Microcystin LR Shows Cytotoxic Activity Against Pancreatic Cancer Cells Expressing the Membrane OATP1B1 and OATP1B3 Transporters

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Abstract. *Microcystin-LR (MC-LR) is a cyanobacterial cyclopeptide, known for its unique ability to cause acute liver injury. Its cellular uptake is facilitated by specific transmembrane organic anion-transporting polypeptides (OATPs) specifically OATP1B1 and 1B3. The objective of the present study was to investigate the expression of OATPs 1A2, 1B1 and 1B3 in pancreatic cancer cell lines BxPC-3 and MIA PACA-2 and assess their role in MC-LR-mediated cytotoxicity by using the novel xCELLigence system and flow cytometry. OATP1B1 and 1B3 were found to be expressed in both cell lines at both the mRNA and protein levels. The cytotoxic effects of MC-LR were proportionally related to the expression of these transporters. Moreover the cytotoxic potency of MC-LR was found superior to gemcitabine. Based on the expression of the organic anion transporting polypeptides 1B1 and 1B3 in pancreatic carcinoma tissue and cell lines and the potent cytotoxicity induced by MC-LR in vitro, we propose that this molecule could be held as structural basis for the development of novel targeted-compounds against pancreatic cancer.*

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Pancreatic cancer is the deadliest cancer with predicted 48,960 new cases and 40,560 deaths in 2015 in the United States (1). Gemcitabine is currently considered the standard first-line treatment for locally advanced and metastatic ductal neoplasms of the pancreas (2, 3). However, the objective response rates for patients with pancreatic cancer treated with gemcitabine is negligible and survival is below 8 months (4).

We recently found that human pancreatic adenocarcinomas overexpress Organic Anion Transporting Polypeptides (OATP) 1B1 and 1B3 (5), while other investigators have shown that OATPs are also up-regulated in colon, liver, breast, prostate and bone cancers (6-10). OATPs are encoded by the *SLC21/SLCO* gene-superfamily, which comprises a total of 11 members (11). They are cell transmembrane influx transporters, that facilitate the sodium-independent uptake of a variety of endogenous and xenobiotic compounds and anticancer drugs (12-14).

Microcystins, which are cyclic peptides synthesized by a variety of cyanobacteria (15) (Figure 1), are known to induce cytotoxic effects by targeting OATPs. Microcystin-LR (MC-LR) is the most commonly occurring variant of microcystins and can be effectively transported intracellular by using OATPs 1A2, 1B1 and 1B3 (16) and induce cytotoxic effects at relatively high concentrations through inhibition of protein phosphatases PP2A and PP1, ROS generation and depletion of cellular GSH levels (17, 18). The cytotoxic potential of

MC-LR in humans was first noticed a few years ago, following an unfortunate fatal medical accident in Brazil when patients with kidney failure on hemodialysis were exposed to dialysis water contaminated with microcystins (19). To our knowledge, this is the first study to investigate MC-LR *in vitro* activity against pancreatic cancer.

Materials and Methods

Cell culture. BxPC-3 and MIA PACA-2 human pancreatic cancer cell lines were used in this study (ECACC Public Health of England, Porton Down, Salisbury, UK). CHO cells were kindly gifted by Professor Bruno Stieger, Department of Clinical Pharmacology and Toxicology, University of Zurich, which served as a negative control for the expression of OATP1B3 (20). Cells were cultured in a humidified CO_2 chamber at 37˚C, 5% CO_2, under normal cell culturing conditions. The complete culture medium used was RPMI-1640 and DMEM (ATCC, Manassas, VA, USA) for BxPC-3 and MIA PACA-2 respectively; both supplemented with 10 % FBS (Invitrogen, Carlsbad, CA, USA). CHO cells were treated with DMEM (Gibco, Grand Island, NY, USA) enriched with 100 U/mL Penicillin/Streptomycin (Gibco, Grand Island, NY, USA), 10 % FCS (Gibco, Grand Island, NY, USA) and 25 mg/500 ml L-Prolin. Human liver tissue-samples were obtained by the University of Ioannina Cancer Biobank Center.

RNA extraction. Quadruplicates samples of 6×10^4 cells (BxPC-3, MIA PACA-2 and CHO-WT) were seeded in 12-well plates and initially cultured for 48 h. Cell total-RNA was extracted using Nucleospin RNA II (Macherey-Nagel, Duren, Germany) with on-column DNA digestion, according to manufacturer's instructions. TRIzol LS (Life Technologies Corporation, Carlsbad, CA, USA) was used for the homogenization of the human tissue sample in a Mini-BeadBeater-1 (Biospec Products, Coventry, UK). The RNA-containing aqueous phase was isolated after Chloroform phase separation followed by an Isopropanol nucleic acid-precipitation step. The resulting RNA pellet was further processed with the Nucleospin RNA II kit as above. RNA eluates' concentration and purity were determined with NanoDrop 2000 (Thermoscientific, Open Biosystems, Lafayette, CO, USA).

