A SELECTIVITY OF SUPER-SHORT PEPTIDE MORPHED HYDROPHILIC AND HYDROPHOBIC DOMAINS TO IMPROVE ANTI-CANCER EFFICACY

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Abstract
The amino acid extends the C-terminal which is tagged onto the VS-9 peptide, a short anti-cancer peptide. Tyrosine, arginine, and lysine have all been noted. The choice of amino acid types at every point stretches reliant on the hydrophobic and hydrophilic residues area, as can be observed in the VS-9 template's helical wheel pattern. The addition of oligopeptide tags should take into account their physiochemical features such as positive net charge, hydrophobicity, and their proportion of hydrophilic amino acids as well as their polar angles. C-terminal NH2- amidation and tyrosine substitutes with arginine or lysine bit enhance the effects against RAW.264.7, hRBCs cells, and human colon cancer test cells. The hydrophobicity (55%), +6 polar positive charges, polar angles (-0.03), and (-0.05) hydrophobicity of the RT2 and CRT2 peptides were effects against four colon cancer cell lines. The morphological assessment revealed good selectivity of RT2 and CRT2 activity in CRC cell lines. Finally, this study found that RT2 and CRT2 unique anti-cancer peptides could potentially be developed in the future for alternative anticancer peptides in clinical applications.

Keywords: VS-9, helical wheel, CRC, hemolytic activity, apoptosis

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DOI: 10.48047/ecb/2023.12.si5a.0456

BACKGROUND

Colorectal cancer (CRC) is a solid malignancy that has uncontrolled proliferating precursors that is one of the most prevalent causes of cancer-related morbidity and death in the world [1,2]. The most successful treatment for CRC is to combine chemotherapy with cytotoxic medications, although patients frequently suffer severe and at times disastrous side effects. As an outcome, the investigators have been looking for an innovative physiologically active biological that has significant cancer-fighting properties and few adverse effects. Bioactive peptides, tiny molecular weight protein fragments encompassing 2-20 amino acid residues, have recently received attention as potential active components in medications and functional foods that benefit health [3,4]. BG-4, a new peptide derived from bitter gourd (Momordica charantia), has been proven to inhibit human colon cancer cells HT-116 and HT-29. CTLEW, a peptide made from walnut residual protein, was discovered to inhibit Caco-2 and HeLa cell growth while triggering both autophagy and apoptosis in MCF-7 cells [7]. Garlic, or Allium sativa, is a well-known herb for its potent therapeutic and preventative health effects. Garlic bioactive components contain anti-bacterial, anti-inflammatory, anti-diabetic, hepatoprotective, anti-fibrinolytic, and anti-cancer activities [8]. According to several systematic reviews and meta-analyses, there is an inverse relationship between garlic consumption and the risk of various cancers [9]. Organosulfur compounds produced from garlic, such as diallyl sulfide, diallyl disulfide, diallyl trisulfide, and S-allyl cysteine, are reported in cell cultures to have anti-cancer potential against several carcinogens [10]. The helical wheel pattern was used during this study to show hydrophilic and hydrophobic active peptides to extend an existing systematic synthetic approach of anticancer peptide design by utilizing hydrophilic and hydrophobic amino acid stretches.

MATERIALS AND METHODS

Peptide design and synthesis

The VS-9 peptide is derived from naturally occurring garlic pulp (Allium sativa) and their amino acid sequence VKLRSLLCS. The amino acid sequence has been split into hydrophobic and hydrophilic sections that are nearly equivalent in size. Tyrosine-Y and R-arginine are hydrophilic amino acids that are used to enhance the hydrophilic area of the peptide. Methionine-M is a hydrophobic amino acid that is used to increase the hydrophobic area of the peptide. The active peptide VKLRSLLCS templet as 1-9 position same and 9-16 position same was applied to amino acid stretches, thus at position 9-16 as same (XZZXZXX) X denotes Methionine-M hydrophobic amino acid and Z designates Tyrosine-Y, R-arginine hydrophilic amino acid. The chosen peptide sequence was synthesized in Eurofins Genomics (Bangalore, India) by the solid phase (S-Fmoc) methods. Each peptide has been synthesized and refined to a greater than 95% purity using Revers-phase HPLC (solid stationary: C18 Colum and mobile phase: 5 to 20% altering acetonitrile in water at run time 5-20min). Findings of molecular weight analysis using ESI-MS data are shown in (**Table 1**) [11,12].

