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Overcoming methotrexate resistance in breast cancer tumour cells by the use of a new cell-penetrating peptide

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Abbreviations:
Ac, acetylated
APA, 4-amino-4-deoxy-N^{10}-methyl pteroic acid
BOP, benzotriazol-1-yloxytris (dimethylamino)phosphonium hexafluorophosphate
CPP, cell-penetrating peptide
DHFR, dehydrate folate reductase
LDH, lactate dehydrogenase
MALDI, matrix assisted laser desorption ionization
MS, mass spectrometry
MTX, methotrexate
RP-HPLC, reversed phase high performance liquid chromatography

ABSTRACT

Resistance to chemotherapy limits the effectiveness of anti-cancer drug treatment. Here, we present a new approach to overcome the setback of drug resistance by designing a conjugate of a cell-penetrating peptide and the cytostatic agent methotrexate (MTX). Two different peptides, YTA2 and YTA4, were designed and their intracellular delivery efficiency was characterized by fluorescence microscopy and quantified by fluorometry. MTX was conjugated to the transport peptides and the ability of the peptide–MTX conjugates to inhibit dihydrofolate reductase, the target enzyme of MTX, was found to be 15 and 20 times less potent than MTX. In addition, in vitro studies were performed in a drug resistant cell model using the 100-fold MTX resistant breast cancer cells MDA-MB-231. At a concentration of 1 μM, the peptide–MTX conjugates were shown to overcome MTX resistance and kill the cells more efficiently than MTX alone. Estimated EC_{50}'s were determined for MTX, MTX-YTA2 and YTA2 to be 18.5, 3.8 and 20 μM, respectively. In summary, cell-penetrating peptide conjugation of MTX is a new way of increasing delivery, and thereby, the potency of already well-characterized therapeutic molecules into drug resistant tumour cells.

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TBTU, 1H-benzotriazole-1-yl)-N,N,N′,
N′-tetramethyluronium
tetrafluoroborate
TFA, trifluoro acetic acid

1. Introduction

Chemotherapy is a harsh treatment—as well as causing unpleasant side effects, many chemotherapeutic drugs are, in themselves, carcinogenic; treatment with these drugs carries a risk of secondary cancer. Many cancer researchers today are therefore committed to producing a new generation of more specific, better-targeted cancer therapies, to improve the quality of life of cancer patients and to minimize the risks of their treatment.

In many cases, tumour cells are resistant to chemotherapy. The mechanisms of drug resistance can occur at many levels including decreased transport, increased efflux, inactivation, alterations in the drug target, increased processing of drug-induced damage, or evasion of apoptosis (for review see [1,2]). Tumours may be intrinsically drug-resistant or develop resistance to chemotherapy during patient treatment; in addition, a cross-resistance may occur rendering the tumour less sensitive to other drugs with different mechanisms of action [2].

This desensitization of tumours treated by chemotherapy limits the effectiveness of anti-cancer drugs, thus if drug resistance could be overcome, the impact on patient survival would be considerable. For example, resistance to chemotherapy is believed to cause treatment failure in over 90% of patients with metastasic cancer [1].

In this study we have focused on methotrexate (MTX), one of the most studied folate antagonist [3]. MTX inhibits dihydrofolate reductase (DHFR), which leads to accumulation of polyglutamated folates, causing further inhibition of thymidylate synthase and glycinamide ribonucleotide formyltransferase. Subsequently, the lack of reduced folate substrates impairs synthesis of purine nucleotides, thereby inhibiting DNA synthesis which leads to decreased proliferation [4].

MTX resistance develops through several mechanisms [5–8], including decreased folate carrier-mediated membrane transport, dihydrofolate reductase gene amplification, specific transcription–translational modifications, and down regulation of intracellular MTX polyglutamation. Antifolate drug development has focused on agents designed to overcome different aspects of MTX resistance [9]. Conjugation of the cytostatic agent MTX, for improved efficiency and/or delivery is not a new concept. In 1978, Ryser et al. [10] conjugated several MTX moieties to a lysine polymer of 70,000 Da. This conjugate killed MTX resistant cells more efficiently than unconjugated MTX. Since then, several similar approaches have been tested; for example albumin-MTX [11], fibrinogen-MTX [12] and lipo-amino acid MTX conjugates [13,14].

In the last decade, reports have accumulated on the subject of a new vehicle for intracellular delivery, namely cell-penetrating peptides (CPP, for review see [15,16]). CPPs are short peptides with the ability to transport relatively large macromolecules across the plasma membrane of a wide range of cell types. In addition, there are reports of successful cell barrier transport and in vivo applications, using these remarkable peptides. Even though these peptides have been successfully applied, their mechanism of cell entry is not completely elucidated. Increasing evidence indicates that there may be several different pathways involved [17,18], depending on the properties of the CPP and its cargo.

To study the effect of CPP conjugation of MTX, a model system of MTX resistant breast cancer cells was selected; the cell line MDA-MB-231 was obtained from a patient who acquired MTX resistance during chemotherapy. It was determined that the tumour cells were defective in transporting MTX ([8] and references therein) and thus less sensitive to MTX toxicity. The basis of the drug resistance was later reported to be due to the lack of expression of the MTX transport protein: reduced folate carrier (RFC).