cDNA synthesis. Complete cDNA libraries were synthesised using Transcriptor first strand cDNA synthesis kit (Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK). A total of 250 ng RNA template from each sample was used in a 10 μL reaction and was reverse transcribed in the iCycler™ Thermal Cycler (Bio-Rad, Philadelphia, PA, USA). Samples were stored at –20˚C for later use.

rtPCR-Absolute quantification. Specific rtPCR primers for OATP1A2, 1B1 and 1B3 cDNAs, were custom-designed using the primer-BLAST (NCBI) on-line tool (21) as follows: For OATP1A2, primers were designed accordingly to enable targeting of both transcript variants (NM_134431 and NM_021094) forward primer 5'- GGGGCATGCAGGATATATGA-3' and reverse primer 3'-TGGAACAAAGCTTGATCCTCTTA-5'. For OATP1B1 (NM_006446) forward primer 5'-ACTGATTCTCGATGGGTTGG-3' and reverse primer 3'- TTTCCAGCACATGCAAAGAC-5'. For OATP1B3 (NM_019844) forward primer 5'-TAATTTGGAC ATGCAAGACAATG-3' and reverse primer 3'-AGTGAAAGA CCAGGAACACCTC-5'. Final working concentrations for each primer pair were set to 0.25 μM, 0.15 μM and 0.25 μM respectively for the three transporters. Standard curves were provided using homologous standards and results were calculated as transcripts (copies) number. Using "LightCycler® 480 SybrGreen I Master" (Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK), a total of 20 ng cDNA template per 10 μL reaction, per sample was used for determining endogenous gene expression. All reactions were carried out in LightCycler® 480 (Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK). Cycling conditions for the real-time quantification of target cDNAs were as follows: A total of 40 amplification cycles, each comprising of a 10 seconds denaturation, annealing and elongation sub-steps; set at 95˚C, 62˚C and 72˚C respectively. Amplification was preceded by a pre-incubation, FastStart Taq DNA polymerase activation and DNA denaturation step, set at 95˚C for a total of 10 minutes. At the end of each assay, product identification was confirmed by melting-curve analysis. For this step, samples were heated at 95˚C and 65˚C for 5 seconds and 60 seconds respectively, followed by a continuous 97˚C sub-step. The endogenous expression of OATP1A2, 1B1 and 1B3 in human liver was used as a positive control.

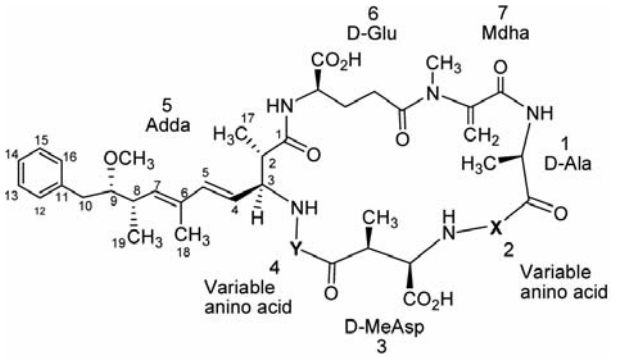

Figure 1. *The chemical structure of Microcystins. Where X and Y positions, resemble variable L-amino acids.*

Western blot analysis. Cells were cultured for 48 h in 6-well plates under normal culturing conditions as described above. Total cell protein extraction and isolation was carried out using TRIzol LS (Ambion® life Technologies™ ,ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions and was eluted in freshly-prepared 1% SDS solution. Equal amounts of total cell lysates (20-60 μg) were incubated in sample buffer for 30 min at 37˚ C and separated by SDS-PAGE (8% separating gels). Proteins were then transferred to nitrocellulose membranes (Hybond-C Extra membrane, Amersham Biosciences). and were blocked for 2 h in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) and 5% non-fat milk at room temperature. A further 2 hour incubation at room temperature followed with primary antibodies: a) mMDQ monoclonal mouse IgG1 1:100 (PROGEN Boitechnik, Heidelberg Germany) targeting 1B1/1B3 as follows: MDQHQHLNKTAES ASSEKKKTRRC for 1B3 (N-terminus) and MDQNQHLNKTAEA QPSENKKTRYC for 1B1 (N-terminus) and b) monoclonal mouse anti-β-actin 1:5000 (Sigma-Aldrich, St Louis, MO, USA), Both antibodies were diluted in TBS-T, 5 % non-fat milk. Goat anti-mouse IgG HRP-conjugated 1:2,000 (Santa Cruz Biotechnology

