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# PEPTIDE-OLIGO CONJUGATES TARGETING RAS AND CYCLIN B1: DESIGN AND AN IN VITRO EVALUATION

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## ABSTRACT

**Background:** cancer is the second leading cause of death worldwide. Peptide-oligo conjugates (POC) are certain molecules that can be used as multifunctional therapeutics. **Aim:** designing POC targeting both Ras family of proteins and Cyclin B1 and evaluating the anticancer activity *in vitro*. **Methods:** Five different sequences of POC were designed (POC 1– 5) and tested against the MCF-7 human breast cancer cell lines and the viability of cells was monitored. **Results:** Two sequences (POC4 and -5) had a potent anticancer activity and resulted in a reduction in viability by 61 and 73%, respectively, when transfected with saponin. **Conclusion:** Using POC improves synergistic targeting of vital cancer pathways and in turn offers a better outcome in cancer treatment.

**KEY WORDS:** Breast Cancer, Cyclin B1, POC, Cyclin B1; Ras.

## 1. INTRODUCTION

Cyclin B1 is a mitotic protein which complexes with Cyclin-dependent kinase-1 (CDK1) to form the Mitosis Promoting Factor (MPF), the main controller of mitosis **(Gavet and Pines, 2010)**. Cyclin B1 is considered a regulator for MPF activity because CDK1 is synthesized homogenously throughout the cell cycle whereas Cyclin B1 is synthesized in late S phase and peaks at M phase. In turn, inappropriate expression of Cyclin B1 may result in uncontrolled cell proliferation. In addition, Cyclin B1 is overexpressed in various tumors including breast

cancer **(Yu *et al.*, 2002; D'Andrea *et al.*, 2007)**. Furthermore, overexpression of Cyclin B1 is associated with poor prognosis **(Aaltonen *et al.*, 2009)**.

Ras oncogenes are among the most frequently mutated genes in cancer. They include 3 isoforms, K, H and N Ras and serve as signal transducers. Ras proteins control vital cellular activities like proliferation, survival and differentiation **(Takashima and Faller, 2013)**. These proteins, in normal, switch between the GTP-bound (active) and GDP-bound (inactive) states, on the other hand, mutations in RAS proteins result in impaired GTP hydrolysis giving rise to a constitutively active Ras **(Pylayeva-Gupta *et al.*, 2011)**. Oncogenic activation of RAS genes is one of the most characterized events in carcinogenesis **(Meng *et al.*, 2013)** and found in 30% of cancers **(Athuluri-Divakar *et al.*, 2016; Baker and Der, 2013)**. K-Ras is the most frequently mutated isoform in Ras family **(Moura *et al.*, 2015)**. In order to be functional, Ras proteins must undergo a post-translational medication (lipidation) which ensures association with the plasma membrane. The first attempt to inhibit Ras oncogenes was blocking its post-translational modification using farnesyltransferase inhibitors (FTI) **(Grabocka *et al.*, 2015)**.

Peptide-oligo conjugates represent a unique category of chimeric molecules composed of a peptide moiety and an oligonucleotide moiety **(Tung and Stein, 2000)**. The peptide moiety functions mainly to enable cell penetration instead of using other auxillary reagents whereas the oligonucleotide moiety functions mainly to interfere with mRNA (e.g siRNA and ASO) or proteins (e.g aptamers).

In the present work we aim to design peptide-oligo conjugates (POC) combining two therapeutic functionalities; targeting Cyclin B1 mRNA through the ASO moiety and/or blocking post-translational modification of Ras proteins though the peptide moiety and evaluate POC for its anticancer activity against the MCF-7 human breast cancer cell lines.