A few anti-cancer or cytotoxic drugs have successfully been delivered by CPPs; the anti-cancer drug doxorubicin has been delivered by using the CPPs penetratin, synB3 [19,20] and pTat [21]. Furthermore, anti-cancer peptides and proteins have also been applied (reviewed in [22,23]). Nevertheless, this study is to our knowledge the first report on delivery of MTX by a cell-penetrating peptide, and taken together with the doxorubicin data from the group of Temsamani [20,24], confirms that CPPs provides a new way to battle drug resistance.

2. Materials and methods

2.1. Synthesis

Briefly, the cell-penetrating peptide component was assembled on the resin by machine and the MTX component (or the fluorophore fluorescein) was coupled to the N-terminus of the peptides. MTX–peptide constructs were cleaved from the solid phase, purified and the correct HPLC fractions were identified by mass spectrometry.

Assembly of the transport peptide component onto solid phase was performed on a Syro multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany). In each reaction vessel, 50 mg Fmoc-protected MBHA Rink-amide resin [(0.56 mmol functional group/g resin)] was loaded. According to the loading amount, the amounts of Fmoc-amino acid, 1H-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and *N*,*N*′-diisopropylethylamine were 4 eq., 4 eq. and 8 eq., respectively. The reaction was performed at room temperature for 30–60 min. The solid phase was washed consecutively and treated with acetic anhydride solution in *N*-methyl-2-pyrrolidone to cap unreacted amino groups. The resin was de-protected with 20% piperidine in *N*,*N*-dimethylformamide (v/v), and washed again. On completion of the whole peptide sequence, the peptide-resin was washed with dichloromethane and dried in vacuum.

2.2. Conjugation and purification

MTX–peptide conjugate was synthesised as described by Nagy et al. [25], adopted to solid phase. In brief: the Gly extended peptide N-terminus was deprotected and reacted with 2 eq. of TBTU activated Fmoc-Glu-O(tBu). After de-protection of the N-terminal, 5 eq. of BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) activated 4-amino-4-deoxy-N^{10}-metyl pteroic acid (APA) in dimethyl sulfoxide was added and the reaction mixture was agitated for 6 h in room temperature. The coupling was repeated with one-third of the original amount of reagents and left over night. Before cleavage, the resin bound MTX-CPP was washed with dimethyl formamide and dichloromethane, and vacuum dried.

After completed assembly of the conjugate, the peptide was cleaved from the solid support, using trifluoroacetic acid (TFA)-scavengers cocktail. Standard reagent K (according to http://www.emdbiosciences.com Fmoc resin cleavage) content was modified to prevent irreversible modification of product by linker. The cocktail mixture contained 82.5% TFA, 5% water, 5% thioanisole, 2.5% 1,2 ethanedithiol and 5% phenol. TFA is a sufficiently strong acid to assist cleavage, yet preserving the integrity of APA. The scavengers were added to minimize alkylation's side-reactions.

Products were purified on RP-HPLC with preparative column (Supelco C18 discovery HSC18–10 μm column, Sigma–Aldrich, US) in water/0.1% TFA (v/v) and acetonitrile/0.1% TFA (v/v) gradient. The fractions were analyzed by MALDI-TOF mass spectrometer (Voyager-DE STR mass spectrometer, Applied Biosystems Framingham, USA).

2.3. Cell culture

The human breast cancer cell line MDA-MB-231 and the human breast cancer cell line MCF-7 (ATCC via LGC, Sweden) were cultured in RPMI-1640 media with Glutamax (InVitrogen, Sweden) supplemented with 5% fetal bovine serum, sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) in air enriched with 5% CO_2 at 37 °C. MCF-7 cells were used for comparison, as they respond to MTX.

2.4. Peptide/conjugate characterization

To confirm that the peptides had cell-penetrating properties, three different detection methods were used: microscopy, fluorometry (quantitative, fluoresceinated peptide) and mass spectrometry (qualitative, unlabeled substance). In parallel, the acute toxicity or membrane disturbance was determined by measurement of lactate dehydrogenase leakage [26]. The data represent the direct action of the substance on cellular membrane integrity.

2.4.1. Characterization of cellular localization

The cells were seeded in NUNC chambers at approximately 2000 cells/well (NUNC at Labdesign, Sweden) 24 h prior to experimentation and incubated for 30 min at 37 °C with the peptides. The cells were washed twice with HEPES buffered Krebs–Ringer solution (HKR) and cell nuclei were stained with DAPI (0.5 μg/ml) for 15 min. Images were obtained with a Leica DM IRE2 fluorescence microscope and a Leica DC350 camera (Leica Microsystems, Sweden) and processed in PhotoShop 5.5 software (Adobe Systems Inc., CA).