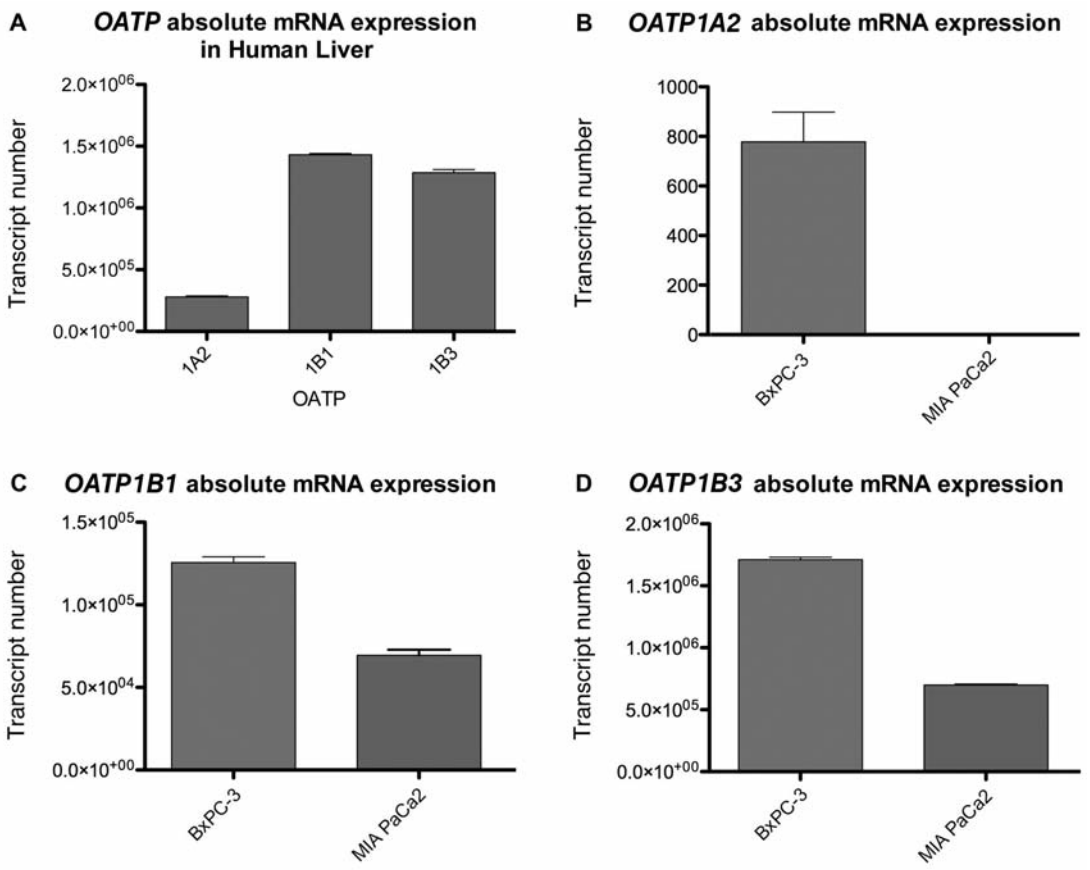

Figure 2. *Absolute quantification of OATPs mRNA in human liver tissue and cancer cell lines using RT-PCR. The expression was measured in absolute transcript number for each sample. (A) Human liver tissue, OATP- 1A2 2.79×10^5 (SEM 8x103), 1B1 1.43×10^6 (SEM 1×10^4), and 1B3 1.29×10^6 (SEM 2.5×10^4). (B) OATP1A2 in BxPC-3 was 7.78×102 (SEM 1.19×10^2) and undetectable for MIA PaCa2 (C) OATP1B1 in BxPC-3 was 1.26×10^5 (SEM 3.5×10^3) and 6.94×10^4 (SEM 3.4×10^3) in MIA PaCa-2. (D) OATP1B3 in BxPC-3 was 1.71×10^6 (SEM 21.3×10^4) and 7×10^5 (SEM 8.4×10^3) in MIA PaCa2 cells.*