## 2 MATERIALS AND METHODS

### *2.1 Designing the Peptide moiety*

The peptides used here were designed to compete with Ras proteins for farnesylation by the enzyme farnsyle transferase (Ftase). In turn the post-translational modification of Ras oncogenes (farnesylation) will be inhibited and consequently Ras proteins will be kept in an inactive state. Two peptides were chosen according to the literature that were found to be farnesylated **(Long *et al.*, 2001)**. The first one is a hexa-amino acid peptide corresponding to

the carboxylic terminus of K-ras (namely TKCVIM) and was found to be potent in competing with Ras proteins for farnesylation. The second peptide is a tetra-amino acid peptide (namely CIIS).

### *2.2 Designing the antisense moiety*

The antisense oligonucleotide specific for Cyclin B1 m-RNA was chosen using the *Sfold* web server **(Ding *et al.*, 2004)** and composed of is 20 nucleotides in length. The web server generated many antisense sequences and arranged them. The best three sequences were chosen; 5`-CCTTTTCAAGAGGTTTTGGT-3`, 5`-AGTAGCTGAAGGTTTTGCTT-3`, 5`-CATTTTGGCCTGCAGTTGTT-3`.

### *2.3 Designing the Peptide-oligo Conjugates (POC)*

Peptide-oligo conjugates were synthesized (Thermo fisher) by linking the amino terminus of the peptide to the 5` end of DNA as illustrated in **Table (1)**.

The TKCVIM peptide was known to inhibit Ftase. For that reason it was coupled with the three ASO. In addition, MIVCKT was extended at the N terminus with a cell penetrating peptide known as TAT peptide to enable intracellular translocation (self-delivery). Then the first sequence which was supposed to have performance better than the other two was used with the other peptide (CIIS).

**Table (1): Sequence of the peptide-oligo conjugates (POC).**

| Name | Sequence |
|---|---|
| POC1 | MIVCKT-Linker-CCTTTTCAAGAGGTTTTGGT |
| POC2 | SIIC-Linker-CCTTTTCAAGAGGTTTTGGT |
| POC3 | MIVCKTQPPRRRQRRKKRG-Linker-CCTTTTCAAGAGGTTTTGGT |
| POC4 | MIVCKT-Linker-AGTAGCTGAAGGTTTTGCTT |
| POC5 | MIVCKT-Linker-CATTTTGGCCTGCAGTTGTT |

### *2.4 Cell culture*

MCF-7 human breast cancer cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 37 ºC incubator at 5% $CO_2$.

### *2.5 MTT assay*

The compounds (POC 1–5) were dissolved in the specified amount of PBS as instructed by the manufacturer to give 1000 nM stock solution. Serial dilutions of the tested compounds were prepared from 0.1 – 1000 nm. In addition, Cisplatin was used as positive control

meanwhile DMSO was used as a negative control. The cells were treated for 48 hours then the viability was determined by MTT assay Kit (Molecular Probes, Cat. No. V13154) according to the manufacturer's instructions and the absorbance was read in a microplate reader at 570 nm.

### *2.6 Saponin-mediated transfection of POC*

Therefore 100 µl of POC (5000 nM) was slightly warmed up at 76 ˚C (melting temperature) and mixed with 100 µl (30 µg/ml) of saponin at (1:1v/v) and then mixture was added to MCF-7 cells ($5*10^4$) and incubated in 5% $CO_2$ at 37 ˚C for 30 min. After incubation, the medium (DMEM) was replaced with fresh medium and incubated for 48 hours and the cell viability was determined by MTT assay.

## 3 RESULTS

### *3.1 Cytotoxicity of the peptide-oligonucleotide against MCF-7*

As shown in **Figure 1**, all tested compounds (POC 1–5) did not exhibit any cytotoxic effect against MCF-7 cells even at high concentrations. On the other hand, cisplatin which was used as a positive control showed a potent toxicity against MCF-7 cells.