2.4.2. Detection of intracellular localization by mass spectrometry

100,000 cells/well of human MDA MB-231 cells were seeded in 6-well-plates 2 days before the experiment. On the day of the experiment, sub confluent cells were washed twice with HKR to remove serum traces. The test substances (10 μM in HKR) were added to the cells and incubated at 37 °C and 300 rpm for 60 min. Samples of 200 μl of extra cellular supernatant were frozen at −20 °C. The cells were washed with 0.05% trypsin/EDTA solution to remove membrane-associated substances. To prepare substances trapped intracellularly, the cells were lysed in 0.1% HCl on ice at 4 °C. Prior to mass spectrometry, the samples were purified by C18 ZipTips (Millipore, Sweden), according to the manufacturer's instructions. The matrix (α-cyanohydroxy cinnamic acid) at 10 mg/ml in 50% AcN in 0.1% H_2O was spotted on the sample plate before the sample. As a positive control, the well documented CPP pVEC (data not shown) was included in the experiment. As an internal standard, ProteoMass$^{TM}P_{14}$R MALDI-MS Standard (from Sigma–Aldrich, Sweden AB) was used (monoisotopic molecular weight 1533.8582 Da). Data were collected from at least three different locations on the spot and with counts >10,000, and accumulated. The mass spectrometry analysis was performed on a Voyager-DE STR (Applied Biosystems Framingham, USA) and processed on the freeware *MoverZ* program (http://65.219.84.5/moverzDL.html).

2.4.3. Detection of peptide translocation by quantitative fluorometry

100,000 MDA-MB-231 cells/ml and well were seeded 1 day before experiments into a 24-well-plate. The cells were washed (2 × 1 ml HKR) and then exposed to 300 μl of 5 μM drug in HKR at 37 °C–30 min–300 rpm (Thermomixer, Eppendorf). The peptide-treated cells were washed and lightly trypsinised: 200 μl of trypsin/EDTA (0.05% T/E 1:2 in HKR), 37 °C–max 1 min–300 rpm. The cells were washed thoroughly and lysed in 250 μl 0.1% Triton X-100 in HKR, 0–4 °C–10 min. The cell lysates were transferred to a black plate for fluorescence readout at 492/520 nm on a Spectramax Gemini (Molecular Devices, GTF, Sweden). The samples were compared to the fluorescence of the added amount of peptide and the total protein amount.

2.4.4. Lactate dehydrogenase leakage

Lactate dehydrogenase leakage was measured to determine any acute membrane disturbance during the translocation studies. Sampling was carried out by multiplexing from assays performed under conditions defined above. After 30 min and at a peptide or conjugate concentration of 5 μM, a 100 μl sample/well in duplicate was collected and detected by the addition of 100 μl CytoTox-ONE assay (Promega Corporation, Madison, WI) incubated for 10 min at room temperature and the luminescence was detected at 560/590 nm in a Spectramax Gemini (Molecular Devices, GTF, Sweden). The samples are presented as percentage of maximum LDH release (100%) from 0.1% Triton X-100 treated cells.

Table 1 – Description of peptides and conjugates used in this study and corresponding membrane disturbance by LDH leakage (for details see Section 2.1–2.2)

Name	Sequence	LDH leakage (%)[b]
YTA2	Acetyl-YTAIAWVKAFIRKLRK-amide	11.6 ± 9.2
MTX-YTA2	APA-γE-G-YTAIAWVKAFIRKLRK-amide[a]	4.4 ± 3.8
YTA4	Acetyl-IAWVKAFIRKLRKGPLG-amide	2.4 ± 1.5
MTX-YT4	APA-γE-G-IAWVKAFIRKLRKGPLG-amide	4.7 ± 2.0
MTX		2.5 ± 2.0

[a] APA-γE-G- represents schematically how MTX was assembled onto the CPP sequence see Fig. 1. Glycine is a linker while APA (4-amino-4-deoxy-N^{10}-methyl pteroic acid)-γE together forms MTX.
[b] LDH leakage at 15 min as percent of total LDH released when cells treated with 0.1% Triton X-100 on MB-231-MDA cells. For comparison pVEC 1.7 ± 0.8, under the same conditions.

2.5. *Enzyme inhibition assay on dihydrofolate reductase (DHFR)*

To characterize the efficiency of the test compounds to inhibit the MTX target enzyme, the dihydrofolate reductase (DHFR) assay was performed in a cell free system. The inhibitory effects of substances on DHFR activity were measured by monitoring NADPH oxidation at λ = 340 nm and in accordance with a procedure adapted from a previously published method by Pignatello et al. [27].

In short, the assay mixture contained 10 mM potassium phosphate buffer (pH 6.0), 2.7 mM KCl, 0.2 mM NADPH (Fluka), test compounds to a final concentration ranging between 10^{-5} and 10^{-9} M, and 0.002 units of bovine liver DHFR (Fluka, Product No. 37294, 8 U/ml) on a 96-plate. After addition of the enzyme, the mixture was incubated at RT for 5 min and the reaction commenced by adding dihydrofolic acid (to a final concentration of 33 μM). The change in absorbance at 340 nm was monitored for 20 min, during which the activity was linear with respect to time.

Results are presented as percent inhibition of the enzymatic activity. IC_{50} values and curve fittings were calculated using GraphPad Prism software (GraphPad Software, CA).

2.6. *Cell viability assay*

The day before drug exposure 15,000 cells/well in 0.5 ml were seeded on 48-well-plates and left to attach over night. The cells were exposed for 30 min at varying drug concentrations in 1% serum media. After exposure, the cells were left to recover in complete media (5% serum RPMI1640) for 2 days.

