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Opioid tripeptides hybridized with *trans*-1-cinnamylpiperazine as proliferation inhibitors of pancreatic cancer cells in two- and three-dimensional in vitro models

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Supporting information for this article is given via a link at the end of the document.

Abstract: According to the World Health Organization, mortality rate among patients with pancreatic cancer will increase in the upcoming years. Gemcitabine is the first choice for treatment of pancreatic malignancy, but rising resistance to this drug decreases the final outcome. Studies on new therapies targeting metabolic pathways, growth factors inhibitors and tumour stroma or tumour stem cells, are currently underway in many laboratories. Here, we report the bioactive properties (cytotoxicity and haemolytic activity) of synthetic peptidomimetics containing opioid tripeptide fragment (Tyr-R^1-R^2-; where R^1 is D-Ala or D-Thr, and R^2 is Phe or Trp) hybridized with *trans*-1-cinnamylpiperazine. These compounds are stable in plasma up to 96 h and exhibit low haemotoxicity and good inhibitory effect on pancreatic cancer cells growth in two- and three-dimensional in vitro models of pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant disease with a very poor prognosis. Originating from epithelial cells of pancreatic ducts, it is the most common type of neoplasm in the pancreas (over 90%) and the seventh leading cause of cancer death worldwide. Early diagnosis is difficult because of non-specific symptoms and most patients remain asymptomatic until advanced stage of the disease. Currently, the most effective treatment is surgical resection. However, due to late diagnosis, only ~20% of patients qualify for surgery.[1] Current chemotherapy consisting of gemcitabine (GEM) or FOLFIRINOX is not very effective and often burdened with undesirable side effects.[2] Constantly growing occurrence of cancer cells natural resistance to chemotherapy, mostly GEM, is also one of the major challenges in cancer treatment. Therefore, there is an urgent need for therapies based on novel anticancer compounds or targeting specific aspects of cancer biology. Peptide-based therapy is one of the possible options. Peptide drugs are predominantly characterized by high specificity and low toxicity, which is a result of very tight binding to their target receptors. The main disadvantage of using peptides as therapeutic agents is their low stability against proteolysis, resulting in a short duration of in vivo activity. A routine practice aimed at reducing this drawback is a design and synthesis of peptidomimetics possessing not only a good activity but also having higher stability in physiological fluids (e.g. plasma) than native peptides.[3,4,5]

It has been shown that compounds with analgesic properties may also have antitumour activity, e.g. Met-enkephalin, one of the endogenous opioid peptides, regulates cell cycle and could inhibit growth of pancreatic cancer cells in vitro and in vivo.[6] Improved selectivity and activity of peptides toward cancer, might be achieved by modification of compound structure using lipophilic moieties. Researchers showed that the linkage or incorporation of lipophilic units to different neuropeptides, including Met-enkephalin, may induce significant inhibition of pancreatic carcinoma cells and increase enzymatic stability of molecule.[7,8] We also have found that one of the best opioid peptidomimetic named biphalin [(Tyr-D-Ala-Gly-Phe-NH)$_2$], in addition to analgesic activity, possess cytotoxic activity against selected tumour cells.[9] Additionally, it has been shown in literature that cinnamic acid derivatives exhibit anticancer properties[10] and may increase membrane penetration.[11]

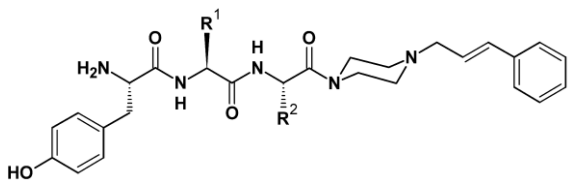


Figure 1. General structure of synthesized peptidomimetics (R^1 = side chain of D-Ala/D-Thr, R^2 = side chain of Phe/Trp).

Based on the above information we hypothesized that hybridization of opioid peptide fragment with cinnamic acid moiety may result in obtaining stabile peptidomimetics with high affinity to μ-opioid receptor (MOR) and antiproliferative effect on pancreatic carcinoma cells. Thus, we prepared a series of compounds based on opioid peptidomimetics described by Lipkowski[12], where peptide fragments are hybridized with cinnamic acid via piperazine linker. Here, we present the syntheses and antitumour activity of peptidomimetics which are analogs of Tyr-R^1-R^2-*trans*-1-cinnamylpiperazine, where R^1 is D-Ala/D-Thr, R^2 is Phe/Trp, which structures are shown in Figure 1.

Results and Discussion

Peptidomimetics synthesis and analysis

Peptide fragments were prepared manually using SPPS methodology following the Fmoc chemistry. 2-Chlorotrityl chloride resin and HCTU/DIPEA protocol were used. Peptides were cleaved from the peptidyl-resin by AcOH/TFE/DCM (1:1:8; v/v/v) mixture to obtain fully protected peptides (all functional groups beside C-terminal carboxy group were protected). The last step of the synthesis consisted of coupling (in solution) of *trans*-1-cinnamylpiperazine with the use of TBTU/HOBt. Crude compounds were purified by RP-HPLC. The isolated pure peptidomimetics were characterized using HPLC-ESI-Q-MS and LCMS-IT-TOF (see Experimental Section and Table S1, S2 in the Supporting Information). The scheme of the peptidomimetics synthesis is presented below (Scheme 1).