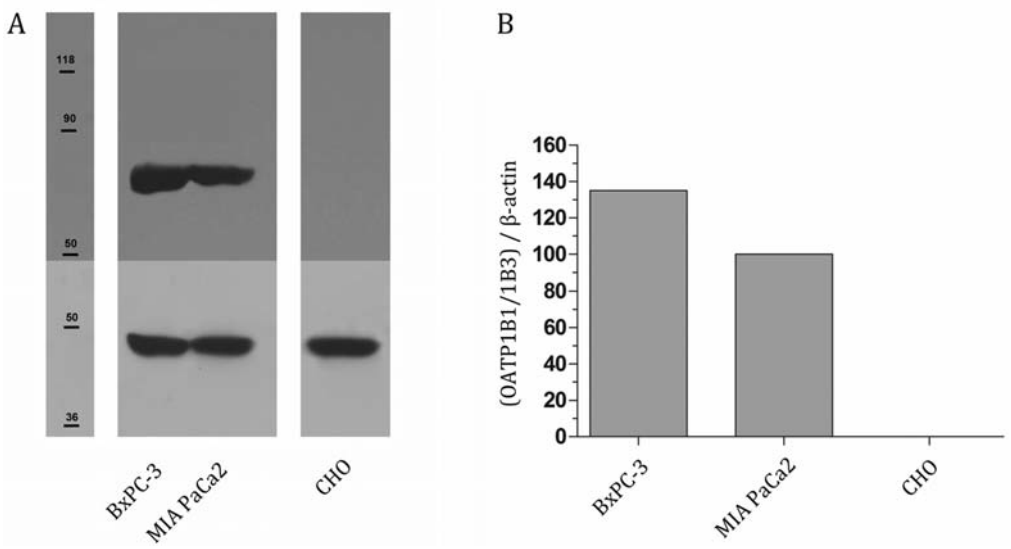

Figure 3. *(A) Protein expression analysis for OATP1B1/1B3 using western blot and (B) densitometric analysis of OATP1B1/1B3 in BxPC-3, MIA PaCa2 and CHO cell lines.*

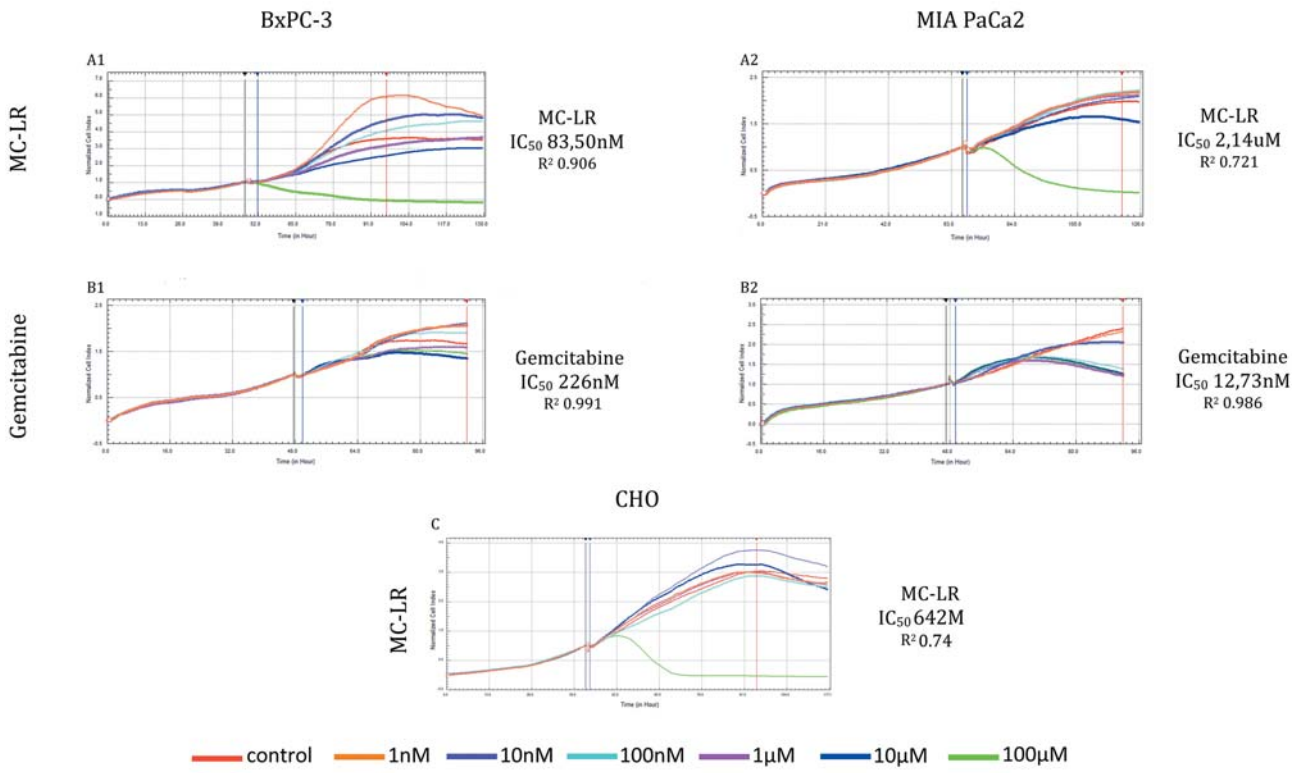

Figure 4. *Effects on cell growth and viability of microcystin-LR and gemcitabine on BxPC-3, MIA PaCa2 and CHO cells, as these were assessed by xCELLigence in real time observation. The toxicity was measured as an IC_{50} value (A1) and (A2) microcystin-LR (B1) and (B2) gemcitabine (C) microcystin-LR toxicity on CHO cell line.*