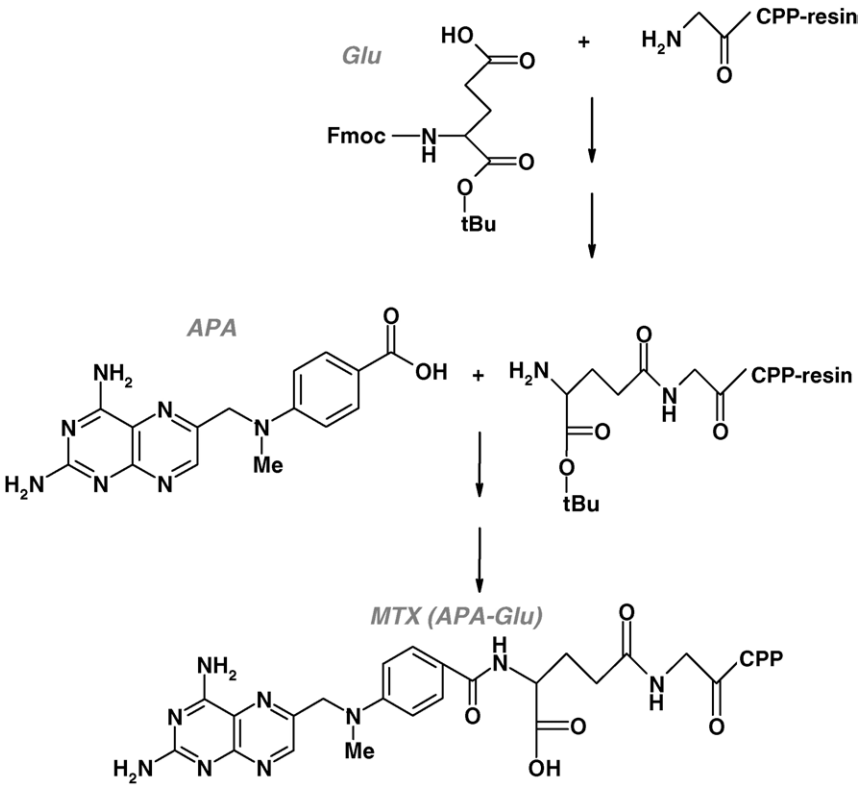

Fig. 1 – Schematic route of MTX conjugation to CPPs. The MTX is built onto the transport peptide; first by addition of a Glu residue, conjugated on the γ-carboxyl group, onto which the APA (4-amino-4-deoxy-N^{10}-methyl pteroic acid) is then conjugated. Then the MTX-CPP is cleaved from the solid support. The sequence of the CPP is shown in Table 1.

CellTiter-Glo™ Luminescent Cell Viability Assay (# G755A Promega Corporation, Madison, WI) was used to detect the amount of viable cells by measuring ATP dependent luminescence. The detection reagent was added in the same volume as the cell media and left at room temperature. After 10 min the samples were transferred from each well onto a white 96 well plate and the luminescence was recorded. CPP-MTX, CPP and MTX were tested separately to produce ''dose–response'' graphs (molar concentrations versus percent of viable cells) from triplicate observations and the EC_{50} values were determined using GraphPad Prism software 4.0 (GraphPad Software, CA). The statistical methods used were paired Student's t-test from three experiments performed in triplicate.

3. Results

The CPPs were designed according to a method outlined in Hällbrink et al. [28]. In short, the transport peptides were designed by extending an amino acid sequence corresponding to a matrix metallo protease-2 substrate [29]. The peptide selected had been produced in a phage library with the aim to find a higher selectivity for matrix metallo protease-2 over several other matrix metallo proteases. The original substrate sequence was SGESLAY-YTA [29], with the hyphen representing the protease cleavage site. The newly designed CPPs were named YTA2 and YTA4 after the substrate's sequence and the order in which they were synthesised. For the amino acid sequences and conjugates see Table 1. In addition, Fig. 1 illustrates the schematic route to MTX conjugation, which is described in more detail in Section 2.

3.1. *Acute toxicity of peptides and conjugates*

Due to the amphipatic nature of cell-penetrating peptides, the peptides and their corresponding MTX conjugates were characterized for their membrane disturbance activities. The membrane disturbance or acute toxicity was detected