Peptide	Sequence	Theoretical molecular Wight (Da)	Measured molecular Wight (Da)	Positive charge	Hydrophobicity (H)[b]	Hydrophobic amino acid content (%)	Polar ngle θ(°)c
VS-9	VKLRSLLCS	931.21	931.28	+2	0.642	55	160
YT1	VKLRSLLCSYMMYMMYYMM-NH2	2458.18	2458.31	+4	0.834	75	160
RT1	VKLRSLLCSRMMRMMRRMM-NH2	2430.23	2430.65	+6	0.339	50	160
YT2	VKLRSLLCSYMMYMMYYMMM-NH2	2589.38	2589.54	+2	0.866	78	140
RT2	VKLRSLLCSRMMRMMRRMMM-NH2	2561.42	2561.48	+8	0.244	47	160
CYT2	VKLRSLLCSYMMYMMYYMM	2457.20	2457.12	+6	0.861	77	160
CRT3	VKLRSLLCSRMMRMMRRMM	2429.24	2429.30	+7	0.319	50	160

Table 1: Designed peptide amino acid sequence molecular weight calculated by ESI-MS/MS, theoretical molecular weight, polar charges, hydrophobicity, and polar angles of all designed peptides.

MTT assay

In a 96-well plate, HT-29, HT-116, SW-620, and SW-480 cell lines ($1.0x10^5$cells/ml) were treated with all synthesized peptides at various peptide concentrations incubated for 24hr at 37°±0.5C in a humidified, with 5% CO_2 environment. Further, the 1 mg/ml MTT solution that had been applied to it earlier for 3 hours at the same conditions. Respectively after the incubation and then addition to 100µl DMSO, each well was then incubated for

a 15min, Then Multiskan GO Microplate (ELISA) multi-Spectrophotometer was used to measure the MTT formazan color intensity at 570 nm to 590nm. The percentage of cell viability compared treated vs control group.

Assay for anti-hemolytic activity

Venipuncture was used to draw human blood in EDTA tubes, and RBCs were separated. All peptide concentrations (0.5 mL) were added to erythrocyte solution (2%, 2 mL) and the volume was increased to 5 mL with 0.85% saline buffer. After incubating the reaction mixtures for 5 minutes at room temperature, 0.5 mL of H2O2 solution in saline buffer was added to induce hemolysis. After 40 minutes at room temperature, the reaction mixture was centrifuged at 250g for 10 minutes to evaluate the amount of hemolysis by measuring the absorbance at 540 nm equivalent to hemoglobin liberation.

Assay for cytotoxicity

Raw 147.6 macrophage cell lines ($1.0x10^5$ cells/mL) were incubated at 37°C for 24 hours in a humidified, 5% CO2 atmosphere before being treated with peptides at varied doses. The formazan color intensity at 570 nm was measured using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, USA). PBS-treated cells were used as the control to determine the percentage of cell inhibition.

AO/EB morphological assay

The qualitative evaluations of live and dead cells by using AO/EB assay. AO/EB was performed as prepared (1mg/ml) using PBS, (final concentrations 100µl/ml cocktail of AO/EB). This experimental procedure was used for the RT2 and CRT2 treated with IC_{50} concentrations and PBS-treated cells as the control group respectively. Further were incubated for 15min with a 100µl/ml cocktail of AO/EB. After examining the cell death was visualized by phase-contrast fluorescent microscope (Nikon TS-100 OLYMPUS, Japan). The dual acridine orange/ethidium bromide staining method was repeated three times n=3.