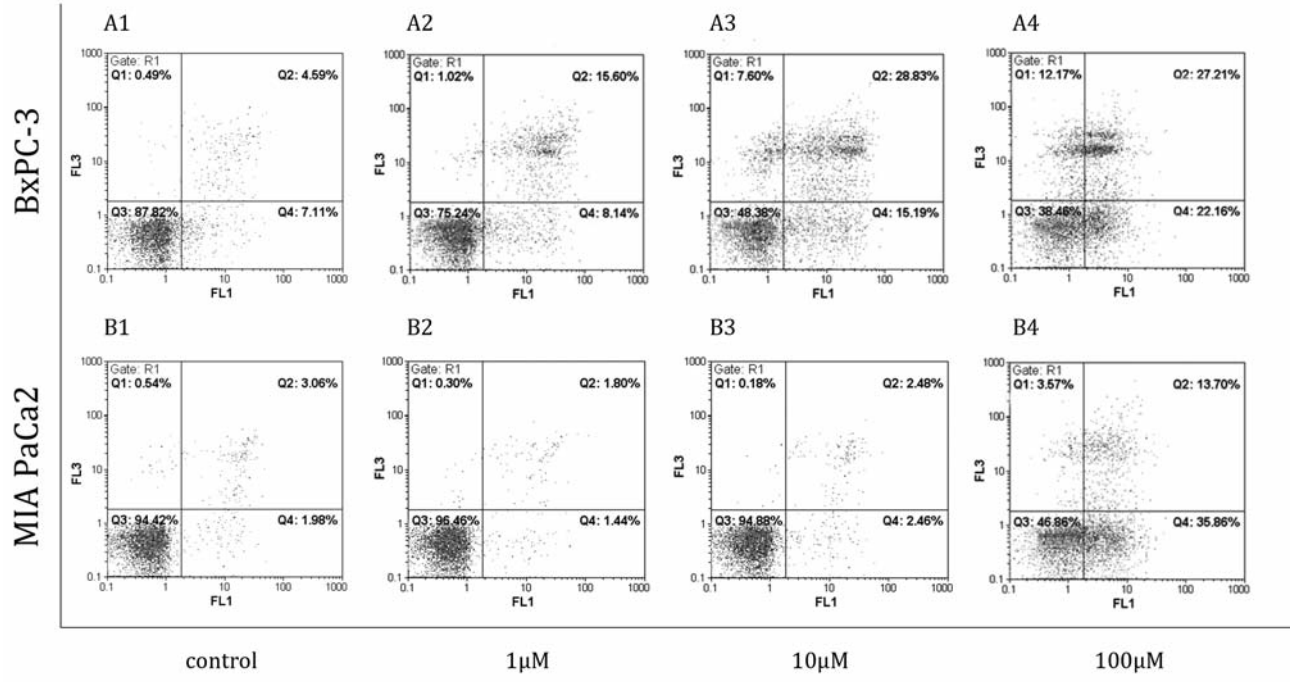

Figure 5. *Flow cytometric analysis (Annexin V/PI method) of the toxic effect of Microcystin-LR on BxPC-3 and MIA PaCa2 cell lines.*