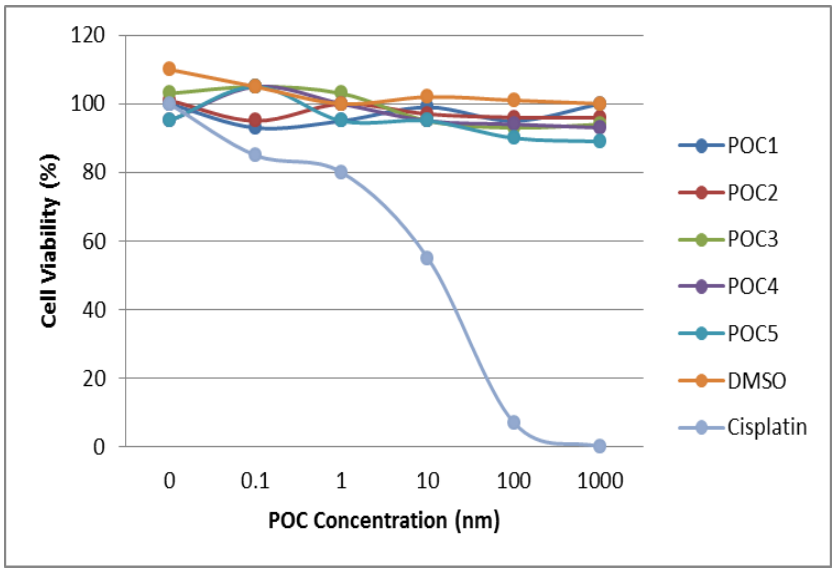


**Figure 1: Cytotoxicity of POC 1–5 against breast cancer cell line (MCF-7).**

### *3.2 Cytotoxicity of POC after heating*

When compounds (POC 1–5) were heated to remove possible secondary structures, POC-3 and -5 showed a slight activity against MCF-7 cells and inhibited the growth of MCF-7 cells

by 24% and 22%, respectively, as shown in **figure 2** whereas POC4 showed a 14% inhibition. By contrast, POC1 and POC2 did not show any toxicity against MCF-7 cells.

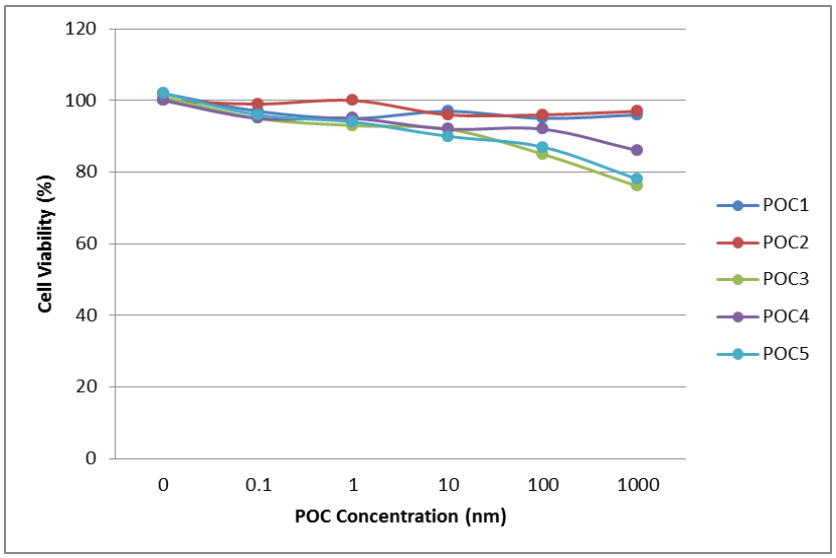


**Figure 2: Cytotoxicity of the heated POC 1–5 against breast cancer cell line (MCF-7).**

### *3.3 Saponin enhance cytotoxic effect of POC*

POC-4 and -5 showed 61 and 73%, respectively, inhibitory activity and saponin was not toxic for 30 min treatment. They have the same peptide moitey but differ in the oligunucleotide sequence. On the other hand, POC1 and POC2 did not any toxicity against MCF-7 cells. In addition, POC3 did not induce toxicity despite the presense of a CPP (TAT) in its peptide moiety. Sponine which used as a negative control did not show toxicity indicating a compatibility for transfecting MCF-7 cells with POC1–5 **(Fig 4 and Fig 5A)**.