Expression of MOR on tested cell lines and peptidomimetics binding affinity to MOR

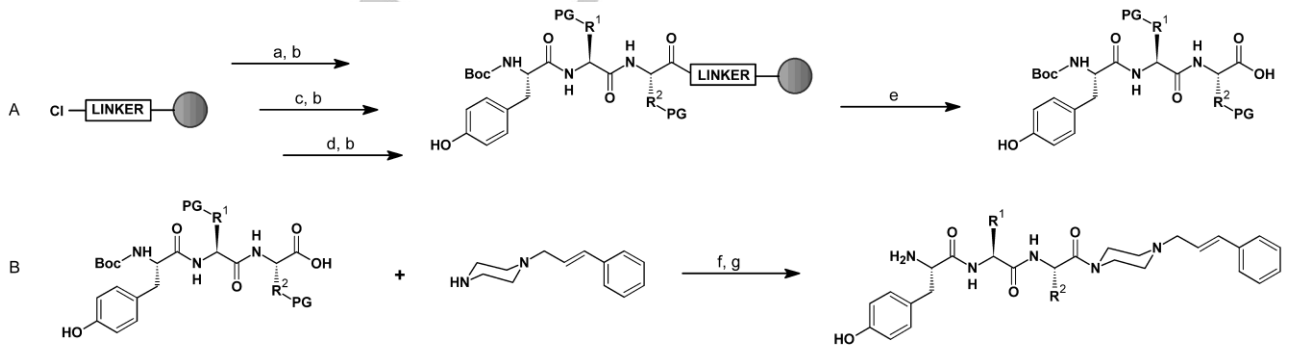
Expression of opioid receptors on the surface of examined cells was carried out with cell-based ELISA. Normal fibroblast (BJ) and three cancer cell lines (PANC-1, MMRC-DS1, MMRC-SS1r) were tested. All cells expressed MOR on their surface at different level (see Table S6 in the Supporting Information). Among tested cells, isolated cells MMRC-DS1 and MMRC-SS1r showed the highest expression of MOR. MOR expression was low for BJ and PANC-1 cells.

Table 1. Binding affinity of synthesized peptidomimetics at MOR.

Compound	Sequence	IC_{50} (nM)[a]
1	H-Tyr-D-Ala-Phe-Cyn	15.1±0.5
2	H-Tyr-D-Ala-Trp-Cyn	77.6±2.8
3	H-Tyr-D-Thr-Phe-Cyn	12.9±0.4
4	H-Tyr-D-Thr-Trp-Cyn	19.5±0.7
5	H-Tyr-D-Ala-Phe-OH	>1000
6	H-Tyr-D-Ala-Trp-OH	977±35.2
7	H-Tyr-D-Thr-Phe-OH	616±14.1
8	H-Tyr-D-Thr-Trp-OH	1000±20.0
Biphalin	(H-Tyr-D-Ala-Gly-Phe-NH)$_2$	1.4±0.7[b]

[a] Displacement of [^{3}H]DAMGO. Cyn = *trans*-1-cinnamilpiperazine. Results are the mean±SEM of three independent experiments carried out in duplicate. [b] Data taken from reference 13.

The binding affinity of peptidomimetics for MORs was determined by the radioreceptor-binding assay and compared to biphalin, a dimeric peptide, which displays a strong affinity for opioid receptors.[13] All peptidomimetics presented good affinity to MOR. Compounds **1**, **3** and **4** showed reasonable high affinity while affinity of analogue **2** was slightly lower. In comparison, binding affinity of **5**-**8**, which are tripeptides without *trans*-1-cinnamyl piperazine (Cyn), was significantly low. The exact data of particular compounds affinities are presented in Table 1. Obtained results show that tripeptide hybridization with *trans*-1-cinnamyl piperazine greatly improves binding affinity to MOR.



R^1 = side chain of D-Ala or D-Thr
R^2 = side chain of Phe or Trp
PG = protecting group

Scheme 1. Synthetic route of peptidomimetics: A) on solid support and B) in solution. Reagents and conditions: a) 2.5 eq Fmoc-Phe-OH or Fmoc-Trp(tBu)-OH, 2.5 eq HCTU, 6 eq DIPEA in DMF, RT, 3 h; b) 20% piperidine in DMF, RT, 20 min; c) 2.5 eq Fmoc-D-Ala-OH or Fmoc-D -Thr(tBu)-OH, 2.5 eq HCTU, 6 eq DIPEA in DMF, RT, 3 h; d) 2.5 eq Boc-Tyr-OH, 2.5 eq HCTU, 6 eq DIPEA in DMF, RT, 3 h; e) AcOH, TFE, DCM (1:1:8 v/v/v), RT, 45 min; f) 1.1 eq TBTU, 1.1 eq HOBt, 5 eq DIPEA in DMF, RT, 12 h; g) TFA, RT, 1h, 46-53%.

The results of tripeptides affinity to MOR was a little surprise for us, because the sequences we used designing our peptidomimetics (Tyr-D-Ala-Phe- or Tyr-D-Ala-Trp-) fulfil typically opioid rules. In the interaction of peptide ligand with opioid receptors N-terminal Tyr is necessary and additionally aromatic amino acid residue in 3 or 4 position is beneficial. In our case only tripeptides hybridized with Cyn moiety possess good affinity to MOR, what suggest that tripeptides are probably too short and the free amino acid residue in position 3 (Phe or Trp) is not a major factor in binding with MOR. The results of affinity of our peptidomimetics indicate that in the bioactive conformation in MOR some interaction of the hybridized fragment of cinnamyl piperazine is involved in the interaction e.g. aromatic ring or nitrogen atom (from piperazine or amide bond).

Peptidomimetics stability in human plasma and haemolytic activity

A stability test of the obtained compounds in human plasma was carried out by monitoring chromatograms changes during 96 h, in a time-course incubation at realistic temperature conditions (37 °C). Analysis was performed with RP-HPLC and HPLC-ESI-Q-MS methods. RP-HPLC analysis is directly quantitative with a UV detector, while mass spectrometry of the degradation products provides data of molecule cleavage sites.