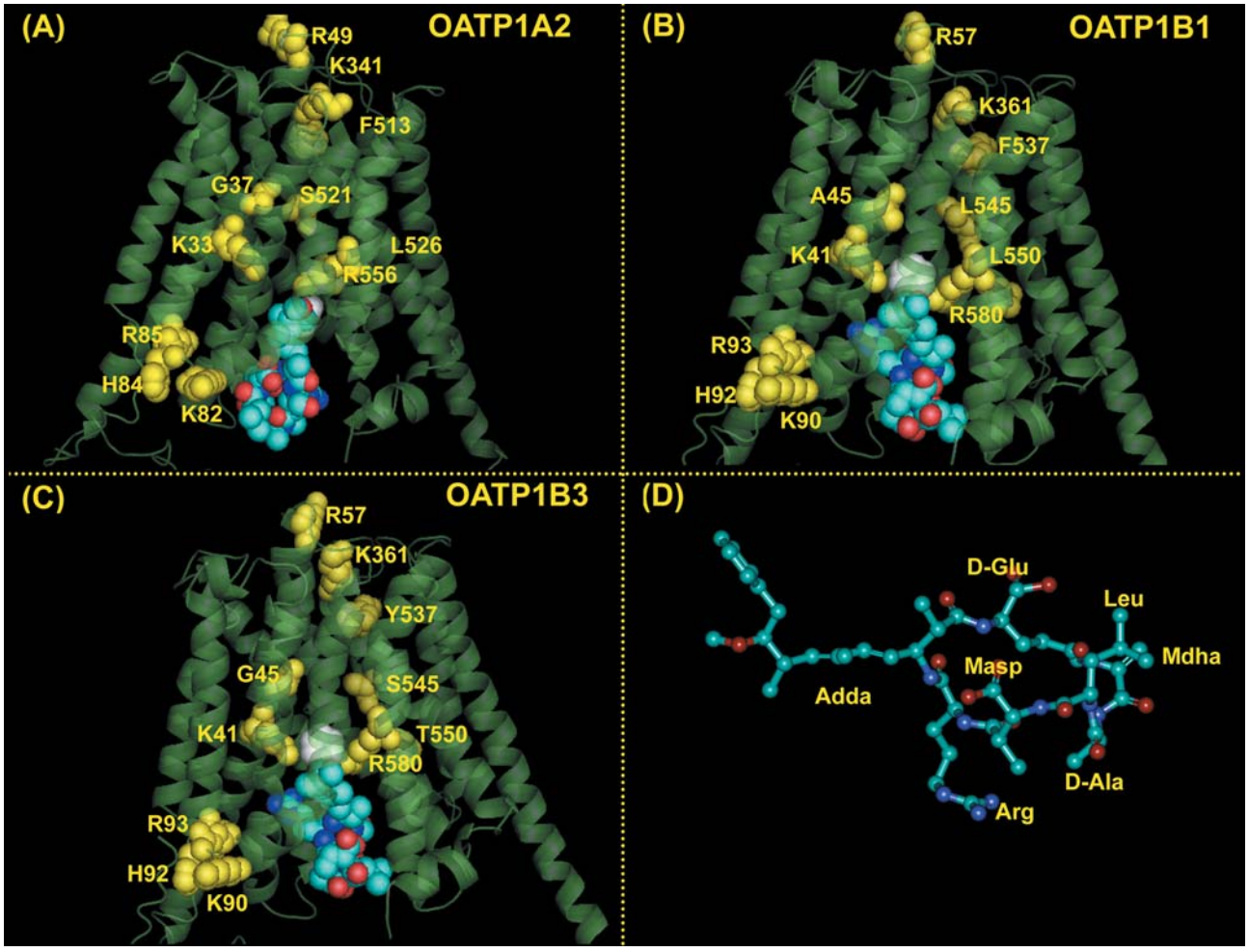

Figure 6. *3-D models of the structures of the complexes of (A) OATP1A2 – MC-LR, (B) OATP1B1 – MC-LR and (C) OATP1B3 – MC-LR. Transporters are shown as ribbons. Residues that have been highlighted from literature to be important for substrate binding and/or translocation pathways (see text) are highlighted in yellow. (D) The 3D structure of MC-LR, retrieved from the X-ray structure (53), is shown in sticks: carbon atoms are colored in cyan, oxygen in red and nitrogen in blue.*

Inc., Dallas, TX, USA) was used as secondary antibody, diluted in TBS-T, 5 % non-fat milk and applied for 1 h at room temperature. Protein identified epitopes, were visualized on X-ray films using the ECL Detection System (GE Healthcare Life Sciences) according to manufacturer's specifications.

MC-LR and gemcitabine toxicity assay. Microcystin-LR (Enzo® Lifesciences, Farmingdale, NY, USA) and Gemcitabine-HCl (Sigma-Anldrich, St Louis, MO, USA) were evaluated for their effect on cell growth, proliferation, morphology and viability on BxPC-3 and MIA PACA-2 cell lines using the xCELLigence RTCA DP system (ACEA biosciences Inc., San Diego, CA, USA), which enables real-time observation. CHO cells, known not to express the OATPs under study neither at gene nor at protein level, were used as the control. IC_{50} values were calculated with RTCA software (ACEA Biosciences Inc., San Diego, CA, USA) based on the normalized dose response curves (DRC) for a time period of 48h post exposure. A total of $5x10^3$ cells per well were seeded in xCELLigence E-plates 16 (Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK) and cultured for 48h prior compound exposure MC-LR and gemcitabine- HCl were used at 1 nM –100 μM on logarithmic scale concentrations respectively. All samples were run in duplicates.

Evaluation of apoptosis. One hundred thousand cells were cultured for 48 h in 24-well plates in complete culture medium (as described earlier in this section) before they were exposed to the following concentrations of the two agents under study: i) 1 μM, 10 μM and 100 μM of MC-LR and ii) 10 nM, 100 nM and 100 μM of Gemcitabine HCl. At the end of the exposure period, cells were collected and washed with serum-containing media followed by an additional cold PBS (Invitrogen, Carlsbad, CA, USA) washing step. Cell pellets were then resuspended in 1x FITC Annexin V Binding Buffer (BD Pharmigen™, San Jose, CA, USA) at a final concentration of $1×10^6$ cells/mL. A 100-μL aliquot ($1×10^5$ cells) of each sample was transferred to a new 5 mL culture tube and 5 μL of