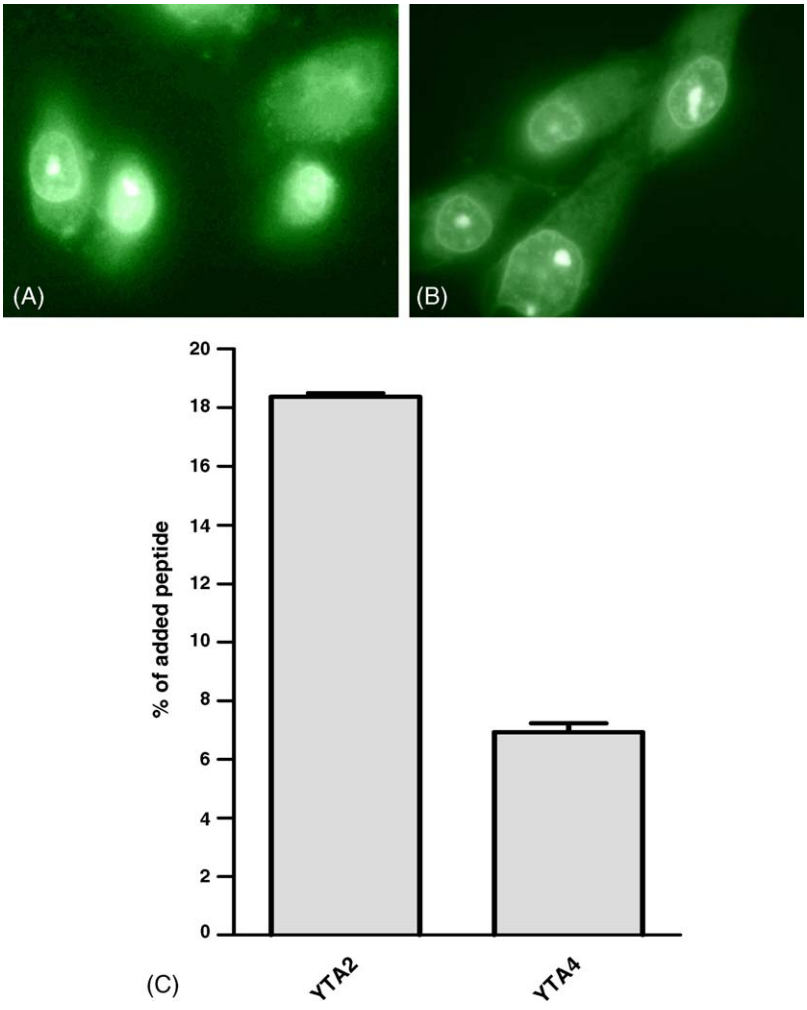

Fig. 2 – Fluorescence microscopy, visualization of fluoresceinyl labeled YTA peptides in MDA-MB-231 cells. Live cells were treated with 1 μM peptide in HKR for 30 min at 37 °C. (A) For fluoresceinyl YTA2, approximately 70% of the cells were heavily stained while for (B) fluoresceinyl YTA4 the staining was comparable to YTA2 but fewer cells (40%) were stained. (C) Quantitative uptake of fluoresceinyl peptides in MDA cells. The amount of peptide found intracellularly in MDA cells, after 5 μM and 30 min at 37 °C, was 22.8 ± 0.9 and 8.8 ± 0.2% of extracellular added peptide for YTA2 and YTA4, respectively. Error bars represent ± S.E.M. (n = 3).

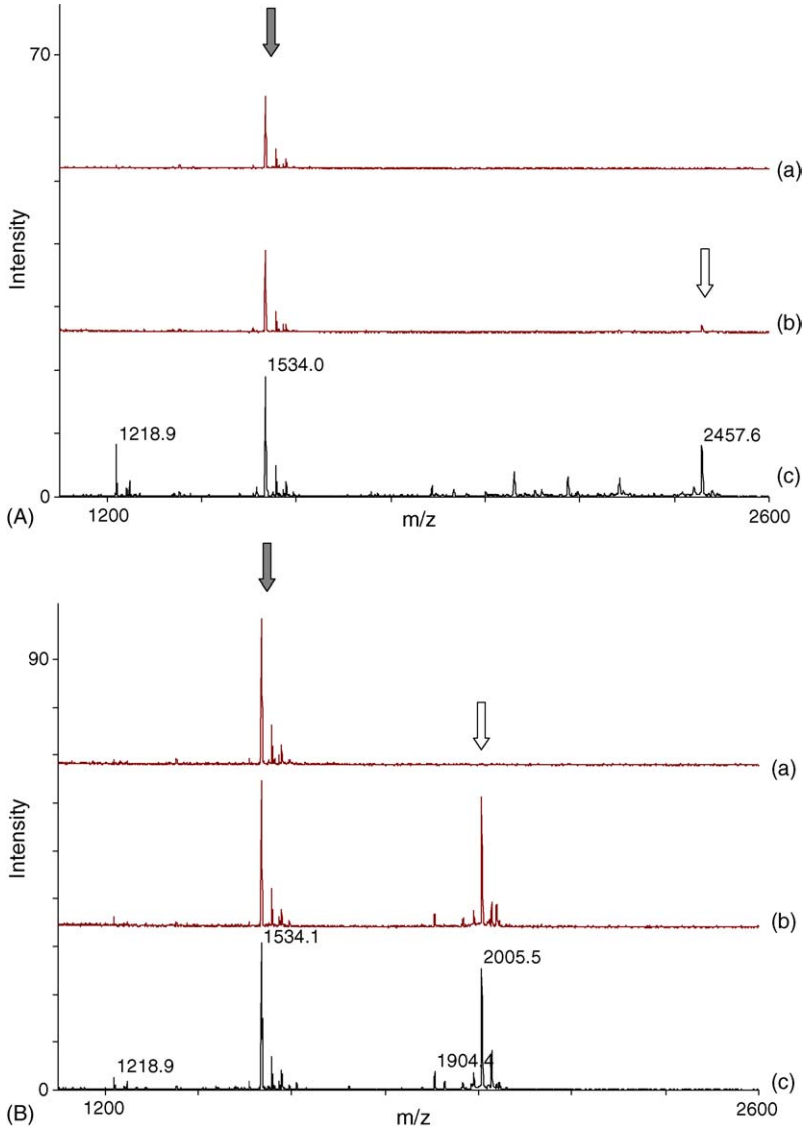

Fig. 3 – MS spectra of intracellular localization of MTX-CPP and CPP, respectively. MS detection of the tested compounds demonstrates that both (A) MTX-YTA2 (MW 2457.6) and (B) YTA2 (MW 2005.5) can be found in the intracellular fraction (spectra b) of the incubated cells after 30 min at 37 °C. The respective spectra are (a) empty spectra with intern standard (filled arrow) at MW 1534.1, (b) intracellular fraction with detected peptide/conjugate (open arrow) and (c) extracellular fraction. In addition, MTX-YTA4 and YTA4 also enters MB-231-MDA cells in an analogous manner (data not shown). MW 1218.9 is a contribution from the cell preparation.