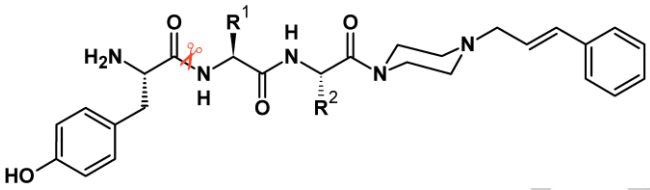


Figure 2. Enzyme cleavage site found in compounds **1** and **2**.

The obtained results have shown that peptidomimetics **3** and **4** were very stable in applied conditions; we observed no degradation at all after 96 h. In case of **1** and **2**, tyrosine was cleaved in minor manner (Figure 2), but we only observed MS signal from truncated peptidomimetics, whereas no signals from tyrosine itself was present on UV and MS chromatograms.

We assume that due to the complicated matrix (plasma), it did not bind to stationary phase and was flushed off the column with several other natural metabolites, hindering its signal on MS detector. Stability results obtained in this experiment suggest that bond between Tyr and D-Ala may be less resistant to enzymatic digestion compared to those between Tyr and D-Thr. Chromatograms and analytical data of degradation products can be found in Supporting Information.

In addition to stability, haemolytic activity of peptidomimetics was evaluated according to Knopik-Skrocka and Bielawski.[14] It was observed that up to concentration of 250 μM, all compounds showed very low haemotoxicity. The level of the degradation of red blood cells was below 3% and was comparable to haemotoxicity induced by GEM (Table 2). According to ASTM E2524-08 guideline, haemolysis < 5% indicates that the test compound does not cause damage to red blood cells.[15]

Table 2. The haemolytic activity of peptidomimetics and the reference drug.

	Haemolysis [%][a]		
Compound	**0 μM**	**100 μM**	**250 μM**
1	0.8±0.1	2.2±0.0	2.4±0.1
2	1.1±0.0	1.7±0.0	2.4±0.0
3	1.2±0.1	1.8±0.1	1.8±0.1
4	0.8±0.1	1.0±0.1	0.9±0.0
GEM	1.9±0.1	1.4±0.1	1.7±0.2

[a] Values are percentage of total haemolysis induced by compounds in several concentrations with respect to control (red blood cells in PBS). The data are expressed as mean±SEM of 3 independent experiments performed in triplicate.

Effect of peptidomimetics on viability of pancreatic cancer cells in vitro (2D model)

Cytotoxicity of peptidomimetics was evaluated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) after 24 and 72 h of incubation. Effect of peptidomimetics on cell viability was concentration-dependent and varied between compounds.

Table 3. Effect of peptidomimetics on cell viability tested on various cell lines after 24 h incubation presented as half maximal effective concentration (EC_{50}).

	EC_{50} [μM][a]			
Compound	**BJ**	**PANC-1**	**MMRC-DS1**	**MMRC-SS1r**
1	81.7±4.0	91.8±2.2	77.8±3.6	154.7±8.7
2	135.6±2.3	175.4±6.4	106.8±9.8	83.9±4.0
3	130.2±2.9	312.7±9.3	241.6±4.1	230.6±5.3
4	221.4±7.5	243.4±2.7	252.9±4.3	188.1±7.8
5	>500	321.1±9.9	>500	>500
6	>500	311.8±9.3	>500	>500
7	>500	>500	>500	>500
8	>500	>500	>500	>500
Cyn	>500	>500	>500	>500
biphalin	>500	>500	>500	>500
GEM	>500	>500	>500	>500

[a] Values are the concentrations of peptidomimetics required to inhibit 50% of cell growth with respect to control (untreated cells), measured with MTS assay after 24 h drug exposure. The data are expressed as mean±SEM of 3 independent experiments performed in triplicate.

Isolated cells MMRC-DS1 and MMRC-SS1r showed higher susceptibility whereas PANC-1 and BJ cells were more resistant

to peptidomimetics. The most interesting compound from synthesized peptidomimetics is **2,** which showed high bioactivity and was statistically significantly less toxic to normal fibroblasts (BJ) than to isolated carcinoma cells. Peptidomimetic **3** significantly decreased cell viability but was more toxic to BJ fibroblasts than to the cancer cells. Compounds **1** and **4** showed similar activity towards both normal fibroblasts and the carcinoma cells, except for MMRC-DS1 and MMRC-SS1r respectively. All peptidomimetics were active after 24 h of incubation (Table 3). Tripeptides (**5**-**8**), Cyn and biphalin did not significantly decrease cell viability, except peptides **5** and **6** for PANC-1 cells. The effect of biphalin on tested cells was similar to results obtained for human glioblastoma T98G cells.[9] Low activity of GEM after 24 h incubation may be attributed to its mechanism of action, interference in S phase of cell cycle.[16,17] In cell culture, effectiveness of GEM increases with post-addition time and its uptake depends on human nucleoside transporters.[17,18] In comparison, the reference drug (GEM) showed higher activity after 72 h of incubation (see Table S5 in the Supporting Information).

Effect of peptidomimetics on viability of pancreatic cancer spheroids (3D model)

Since 2D model is not mirroring cell-to-cell interaction, 3D models were developed to better understand cells interactions, proliferation, differentiation with delivery and distribution of tested compounds within tissue in in vitro conditions.[19] The sphere-forming ability of cell culture depends on the cell type. In our studies we were able to obtain spheroids only from isolated carcinoma cells MMRC-DS1 and MMRC-SS1r. Spheres were not obtained from BJ fibroblasts, while PANC-1 cells formed irregular aggregates (Figure 3).