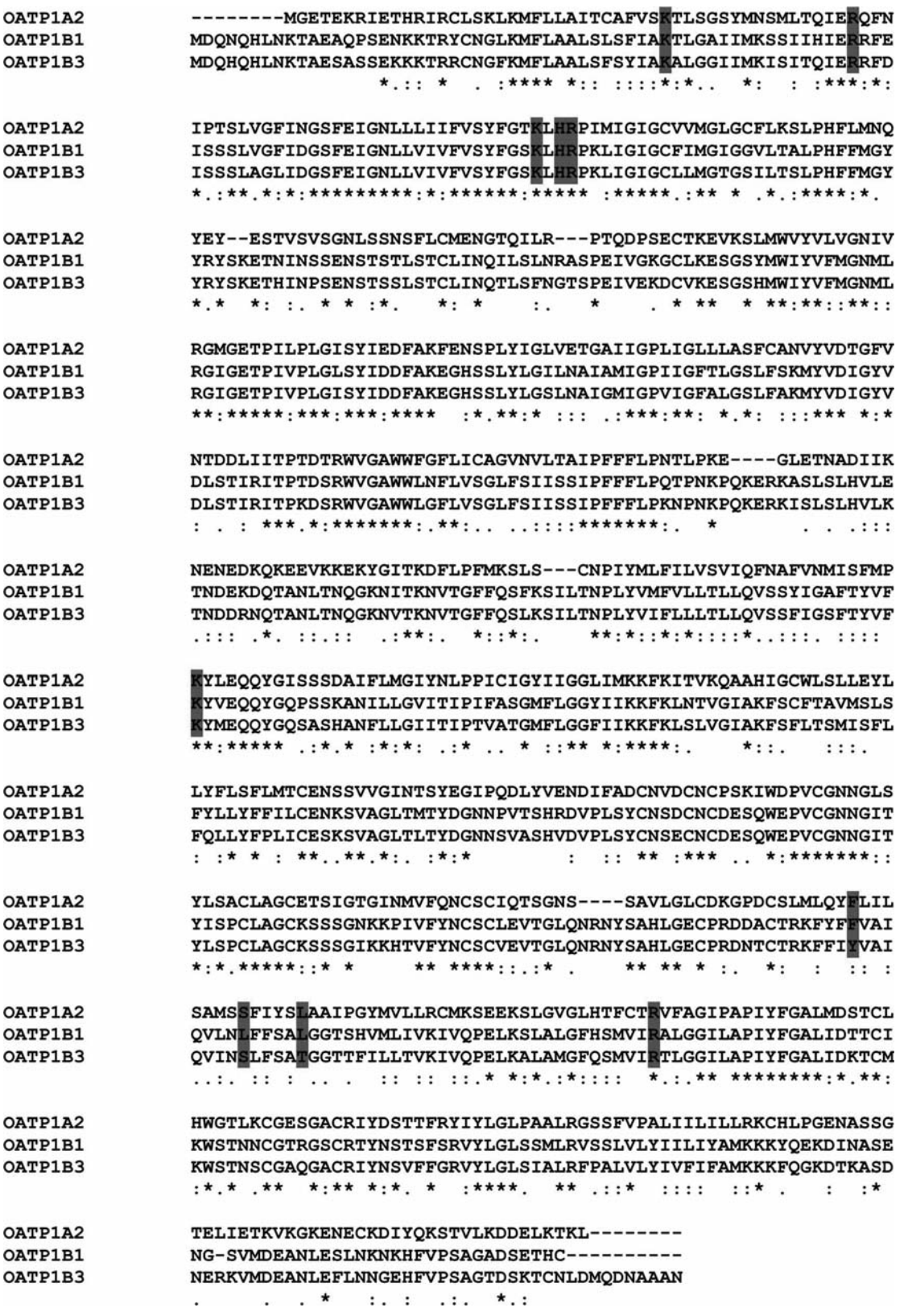

Figure 7. *Sequence alignment of OATP1A2, OATP1B1 and OATP1B3. Residues characterized as important from the literature (see text) in transport activity and/or binding capacity are highlighted.*

each: Annexin V (BD Pharmigen™, San Jose, CA, USA) and PI (Invitrogen, Carlsbad, CA, USA), (final concentration 50 μg/mL) were added and incubated at room temperature for 15 min in dark. 1x FITC Annexin V Binding Buffer was added to each sample to a final volume of 1 mL Flow cytometric analysis for FITC Annexin V binding and PI staining (10,000 events per sample) was performed on a Partec ML flow cytometer (CyFlow®ML, Partec, Munster, Germany) equipped with an argon laser, using the FL1 and FL3 detection channels respectively. Viable Cell Fraction at 48h (VCF48) is defined as the sum of Annexin V and PI (Propidium Iodide) negative events, and is given as a percentage of the total cell population (viable or no measurable apoptosis) (22). Data were analyzed using the FloMax software.

Structural modeling and docking. Structural templates for homology modeling were detected using the protein structure prediction server at the BioInfoBank Institute (23). The individual prediction methods combined by the server consistently predicted that the glycerol-3-phosphate transporter (24), share a significant structural similarity to OATP1A2, OATP1B1 and OATP1B3. Homology modeling was performed for the three transporters based on this template using Modeller 9.11 (25). Due to the limited sequence homology of the loop regions, modeling was restricted to the transmembrane helices. The 3-dimensional structure of microcystin-LR was obtained from the Protein Data Bank (26). The molecular docking of MC-LR to OATP1A2, OATP1B1 and OATP1B3 was conducted using AutoDock Vina,

Statistics. GraphPad Prism version 5 was used for graph plotting and data analysis. The compounds were sorted by binding affinity and visually analyzed using PyMOL software v1.3 (27).