Statistical analysis

All the experiments were repeated thrice and analyzed by one-way ANOVA method using Prism (version 8.0.1) software. Data with *** p> 0.01, **p> 0.1, and *p>0.5 was considered to be statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity of designed all peptides shown in 100% activity **(Table 2),** a peptide which has a good anti-cancer activity against four human colon cancer cell lines which is HT-29, HT-116, SW-620, and SW-480, Respectively YT1, RT1, YT2, RT2, and CRT2 were 25 to 100µg/ml. the RT1, RT2, and CRT2 peptides can induce cell inhibition of colon cancer cell lines HT-29, HT-116, SW-620, and SW-480, Similarly, RT1 and RT2 peptides show good cytotoxic effects in four cell lines (**figure 1**), further the therapeutic resulted indicated to increasing the peptide amphiphilicity by increasing of polar charges, hydrophilic amino acid addition, and polar angles factor maybe could support for this activity. The YT1 tyrosine Addition slightly decreases the cytotoxic activity of HT-29 and HT-116 cell lines which may cause positive residues and their polar angle (160) as shown in **(figure 2)** and RT1, RT2, and CRT2 peptide means amphiphilic nature (55%), the hydrophobic and polar index (PI) of tyrosine and arginine are different (0.10 and 0.04) respectively, the RT1 and RT2 peptide amino acid sequence as same. Accordingly aimed not C-terminus only, aimed both side chains but the results were found to be the same anti-cancer activity compared then YT1, YT2, and CYT3. Similarly, previous results reported C-terminal peptides to increase the cytotoxic activity of asurin 2.2,2.3 and other peptides [13] [14]. The tyrosine replacement arginine shows improve anticancer activity compared then YT1, YT2 CRT2, and CRT3peptide. This may cause due to higher positive charge –NH2 and then more appropriate hydrophilic nature fact of RT2 and CRT2 as shown in (**figure 1**). The RT2 and CRT2 addition to the hydrophobic fact tends to be more interrupted by the lysine position at 5 and aimed position was 16 resulting in the size of the hydrophobic fact being smaller. were selected as in vitro cell models to explore the broadly inhibiting effects of RT2 and CRT2 on colon cancer cells, four colon tumor cell lines were exposed to various doses of RT2 and CRT2 for 24 hours, and the MTT test was used to gauge the viability of the treated cells. According to the findings **(Figure 2)**, RT2 and CRT2 exhibited a wide inhibition on all of these different colon cancer cell lines in a concentration-dependent manner [15].

Peptide	Sequence	HT-29	HT-116	SW-620	SW-480
VS-9	VKLRSLLCS	>80	>80	>80	>80
YT1	VKLRSLLCSYMMYMMYY-NH2	56	>100	>100	>100
RT1	VKLRSLLCSRMMRMMRR-NH2	54	57	56	59
YT2	VKLRSLLCSYMMYMMYYMM-NH2	91	86	>91	>91
RT2	VKLRSLLCSRMMRMMRRMM-NH2	23	18	11	26
CRT2	VKLRSLLCSRMMRMMRRMMM	32	29	21	25
CRT3	VKLRSLLCSRMMRMMRR	>100	>100	X	X

Table2: IC_{50} values of all designed peptide against HT-29, HT-116, SW-620 and SW-480 cell lines

