dimethylsulphoxide (DMSO) at a concentration of 100 mM to serve as stock solutions. The final concentrations in contact with the cells were 0, 1, 10, 25, 50, 75, 100 and 250 µM.