Results

Quantification of OATPs mRNA in pancreatic cell lines. The transcript expression was very minimal for OATP1A2, fair for OATP1B1 and predominant for OATP-1B3 expression in cell lines (Figure 2). The OATP1B1 expression in BxPC-3 was 1.82-fold higher to that of MIA PaCa-2. The human liver tissue was found to have the most prominent expression with regards to OATP1B1, 11.35-fold when compared to BxPC3 cells (Figure 2). The OATP1B3 mRNA in BxPC-3 cells was found to be 2.44-fold greater than its expression in MIA PaCa2 cells and 33.1% stronger when compared to the native transporter expression level occurring in human liver (Figure 2).

OATP protein expression. The expression was stronger in BxPC-3 (Figure 3A). Further densitometric analysis showed that the protein expression was 38 % higher in BxPC-3 when compared to that of MIA PACA-2 cells (Figure 3B).

MC-LR and gemcitabine toxicity assessment. Microcystin-LR with an IC_{50} of 83.50 nM (R^2=0.906) appeared to be 25.63-fold more efficient on inhibiting cell proliferation in BxPC-3 compared to MIA PaCa2 cells (IC_{50}=2.14 μM, R^2=0.721) (Figure 4A1 and A2). Furthermore, MC-LR presented to have a 2.71-fold greater stronger inhibitory effect on BxPC-3 compared to gemcitabine (IC_{50}=226 nM; R^2=0.991) (Figures 4A1 and 4B1). In contrast, gemcitabine (IC_{50} 12.73 nM; R^2=0.986) was found to be 168.11-fold more toxic than MC-LR for MIA PaCa2 (Figures 4A2 and 4B2). As expected, MC-LR had no toxic effect on the negative control cell line CHO with an IC_{50} of 642M (R^2=0.740).

Flow cytometry. Baseline VCF48 values for the control-untreated BxPC-3 and MIA PaCa2 samples, were 87,82% and 94,42% respectively (Figure 5A1 and B1).

Cytotoxicity. Microcystin-LR treatment showed a dose-dependent, apoptotic effect for BxPC-3 cells. The (Q3) VCF48 decreased with increasing toxin concentrations. BxPC-3 VCF48 was 75.24%, 48.38% and 38.46% for 1μM, 10 μM and 100 μM of MC-LR respectively (Figure 5A). MIA PaCa2 showed toxic response to MC-LR only at the highest 100 μM concentration with VCF_{48} 46.86%. The 13.70% and 35.86% of the cell population of MIA PaCa-2 treated with 100 μM MC-LR were found to have entered early and late apoptosis respectively (Figure 5),

Structure modeling and docking. It has been previously demonstrated that the highly conserved positively charged amino acids Lys41 and Arg580 are pivotal to the transport activity of OATP1B3 (28). In another study the extracellular and intracellular conserved amino acids for OATP1B1 had been assigned as important for the mediated transport of various model substrates (29). The relevant conserved residues are highlighted in the 3D models of the 3 receptors in Figure 6. A sequence alignment of the 3 OATPs is demonstrated in Figure 7, where the amino acids that have been determined as important in transport/substrate binding are highlighted.

Discussion

Currently no optimal therapy exists or targeted therapy has been developed for pancreatic cancer. Following the identification of OATP 1B1 and 1B3 as potential targets in pancreatic cancer, we herein demonstrated that OATP1B1 and 1B3 targeting MC-LR, can cause inhibition of proliferation of pancreatic cancer cells in a dose-response mode. We found anti-proliferative and pro-apoptotic effects to be proportionally related to the expression of these transporters suggesting an essential role for OATP expression in the process of MC-LR induced cancer cell damage. Moreover, direct comparison of the inhibitory effect of MC-LR and gemcitabine, has shown a noticeable advantage of the former.

Eriksson *et al*., were the first to suggest that MC-LR uptake required a transporting system to be carried intracellularly across the cell membrane (30). We consider that

overexpression of OATPs in pancreatic cancer (5) offers an opportunity to develop novel targeted agents against pancreatic cancer. Development of OATP targeting compounds that would be based on the chemical structure of MC-LR, which has some unique physicochemical properties such as high water-solubility, resistance to chemical hydrolysis or oxidation at near-neutral pH and its stability in pH alterations, appears to be a feasible and promising option in this direction (31, 32). Challenges of potential organ-specific toxicity remain to be resolved by proper chemical modifications.

Conclusion

The lack of any effective treatment for pancreatic cancer makes the discovery of novel targeted-therapies a priority in this field of cancer research. Cyanotoxins is a rich source of naturally-occurring bioactive compounds with potential cytostatic effects making them potential candidates for further drug design. Our results indicate that MC-LR deserves further investigation as a model molecule for the development of novel targeted cancer therapeutics with activity against pancreatic cancer and possibly other tumors that express specific OATPs.

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