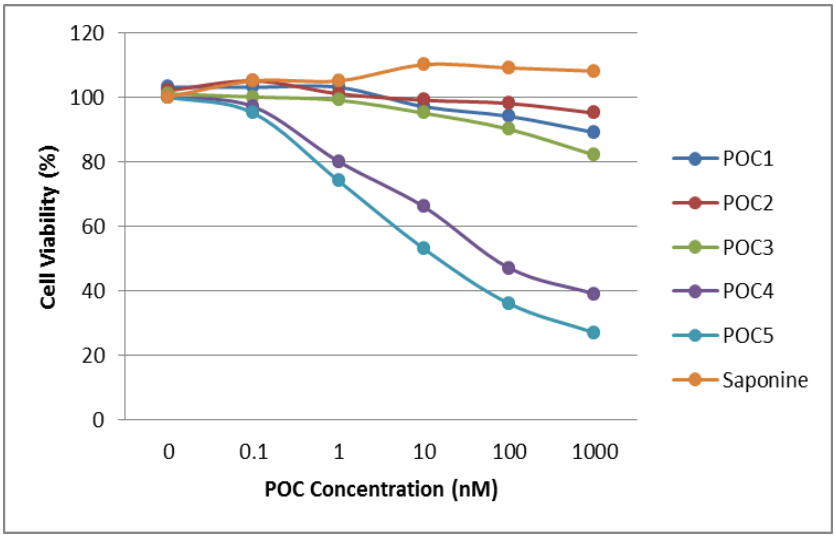


**Figure 3: Cytotoxicity of POC 1–5 and saponine against breast cancer cell line (MCF-7).**

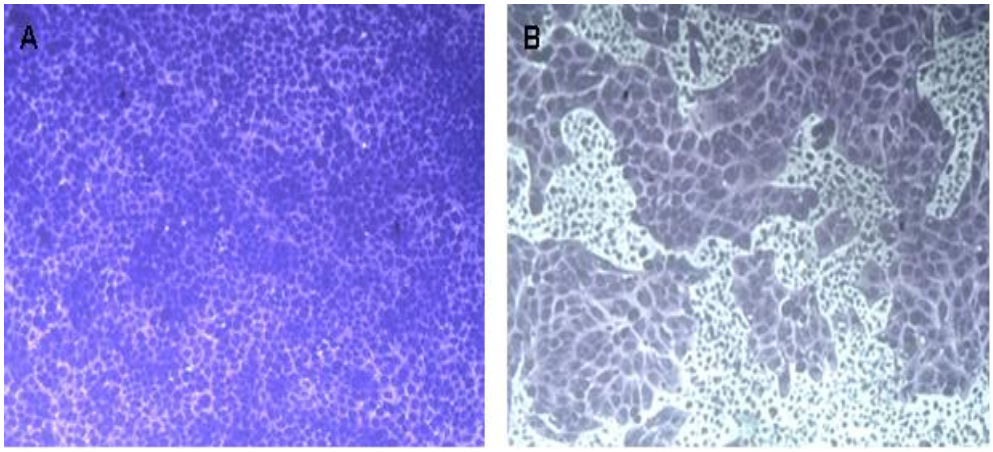


**Figure 5: MCF-7 cell stained with crystal violet. A, Saponin (30 µg/ml); B, Saponin (30 µg/ml) + POC-5.**

## 4 DISCUSSION

In the present work, five different peptide-oligo conjugates (POC) were designed and evaluated for anticancer activity. The five POCs are targeting Cyclin B1 which is considered a potential cell cycle target and Ras proteins which are frequently mutated in many types of cancer. The mechanism of the designed compounds depends on interfering with the Cyclin B1 mRNA and preventing its translation and/or competing with Ras proteins for post-translational modification (farnesylation) by the enzyme Ftase.