by measuring drug induced lactate dehydrogenase (LDH) leakage (see Table 1). In summary, one of the CPPs (YTA2) had a minor effect on the membrane integrity; however, this effect was lower in comparison with other well-characterized CPPs [26]. In contrast, when the CPP was conjugated to MTX, the effect of membrane disturbance was lost. The rest of the peptides and conjugates did not have any significant effect on LDH release (Table 1).

3.2. *Cellular translocation*

To verify that the designed peptides would function as CPPs, three different methods were used: quantitative fluorometry, fluorescence microscopy and mass spectrometry (MS).

To be able to evaluate the cell-penetrating abilities of the new peptide sequences; we utilized two methods in parallel. Microscopy (Fig. 2A and B) together with quantitative fluorometry (Fig. 2C) gave an overview of the translocation abilities of the fluoresceinyl peptides. Visualization of the cellular translocation by microscopy showed that both fluoresceinyl-labeled YTA2 and YTA4 entered MDA-MB-231 cells (Fig. 2A and B). Using 10 randomly selected visual fields it could be estimated that approximately 70% of the cells were stained when treated with YTA2 and 40% with YTA4; to conclude, YTA2 seemed to be more efficient than YTA4. Quantitative fluorometry confirmed that YTA2 was the more efficient CPP (Fig. 2C).

The MS detection is a qualitative way to detect the peptides and conjugates in cell lysates. The advantage of MS is that

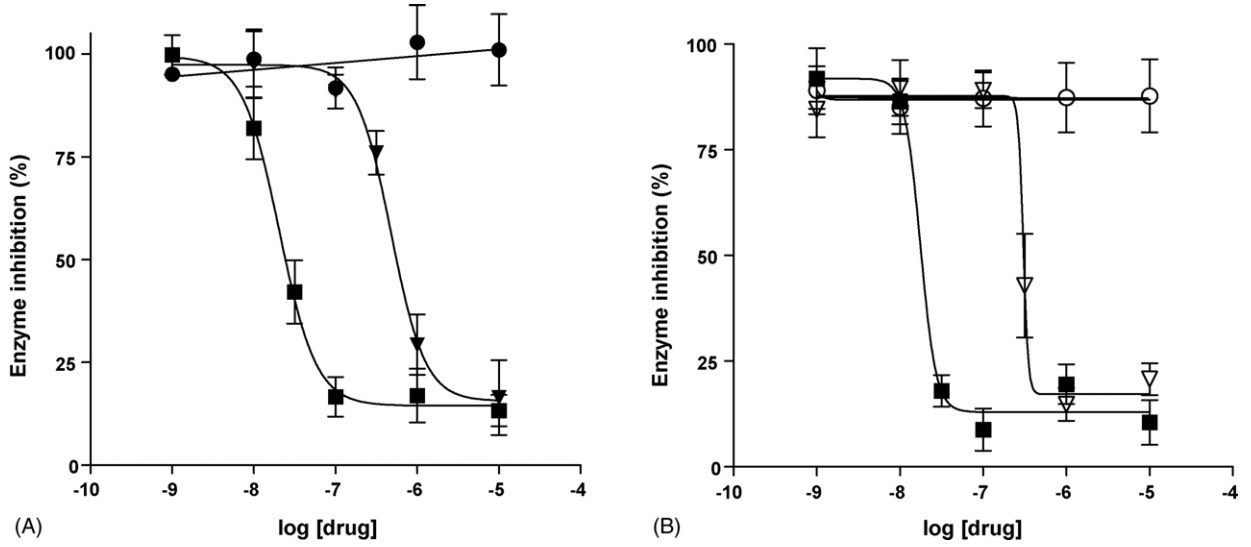

Fig. 4 – Inhibition of DHFR enzyme activity by the two MTX-CPP conjugates as compared to MTX. Inhibition of DHFR enzyme activity was detected as a reduction of NADP+ by absorbance. The data, from three experiments performed in triplicate, are presented as percent of enzyme activity (without inhibition) against log of drug concentration. IC_{50} for MTX (filled squares) is 0.019 μM, (A) MTX-YTA2 (filled arrow head) with IC_{50} of 0.30 μM and YTA2 alone (filled circles), (B) MTX-YTA4 (open arrow heads) with IC_{50} of 0.48 μM and YT4 alone (open circles). Error bars represent ± S.E.M. ($n = 3$).

there is no need for a fluorophore or other label, which is useful since it has been shown that the cargo may influence the uptake efficiency of the CPP [30]. Fig. 3A illustrates that MTX-YTA2 entered MDA-MB-231 cells; the spectrum shows the intracellular fraction after purification. In a similar manner, YTA2 without any cargo entered the cells (Fig. 3B).

3.3. *MTX target enzyme inhibition*

In order to determine the potency of MTX when conjugated to the peptide, the MTX-CPP and CPP (as a negative control) were tested for their ability to inhibit DHFR in a cell free system (see Fig. 4).