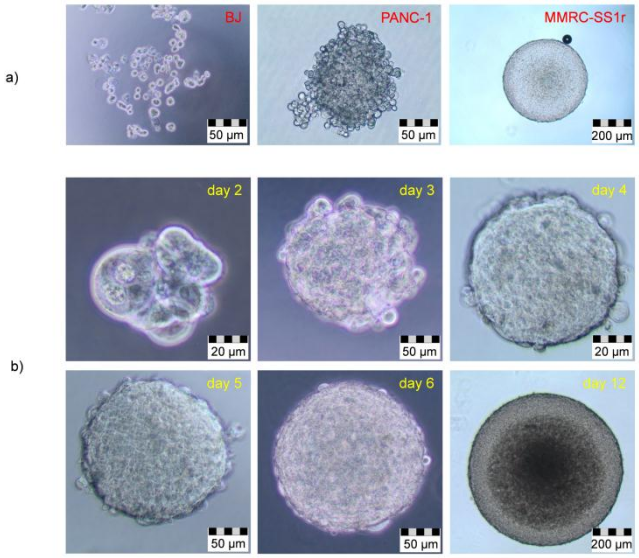


Figure 3. a) BJ aggregated cells, PANC-1 aggregate and MMRC-SS1r 5-days old spheroid; b) formation and growth of MMRC-DS1 spheroid.

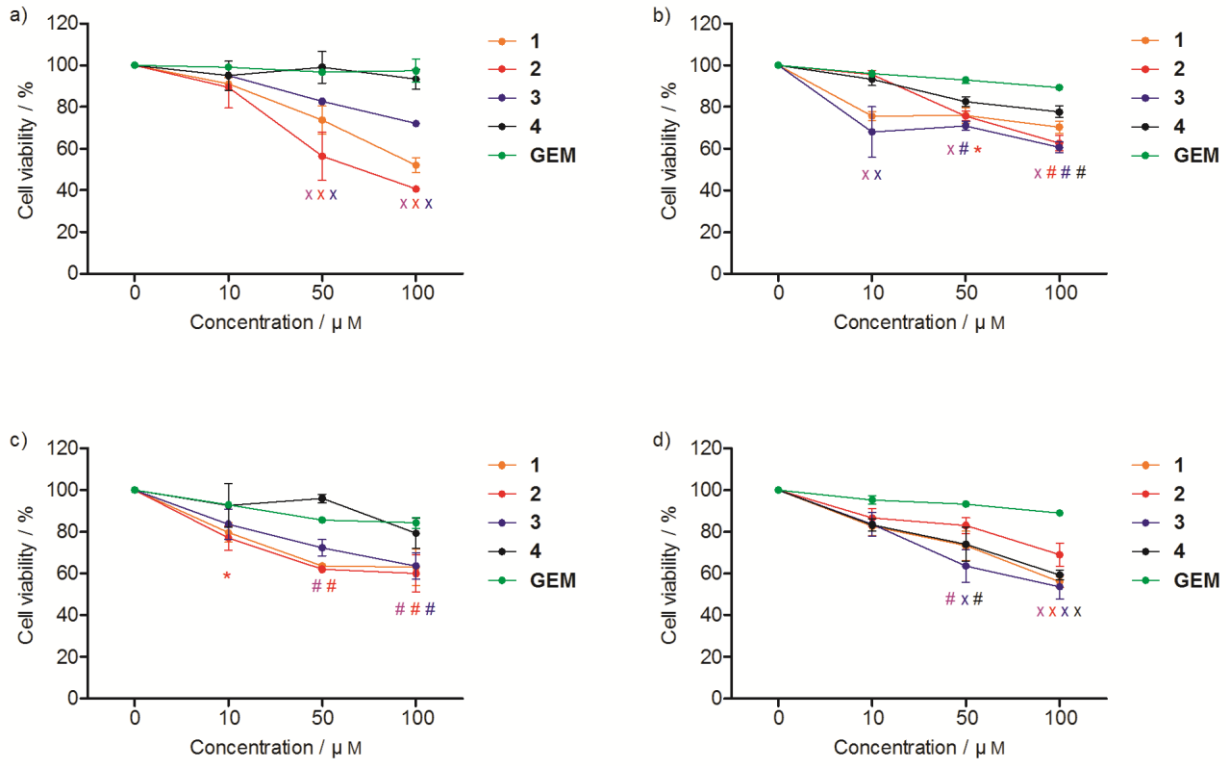


Figure 4. Viability of a) MMRC-DS1 after 24 h, b) MMRC-DS1 after 72 h, c) MMRC-SS1r after 24 and d) MMRC-SS1r after 72 h spheroids treatment with peptidomimetics and the reference drug (GEM) in various concentrations. The data are presented with reference to GEM; $p<0.05$ (*); $p<0.01$ (#); $p<0.001$ (x). The data are expressed as mean±SEM of 3 independent experiments, each performed in triplicate.

