Aminopeptidase N (APN) is one of the important enzymes highly expressed in metastatic cancers, thus employed as a marker to target tumor cells. CPG2-CNGRC fusion protein is produced to target high APN expressing cancer cells, which with the produg results in high toxic effect. Since PEGylation of CPG2 has shown an improved favorable in vitro stability and immunotoxicity, we performed a site-directed PEGylation (thiol group directed) of the CPG2-CNGRC fusion protein and examined the effect of PEGylation on the resulting fusion protein’s therapeutical efficacy. CPG2 kinetic activity was substantially enhanced following PEGylation of the single fusion protein (PEG-CPG2). The effect of PEGylation on the produced PEGylated fusion proteins to their cellular marker (APN) was notably reduced in case of the double fusion protein compared to non-PEGylated ones. Moreover, the cytotoxic effect of methotrexate and ZD2767P (produg) in association of the PEGylated fusion proteins was investigated and found that the cytotoxic effect of produg with PEGylated single fusion protein was improved significantly (low cell survival). Similar finding was found following MTX treatment in a subpopulation where lower binding and kinetic activity of the PEGylated double fusion proteins resulted in higher MTX toxic effect (lower cell survival) in comparison with X-CPG2 (CNGRC-peptide “blue” fused at N terminus) and PEGylated MTX. Thus, although PEGylation is known for its usually favorable effect on the protein/drug pharmacodynamics, our results indicated that with our different fusion proteins (single and double fusion proteins) PEGylation did not improve it similarly.

**INTRODUCTION**

The use of anticancer agents have been facing several drawbacks as their poor solubility, short in vivo half-life, low specificity to the tumor cells resulting in low therapeutic efficacy and serious side effects (1). PEGylation (conjugation of protein and/or drug with Polyethylene glycol (PEG) polymer) is well known approach widely used lately to improve pharmacokinetics and therapeutic properties of peptides and drugs used in cancer therapy. Carboxypeptidase G2 (CPG2) and Carboxypeptidase G3 (CPG3) are two double fusion proteins which are produced by the fusion of APN to CNGRC and P. In the current study we combined the two approaches and applied PEGylation to the previously generated fusion proteins (single “X-CPG2” and double “X-CPG2-X”) producing PEGylated fusion proteins (PEG single “PEG X-CPG2” and PEG “PEG X-CPG2-X” double fusion proteins) as a proof of the effectiveness of PEGylated fusion proteins as potential therapeutic compounds used in combination of a produg for targeted cancer therapy.

**RESULTS**

**Enhanced CPG2 enzymatic activity and stability of the PEG CPG2 fusion proteins:**

![Fig. 3](image1)

**Fig. 3** Circular dichroism spectra of CNGRC, fusion proteins before and after PEGylation. (A) The combination of UV spectra of CNGRC “WT”, single “X-CPG2” and double “X-CPG2-X” fusion proteins. (B) The combined UV spectra of PEG CPG2, PEG X-CPG2 and PEG X-CPG2-X fusion proteins. (C) Table presents the results of CDNN deconvolution analysis of the PEGylated and non-PEGylated CPG2 fusion proteins CD spectra, showing their secondary structure composition. Darker shades indicate an increase in the composition percentage.

**Lowered Ex-vivo immunotoxicity of the PEGylated CPG2 fusion proteins:**

![Fig. 6](image2)

**Fig. 6** Ex-vivo cell proliferation assay. The immunotoxicity of PEGylated and non-PEGylated CPG2 fusion proteins was studied. PBLs from normal healthy donors were co-incubated with the PEGylated fusion proteins “X-CPG2” and X-CPG2-X, respectively, whereas lanes 2, 4 and 6 show the bands for PEGylated proteins (WT, X-CPG2 and X-CPG2-X respectively). Whereas lanes 2, 4 and 6 show the bands for PEGylated proteins (WT, X-CPG2 and X-CPG2-X respectively), whereas lanes 2, 4 and 6 show the bands for PEGylated CPG2 proteins (PEG CPG2, PEG X-CPG2 and PEG X-CPG2-X respectively). PEG: polyethylene glycol; CPG2: Carboxypeptidase G2, X: CNGRC peptide.

**REFERENCES**