Inhibition of DHFR was detected as a reduction of NAPD+ by measuring the absorbance at 595 nm. The data are presented as percent of enzyme inhibition against log of drug concentration (see Fig. 4). From the graph, the IC_{50} values were calculated: IC_{50} for MTX is 0.019 μM, MTX-YTA2 0.30 μM and MTX-YTA4 0.48 μM.

In comparison with MTX alone, the YTA2 and YTA4 conjugates were approximately 15 and 20 times, respectively, less potent. This is similar to other drugs that function in a similar manner to MTX. For instance, the thymidylate synthase inhibitor AG331 by McGuire et al. [31] is about 10 times less potent as compared to MTX, but with other improved properties.

3.4. *Effect on CPP conjugation on cell viability in MTX resistant cells*

Since YTA2 was shown to be both a more efficient CPP (Fig. 2) as well as more potent in DHFR inhibition (Fig. 4), we decided to continue with only YTA2 in the cell viability studies. The MDA-MB-231 cells were confirmed to be MTX resistant with an EC_{50} of 18.5 μM. In comparison, MTX-sensitive cells (MCF-7) gave an EC_{50} of 0.2 μM in the same assay, which indicates a 100-fold relative resistance (data not shown). There is no clear definition of how many times less sensitive a cell line should be to be defined as drug resistant; however, for comparison, Pignatello et al. have shown a relative resistance of 100–150-fold [14].

In the cytotoxicity studies, MTX-YTA2 demonstrated enhanced cytotoxicity compared to MTX alone, an observation that was statistically significant ($p < 0.05$) already at a peptide and MTX concentration of 1 μM. Estimated EC_{50}'s in MDA-MB-231 cells for MTX, MTX-YTA2 and YTA2 are 18.5, 3.8 and 20 μM, respectively (Fig. 5B). However, the CPP component had a minor effect on cell viability at the highest concentrations tested (10 μM), which may have been attributable to the effect on membrane disturbance (Table 1).

In conclusion, conjugation of MTX to a CPP overcomes the transport resistance of human breast cancer cells in culture, and is demonstrated here by MTX-CPP (65% reduction in cell viability at 10 μM) being more toxic than MTX (5% reduction in cell viability at 10 μM) alone.

4. Discussion

The aim of this study was to investigate whether a CPP-drug conjugate could be designed, with the ability to enter tumour cells with acquired influx resistance, and be able to kill MTX-resistant cells more efficiently than MTX alone. The advantage of this approach is the flexibility of the conjugate; any small anti-cancer drug may be attached to the transport peptide, thereby using a new route to enter cancer cells without dependence on the tumour cells' difference in expression of transporter proteins. MTX was selected as a model compound

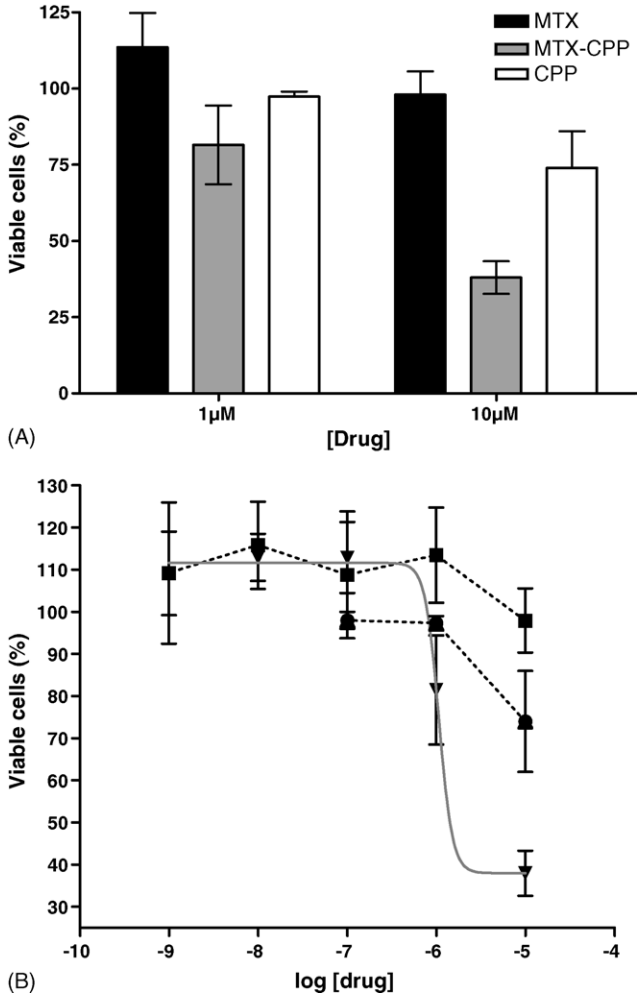

Fig. 5 – Effect on cell viability by 30 min exposure of the test compounds to MB-231-MDA cells. (A) MTX-YTA2 (gray bar) is more cytotoxic than MTX (black bar) alone, an observation that is statistically significant ($p < 0.05$) at a peptide and MTX concentration of 1 μM. The CPP component (white bar) has a minor effect at the highest concentrations tested (10 μM). Error bars represent ± S.E.M. ($n = 3$). Reduction in cell viability, using the Promega technology of Cell-Titer Glow ATP detection assay, was used to quantify cytotoxicity. (B) Estimated EC_{50}'s for MTX (squares), MTX-YTA2 (arrowhead, gray line) and YTA2 (circle) are 18.5, 3.8 and 10 μM, respectively.

since it was compatible with the peptide synthesis procedure and could easily be tested in in vitro cell models [8].