Spheroids were treated with peptidomimetics and the reference drug (GEM) for 24 and 72 h. Effect of peptidomimetics and GEM on the cell viability was similar for spheres obtained from both isolated cell lines. However, the activity of GEM was lower after both 24 and 72 h incubation periods in comparison to the activity of peptidomimetics. Low activity of reference drug can be the result of poor penetration abilities or cellular resistance to GEM. Penetration of GEM into tumour tissue is slow and concentration independent, but decreases with increasing thickness of the tumour cellular layers.[20]

In comparison to the effect of the reference drug, compounds **1**, **2** and **3** showed higher activity both after 24 and 72 h incubation. It was observed that after 72 h incubation, **1**-**3** lost some of their activity and **4** became significantly more active (Figure 4).It is possible, that the relative better effect of peptidomimetics on cells may be attributed to lipophilic part of peptidomimetics, namely *trans*-1-cinnamylpiperazine, or high affinity of the synthesized compounds to opioid receptors. It was suggested in literature that membrane permeability is crucial for cytostatic effects on tumour cell lines, thus increasing hydrophobicity and lipophilicity should be desired.[8,21] Cinnarizine, a drug that contain *trans*-1-cinnamylpiperazine conjugated with diphenylmethane, is an example of compound with high lipophilicity that can easily cross blood-brain barrier by diffusion. This would also explain the highest activity obtained for **1**, due to the hydrophobic aromatic moieties (cinnamic acid and phenylalanine) and alanine side chain.[22] The other possible mechanism of action of our peptidomimetics could be with the use of opioid system to enter cancer cells. Since all our compounds express good affinity to MOR, while all tested pancreatic cancer cells express opioid receptors, therefore studied peptidomimetics could be internalized into the cancer cells through these receptors. However, more experiments and data are required to determine the mechanism of action of the synthesized peptidomimetics.

2D and 3D models comparison

It was observed that cells sensitivity to therapeutic agents differs between 2D and 3D models. In comparison to 3D models, cells in 2D cultures are grown in abnormal microenvironment which may result in cells being less selective.[23,24] We observed differences in cellular response in 2D and 3D models after treatment with peptidomimetics **1**-**4** and GEM. For both MMRC-DS1 and MMRC-SS1r cells treated with compound **1** for 24 h, no significant difference in cell viability between 2D and 3D models was observed. However after 72 h incubation, spheroids (3D) showed higher resistance than 2D culture. Interestingly for compound **2** cellular response after 24 h incubation differed between cell lines. For MMRC-DS1 cell viability was similar in both models (Figure 5A), however for MMRC-SS1r cells sensitivity depended on compound concentration (Figure 5B). After 72 h of spheroids obtained from both lines treatment were more resistant in comparison to 2D culture (see Figure S16 and Figure S17 in the Supporting Information). After treatment with peptidomimetic **4** for 24 and 72 h, cells displayed similar response in 2D and 3D models. When treated with GEM for 24 and 72 h, MMRC-DS1 cells cultured in 3D model were more resistant than cell in 2D culture (Figure 5A). However, MMRC-SS1r cells growing in 2D model were more resistant than MMRC-SS1r spheroids after 24 h incubation with GEM (Figure 5B). Similar results were obtained for MMRC-SS1r treated with compound **3** for both 24 and 72 h and for MMRC-DS1 cells treated with **3** for 72 h (see Figure S16 and Figure S17 in the Supporting Information). Adcock *et al.* observed similar results for CAL27 cells where cells cultured in monolayer were more resistant to drugs than cells in 3D models.[25] These results are in opposite to observations reported by other research groups. Many studies indicates that cells in 3D culture are more resistant to chemotherapeutical agents in comparison to 2D model.[24,26] Loessner *et al.* reported that treatment with paclitaxel reduced cell viability by 80% in 2D culture and only by 40 - 60% in 3D model.[24] Higher resistance to therapeutical agents in 3D can be attributed to cell-to-cell interaction, poor diffusion of the agent through cellular layers and hypoxia, which can lead to activation of genes responsible for cell survival.[27] Higher drug susceptibility of spheroids comparing to monolayer culture may be attributed to higher proliferation rate in 3D model as some drugs require active cell proliferation.[26]

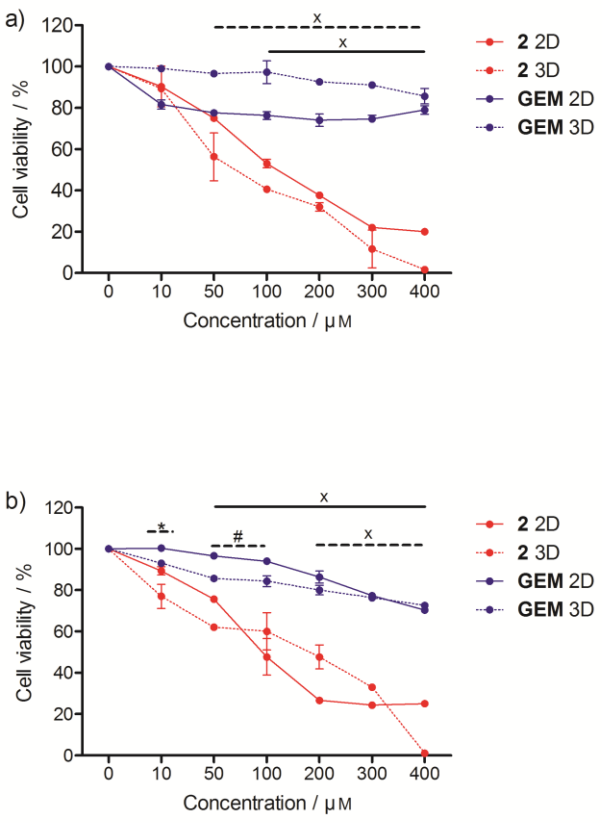


Figure 5. Viability of a) MMRC-DS1 and b) MMRC-SS1r cell cultured in 2D and 3D models after 24 h of treatment with APL2 and GEM in various concentrations. Data are presented with reference to GEM. Statistical significance for 2D model in presented as continuous line, for 3D model as dotted line; p<0.05 (*); p<0.01 (#); p<0.001 (x). The data are expressed as mean±SEM of 3 independent experiments, each performed in triplicate.