One of the compounds was designed to have a self-transfection moiety, the HIV-1 TAT peptide. TAT peptide is derived from the basic domain of HIV-1 Trans-Activator of Transcription protein (residues 48-60) and belongs to a class of molecules known as cell penetrating peptides (CPP) that are used to deliver cargo molecules like proteins, nucleic acids and peptides into different types of cells **(Milletti, 2012)**. Tat peptide was the first discovered CPP then other CPP were discovered including penetratin, transportan and polyarginines (**Bechara and Sagan, 2013)**.

When POCs were used directly, there was no obvious anticancer effect. At first, the hypothesis that POC did not enter the cells was excluded because at least POC3 shall enter because it contains a CPP domain in its peptide moiety. This was suggested to be as a result to being folded into secondary structures. When POCs were heated to remove any secondary structure; a slight improvement was observed with POC3 and POC5. This made us to rethink about the whether the compound had entered the cells or not.

Saponine is a natural product that acts as a detergent, and could be used to induce reversible perforation in the cell membrane in about 30 minutes **(Bachran *et al.*, 2006)**. In the present work we used the saponine for transfecting cells with POCs an obvious anticancer effect was observed for POC4 and POC5. These results may explain that, when POCs were used in the first experiment (without heating or transfection), the compounds did not enter the cells. In addition, the action of the TAT peptide as a self-transfecting domain was abolished by the negative charges of oligonucleotide backbone. Furthermore the negative charge that seems to dominate the overall charge of POCs and impedes the penetration of cell membrane which is negatively charged.

First efforts for blocking the mutated, and in turn continuously active, Ras directed towards preventing its functional localization into the inner surface of plasma membrane. Substrate and non-substrate peptides based on CAAX sequence were used as competitive inhibitors for the enzyme Ftase which is responsible for post-translational modification of Ras **(Reiss *et al.*, 1990)**. These peptide inhibitors and their peptidomimics showed promising results with H-Ras dependent tumors but not K and N-Ras which have an alternative prenylation **(Karnoub and Weinberg, 2008)**.

Cyclin B1 have been previously targeted using transient (siRNA) or stable (shRNA) strategies. Although shRNA offers complete depletion of its target, but exhibit a slower dynamics because the vector encoding the shRNA needs to reach the nucleus in order to be transcribed by the cellular machinery **(Yuan *et al.*, 2006)**. By contrast, siRNA and antisense oligonucleotides (ASO) act directly in the cytoplasm by overlapping with its targeted mRNA **(Androic *et al.*, 2008)**. Both transient and stable strategies work by blocking the translation of mRNAs either through steric hindrance or RNase H- dependent degradation **(Moreno and Pêgo, 2014)**.

There is no POC have been used for targeting Cyclin B1 to date. Instead, non-covalent complexes of CPP/ASO were applied. For instance, Morris *et al.*, designed a CPP (Pep-2) for delivery of a modified ASO (Pep-2/HypNA-*p*PNA chimera or Pep-2/PNA) *in vitro*. In that study, Pep-2/PNA inhibited the proliferation of MCF-7 cell lines by 64% in agreement with our results **(Morris *et al.*, 2004)**. The same group developed another CPP (Pep-3) for delivery of ASO analogous *in vitro* and *in vivo* **(Morris *et al.*, 2007)**. Pep-3/ASO analogous non-covalent complexes inhibited the proliferation of Jurkat T cells and the hard-to-transfect HUVEC cells also by about 70% in agreement with our results.

In conclusion, mono-targeting cancer therapeutics are often exposed to drug resistant and thus multifunctional drugs may offer a better outcome. POC combine the functionalities of two molecules, a peptide moiety and an ASO moiety. It offers specific targeting via ASO domain accompanied with another functional peptide which may be a CPP or therapeutic peptide (TP). The present work shows that POC4 and POC5 have a good anticancer activity which needs to be further studied *in vivo* to evaluate the effect of physiological environment on its activity and stability.

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