Two CPP-MTX conjugates were selected for synthesis and evaluation as potential candidates; YTA4 was designed to ease synthesis; however, it was demonstrated to be less active than the original YTA2 peptide (Figs. 2 and 3). YTA1 and YTA3 were not cell-penetrating peptides according to the three evaluation methods described for cellular translocation studies.

When using the LDH leakage to observe the direct effects on the cell membrane, the endpoint must be carefully chosen; measuring more than 30–60 min after addition of the test substance, the LDH enzyme activity may be lowered through degradation [32]. Therefore, the effects of peptides on membranes were scanned at 5, 15 and 30 min at two different concentrations (5 and 20 μM) but only data from 15 min incubation are shown here for clarity (Table 1). Contrary to the LDH data, YTA2 has lower haemolytic activity (data not shown) which indicates that YTA2 may be selective for cancer cells over healthy blood cells; this activity will be further explored. For a comparison of several CPPs and their membrane activities see Saar et al. [26].

In our experience, 5 μM is sufficient to detect uptake of a relative efficient CPP without membrane toxicity. A higher concentration (20 μM) will show which peptides are toxic, as well as increasing sensitivity to CPPs with lower efficiency.

The choice of cell line was important because a transport resistance was crucial in this study; however, as shown recently, transport resistance covers a major part of drug resistance in MTX-treated tumours [33]: The human breast cancer isolate MDA-MB-231 proved to possess the qualities needed. The cell line was derived from a MTX-treated patient, who became resistant during treatment ([8] and references therein), which makes this cell line a unique model for the study of MTX resistant breast cancer. It lacks expression of reduced folate carrier (RFC), which is the major route for the cellular uptake of MTX. Other MTX resistant cell models have been applied for similar studies, such as CCRF-CEM [34] and ZR-75-MTXr [35], both of which have been rendered resistant by drug exposure in vitro.

The key aim of these experiments was to overcome MTX transport resistance by conjugating MTX to a CPP. To ensure reliable results, these experiments have been carried out numerous times (with new sets of cells and two different synthesis batches of the MTX-YTA2 peptide) to rule out the inherent variations in the behaviour of cultured cells and variances in batch synthesis.

Generally, when testing drugs effects on cell viability, the drug exposure time is between 24 and 72 h [14,20], but since we are studying peptides, we decided on a much shorter exposure time. The reason for choosing 30 min exposure was to minimize effects of degradation products and to mimic the short exposure (systemic circulation of minutes) of future animal studies as well as copying the quantitative uptake experiments. Preliminary experiments suggest that longer exposure times (24–48 h) results in higher effects of the MTX-CPP conjugate (data not shown).

The CPP effect on cell viability correlates with the effect of the peptide in the membrane disturbance LDH assay. Furthermore, when the CPP is conjugated to MTX the lytic activity is lowered, thereby the lytic contribution of the CPP in the conjugate may be disregarded as a contribution of the conjugates effect on MDA-MB-231 viability.

In comparison with MTX alone, the YTA2 conjugate was approximately 15 times less potent in inhibiting DHFR. Yet, in the cell viability the comparison of the EC_{50} values of MTX (18.5 μM) and MTX-YTA2 (3.8 μM) gives an improvement of potency of about five-fold. This may be due to the accumulation of CPP intracellularly which has been reported earlier [36]. In contrast to our data Pignatello et al. [13] showed an increase in DHFR inhibition upon lipophilic alkyl amino acid conjugation of MTX. To speculate, this may reflect the difference in mechanism of MTX translocation into the tumour cells, i.e. the

increase in lipophilicity increases the membrane diffusion of MTX while the CPP conjugation uses the suggested pathways of endosomal release and penetration.

Recent studies by Temsamani and co-workers [20,24] showed that the anti-cancer drug doxorubicin and its potent analogue 2-pyrrolinodoxorubicin overcame multidrug resistance by peptide conjugation. They demonstrated that the peptide conjugation of the small drugs made them unrecognisable by the efficient efflux pump P-glycoprotein, thereby increasing the accumulation and cytotoxicity of these drugs. Not only peptide vectorization of anti-cancer drugs may well lead to a higher potency and lowered systemic toxicity [24], but also albumin conjugation. For instance, MTX conjugated to human serum albumin has been entered into clinical trials in the battle against both cancer [37] and rheumatoid arthritis [38].

To summarize, it can be concluded that MTX can be successfully delivered by the new cell-penetrating peptide YTA2 into tumour cells. Furthermore, the conjugate (MTX-CPP) can overcome the MTX resistance in breast cancer cell line MDA-MB-321, thereby restoring the effect of MTX and rendering the tumour cells once again sensitive for chemotherapy.

To our knowledge, this is the first delivery of MTX by a cell-penetrating peptide, and taken together with the doxorubicin data from the group of Temsamani [20,24], this confirms that CPPs provides a new way to battle drug resistance in cancer.

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