Conclusions

In summary we have designed and synthesized peptidomimetics which are composed of opioid tripeptides and lipophilic *trans*-1-

cinnamylpiperazine. Affinity experiments proved that our compounds bind effectively to MOR which are expressed in all tested cell lines. The lipophilic fragment of the peptidomimetics (*trans*-1-cinnamylpiperazine) may affect the binding affinity to MOR and increase penetration abilities of synthetized compounds. It has been showed that this moiety was already used for synthesis of bile acid derivatives, where obtained compounds had pro-apoptotic activity for various human cancer cells (GBM, KMS-11, HCT-116).[28] It has been reported that the lipophilicity of the anticancer compounds is suspected of correlation with increased biological activity and cytotoxicity.[29,30] Thus, this molecular property is important factor in the drug design process, due to the formation of additional binding sites in ligand-receptor complex. Obtained peptidomimetics were resistant to enzymatic hydrolysis in human plasma and displayed very low haemolytic activity. In 2D pancreatic cancer in vitro model, peptidomimetics showed higher antiproliferative activity than the reference drug (GEM) after 24 h incubation, while after 72 h treatment activity was similar for all studied compounds. In 3D pancreatic cancer in vitro model, the reference drug did not influence viability of spheroids. In contrast, three of our peptidomimetics (**1**-**3**) significantly decreased viability of spheroids after 24 and 72 h incubation. In case of **4**, its activity increased after 72 h of treatment. Higher bioactivity of peptidomimetics, compared to GEM, might be attributed to better penetration of tumour tissue in 3D in vitro model. There are hypothesis describing opioid ligands inhibitory effect on carcinoma cells. The effect of opioids on tumour growth is discrepant. Both growth-promoting and growth-inhibiting effects have been reported.[9,31,32,33] Activation of MOR may also lead to endocytosis and internalization of conjugated ligand displaying antiproliferative effect through different cell signalling pathways.[34] Beside MOR activation, antiproliferative effect may be induced by stimulation of opioid growth factor receptor (OGFr). It has been showed that Met-enkephalin interacts with OGFr and inhibits DNA synthesis by modulating cyclin-dependent inhibitory kinase (CKI) pathways. [35,36] To perform structure adjustment and understand the mechanism of action of peptidomimetics **1**-**4**, further studies are intended including apoptosis/necrosis, binding to other opioid receptors (DOR, KOR, OGFr) and conformation-activity relationships. Based on the obtained data, we conclude that our peptidomimetics could be promising candidates for further development as prospective anticancer drugs, but additional modifications to improve both activity and selectivity are necessary.

Experimental Section

Synthesis of peptidomimetics 1 - 4: The synthesis of peptides was carried out manually on 2-chlorotrityl chloride resin (500 mg, 0.83 mmol/g) by solid phase method following the Fmoc chemistry. Resin was loaded with Fmoc-Phe-OH (403 mg, 1.04 mmol) or Fmoc-Trp(Boc)-OH (548 mg, 1.04 mmol). Coupling was carried out with 2.5 eq of amino acids in the presence of 2.5 eq of HCTU (430 mg, 1.04 mmol) and 6 eq of DIPEA (422 µL, 2.5 mmol) in 5 mL DMF (3 h). Fmoc deprotection step was done using 20% piperidine in DMF (20 min). Completion of coupling and deprotection was checked using Kaiser test. After completing the peptide synthesis, peptides were cleaved from the the resin without deprotection using 5 mL AcOH:TFE:DCM (1:1:8, v/v/v) preserving all the protection groups (45 min). A presence of compounds was confirmed by HPLC-ESI-Q-MS.

Crude peptides (0.30 mmol) were coupled in 5 mL DMF with 1.1 eq of *trans*-1-cinnamylpiperazine (67 mg, 0.33 mmol) using 1.1 eq of TBTU (106 mg, 0.33 mmol), 1.1 eq of HOBt (51 mg, 0.33 mmol) and 5 eq of DIPEA (254 µL, 1.5 mmol). Reaction was carried out for 12 hours in room temperature. Crude product was precipitated, filtered and washed with distilled water until the neutral pH was achieved. Crude compounds were dissolved in TFA, allowing removal of all protecting groups (1 h). TFA was removed and crude product was evaporated 3 times with toluene and 2 times with diethyl ether. Structures of peptidomimetics was confirmed by HPLC-ESI-Q-MS and crude compounds were purified by preparative RP-HPLC on C_{12} column with water/acetonitrile gradient containing 0.1% TFA. Peptide fractions were collected, lyophilized and analysed by HPLC-ESI-Q-MS, LCMS-IT-TOF and NMR.

1-[L-tyrosyl-D-alanyl-L-phenylalanyl]-4-[(2E)-3-phenyl-2-propen-1-yl]piperazine (1) (153 mg, 53%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{34}H_{41}N_5O_4$: 584.3231, found: 584.3214.
1-[L-tyrosyl-D-alanyl-L-tryptophyl]-4-[(2E)-3-phenyl-2-propen-1-yl]piperazine (2) (150 mg, 49%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{36}H_{42}N_6O_4$: 623.3340, found: 623.3321.
1-[L-tyrosyl-D-threonyl-L-phenylalanyl]-4-[(2E)-3-phenyl-2-propen-1-yl]piperazine (3) (154 mg, 51%): purity: >99%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{35}H_{43}N_5O_5$: 614.3337, found: 614.3333.
1-[L-tyrosyl-D-threonyl-L-tryptophyl]-4-[(2E)-3-phenyl-2-propen-1-yl]piperazine (4) (146 mg, 46%): purity: >99%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{37}H_{44}N_6O_5$: 653.3446, found: 653.3416.

Synthesis of peptides 5 - 8: The synthesis of peptides was carried out manually on Wang resin preloaded with Fmoc-Phe-OH (397 mg, 0.63 mmol/g) or Fmoc-Trp(Boc)-OH (455 mg, 0.55 mmol/g) by solid phase method following the Fmoc chemistry. Coupling was carried out with 2.5 eq of amino acids in the presence of 2.5 eq of HCTU (261 mg, 0.63 mmol) and 6 eq of DIPEA (253 µL, 1.5 mmol) in 5 mL DMF (3 h). Fmoc deprotection step was done 20 min using 20% piperidine in DMF. Completion of coupling and deprotection was checked using Kaiser test. After completing the peptide synthesis, peptides were cleaved 3 h using TFA:H_2O:TIS (95:2.5:2.5, v/v/v). A presence of compounds was confirmed by HPLC-ESI-Q-MS and crude peptides were purified by preparative RP-HPLC on C_{12} column with water/acetonitrile gradient containing 0.1% TFA. Peptide fractions were collected, lyophilized and analysed by HPLC-ESI-Q-MS and LCMS-IT-TOF.

L-tyrosyl-D-alanyl-L-phenylalanine (5) (105 mg, 79%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{21}H_{25}N_3O_5$: 400.1867, found: 400.1853.
L-tyrosyl-D-alanyl-L-tryptophan (6) (109 mg, 77%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{23}H_{26}N_4O_5$: 439.1976, found: 439.1977.
L-tyrosyl-D-threonyl-L-phenylalanine (7) (105 mg, 77%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{22}H_{27}N_3O_6$: 430.1973, found: 430.1977.
L-tyrosyl-D-threonyl-L-tryptophan (8) (109 mg, 72%): purity: >98%; ESI-HRMS: *m/z* [M+H]+ calcd for $C_{24}H_{28}N_4O_6$: 469.2082, found: 469.2084.

Receptor binding assay: Receptor binding assays were performed as described previously[37] with some modifications. Crude membrane preparations, isolated from Wistar rat brains, were incubated at 25 °C for 60 min with 0.5 nM [^{3}H]DAMGO in a total volume of 1 mL of 50 mM Tris–HCl (pH 7.4) containing bovine serum albumin (BSA) (1 mg/mL), bacitracin (50 mg/mL), bestatin (30 mM) and captopril (10 mM). All reactions were carried out in duplicate, at 10 mM peptide concentration. Incubations were terminated by rapid filtration through GF/B Whatman glass fiber strips, using Brandel 24 Sample Semi-Auto Harvester. The filters were washed with 2 mL of ice-cold saline solution and the bound radioactivity was measured in the liquid scintillation counter MicroBeta LS, TriLux (ParkinElmer). Nonspecific binding was determined in the presence of naltrexone hydrochloride (10 mM). Receptor binding experiments were repeated three times. The data were analysed by a nonlinear least square regression analysis computer program Graph Pad Prism 5.0. Compound potency was expressed as IC_{50} values.

Blood collection and plasma preparation: Human blood from four healthy donors was directly drawn into evacuated tubes with lithium heparin. Tubes were spun immediately and centrifuged at 2500 g for 10 min at 4 °C. Plasma samples were pipetted out of the blood collection tubes into 15 mL falcon tube to pool obtained fluids, collected in 2 mL tubes and frozen at -80 °C until use.

Peptide degradation by sera and plasma: Human blood plasma in 1.5 mL tube was incubated in thermomixer (350 rpm) for 20 mins at 37 °C to preactivate biological fluid. A solution of tested peptide (5 μM) in water was spiked (1:1 v/v) and incubation was continued. At specific time intervals from 0 up to 96 h, 100 μL of the mixture was collected and quenched by adding 100 μL of 98% EtOH. The obtained suspension was shaken for about 1 min on a vortex (3000 1/min) and then centrifuged for 10 min at 4 °C (11000 g) to remove precipitated proteins by pelleting. 100 μL of supernatant was collected in 1.5 mL tube and 100 μL of water was added. Each sample was frozen and lyophilized.

The dried samples were re-suspended in 100 μL of water just before analysis. Each sample was subjected to HPLC analysis, followed by HPLC-ESI-Q-MS analysis for selected samples. Endomorphin-2 was incubated with plasma to check plasma enzymes activity before performing the peptide stability test.

Haemolysis assay: Compound-induced haemolysis was measured with modified Knopik-Skrocka and Bielawski,[14] procedure. Briefly, human red blood cells (RBC) were obtained from healthy volunteers with known haematocrit. Samples from each person were prepared separately. Samples were centrifuged at 2500 rpm for 10 min and plasma was carefully removed without disturbing the pellet. Samples were resuspended in PBS (pH 7.4, RT) and centrifuged at 2500 rpm for 10 min. This step was repeated three times. Blood samples were diluted in PBS according to each donor haematocrit. Suspension of 10% RBC was used to prepare 2% suspension and 100% haemolysis control. Final suspension of 2% haematocrit was incubated with serial concentration (0-1000 μM) of peptidomimetics in 1:1 ratio for 60 min at 37 °C. Next, samples were centrifuged at 4500 rpm for 5 min and 100 μL of supernatant from each sample was transferred to new well. Optical density (OD) was measured at 540 nm. A value of 100% haemolysis was determined by incubation of 10% in distilled water (ratio 1:9). For negative control (0% haemolysis), 2% RBC suspension was incubated with PBS (ratio 1:1) without compound. Value of peptidomimetic-induced haemolysis was calculated:
Haemolysis [%] = $(A – A_b) / (A_{100\%} – A_{0\%})$,
where: A - absorbance of the sample incubated with peptide, A_b - absorbance of blank sample, $A_{100\%}$ - absorbance of reference (100% haemolysis), $A_{0\%}$ - absorbance of 1% haematocrit incubated with PBS (0% haemolysis).

Cell lines and cell culture: Human pancreatic cancer cell line PANC-1 was purchased from ECACC. Human fibroblast cell line BJ was purchased from ATCC. BJ cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin-streptomycin. PANC-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) h.i. FBS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin. All cell lines were kept at 37 °C in a humidifying atmosphere at 5% CO_2. In addition, cancer cells (MMRC-DS1, MMRC-SS1r) isolated from pancreatic tumour tissue samples were used for experiments. Tissue samples were obtained from patients undergoing tumour resection. Isolated cell were maintained in DMEM supplemented with 10% (v/v) FBS, 2mM L-glutamine and 1% (v/v) penicillin-streptomycin. All experiments were approved by the Bioethics Committee of Central Clinical Hospital of the Ministry of the Internal Affairs in Warsaw. Description of cell isolation and characterization are available in Supporting Information. All cell culture reagents were purchased from Sigma. All experiments were approved by the ethical committee of Central Clinical Hospital of the Ministry of the Interior in Warsaw (Decision No. 64/2017).

Enzyme-linked immunosorbent assay: Qualitative analysis of the presence of opioid receptors was determined with colorimetric cell-based ELISA for MOR (Antibodies-online.com ABIN1381370). Cells were seeded in 96-well plates at density of 2×10^4 and grown for 24 h. Next, cells were fixed with 4% PFA and treated according to manufacturer protocol. Samples OD was measured at 450 nm. The insensitivity of colour produced was directly proportional to the amount of receptors on cell surface.

Cells proliferation assay: Cell viability was determined with colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) metabolic activity assay (G3581, Promega). Cells were seeded in a 96-well plate at densities at 5×10^3 (PANC-1, MMRC-DS1, MMRC-SS1r) and 3×10^3 (BJ) cells/well and grown for 24 h (37 °C, 5% CO_2). Cells were exposed to peptidomimetics, GEM (LC-Labs) (in PBS; final concentrations: 0-500 μM) for 24 h. Untreated cells served as a negative control (equivalent to normal viability). Next, 20 μL of MTS solution was added into each well and incubated for 1 h at 37 °C, 5% CO_2. Samples OD was measured at 490 nm. During the experiment medium was not changed. The amount of colour produced was directly proportional to the number of viable cells. Cell viability was expressed as a percentage of untreated control.

Tumour spheres culture: Spheroids were obtained with hanging drop assay. Cancer cells PANC-1, DS1, SS1r were harvested with trypsin/EDTA, centrifuged and counted. Cell concentration was adjusted to 4×10^4 cell/mL for all cell lines. Next 10 μL drops of fresh culture medium were applied on inverted 60 mm Petri dish lid and 10 μL of previously prepared cell suspension was added into each drop. 4-5 mL of sterie, distilled water was placed in the bottom of the dish. Lid was then carefully inverted on PSB-filled bottom and cells were incubated at 37 °C. After 3 days 10 μL of medium was carefully removed and 10 μL of fresh medium was added. Spheroids were incubated for another 2-3 days. Growing spheroids were examined daily by phase contrast microscopy. Passages from 3 to 5 were used for this experiment.

Spheroids viability assay: CellTiter-Glo® 3D Cell Viability Assay (G9681, Promega) based on quantitation of the ATP present was used to determine the viability of spheroid-forming cells. 5-day old spheroids (ø300-400 μm) were used for this experiment. Before addition of the peptides, medium was carefully removed without disturbing spheroids and 22.5 μL of fresh medium was added. Next, 2,5 μL of peptidomimetics GEM (in PBS) at final concentrations: 0-500 μM were added to each drop and incubated for 24h. Untreated spheres served as control. Next, spheroids were gently transferred to opaque-walled 384-well plate and 25 μL of CellTitter Glo®3D was added into each well and mix vigorously for 10 min. Plate was incubated for additional 25 min (RT) in darkness and luminescence was recorded.

Statistical analysis: All experiments were prepared in triplicate and were repeated three times. Quantitative data are expressed as mean ± Standard deviation (SD). Statistical analyses were performed with analysis of variance (ANOVA), followed by the Bonferroni's multiple comparison. The data were analysed by computer program Prism Graph Pad 5.0.

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Keywords: pancreatic cancer • peptidomimetics • spheroids • opioid receptors • anticancer drugs

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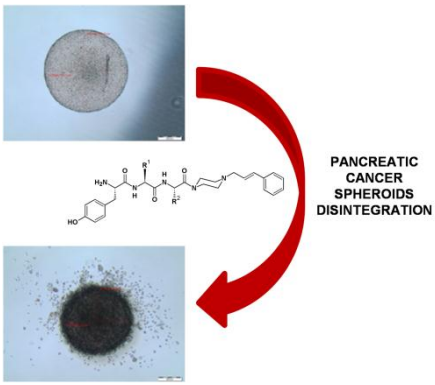

Pancreatic cancer is a deadly disease resistant to most chemotherapeutics. We have synthesized a series of peptidomimetics which are hybrids of opioid tripeptides and *trans*-1-cinnamylpiperazine. These compounds showed good bioactivity against pancreatic cancer cells in in vitro models. They were especially highly efficient against spheroids obtained from isolated carcinoma cells. Moreover, presented hybrids are stable in plasma up to four days and exhibit very low haemotoxicity.