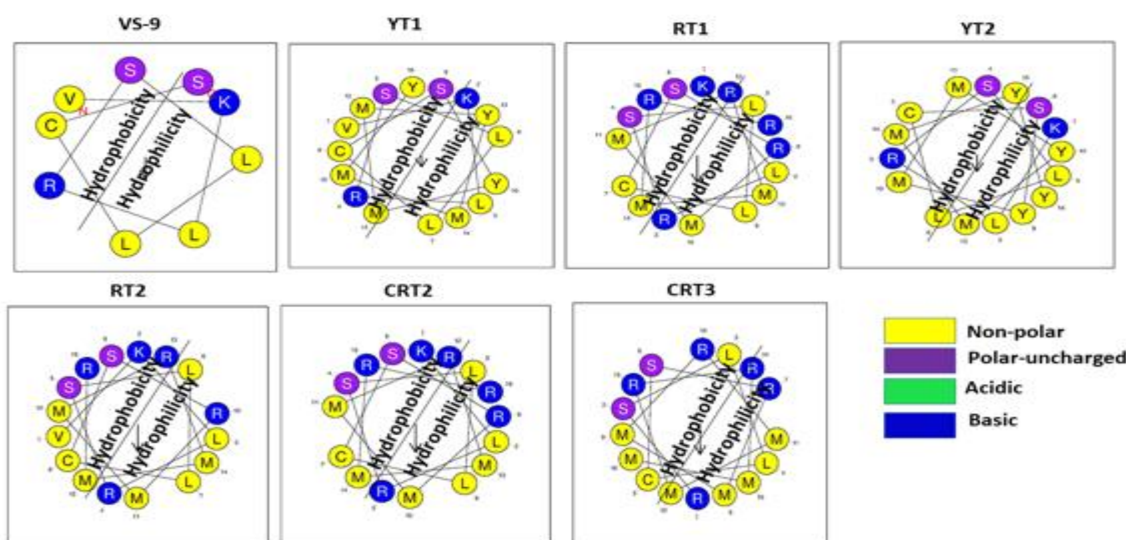

Figure 1: designed peptide helical good pattern and VS-9 template partition into Hydrophobic, and hydrophilic as equal size.

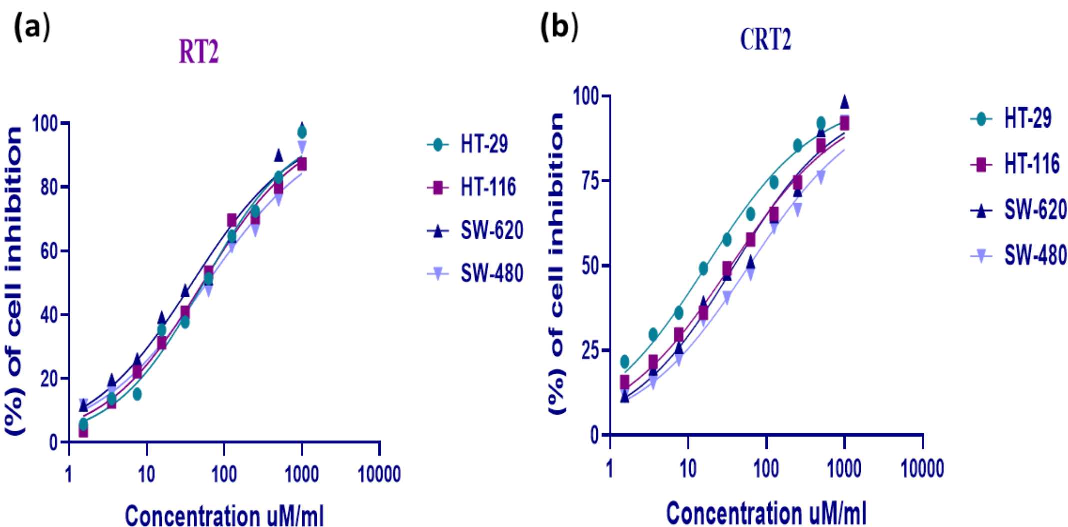

Figure 2: Cytotoxic effect of RT2 and CRT2 peptide on colorectal cancer cells. **(1a, b).** Ht-29, Ht-116, SW620 and SW-480 cells with RT2 and CRT2 peptide for 24hr**.** The data represented as mean ± standard deviation of n=5 independent experiments

All the designed peptides further evaluate toxicity against human red blood cells (hRBC), the peptides show minimum hemolytic activity at low concentrations to produce 2% as shown in **(figure**

3a) RT2 and CRT2 show lower hemolytic activity followed by RT1. However, the hemolytic activity is almost the same. YT1 shows clear toxicity than RT2, and CRT2 at the same treated concentrations **(figure 4)**. Therefore, the RT2 and CRT2 show % of hemolytic activity lower than the YT1, YT2, and CRT3 peptides. RT2 and CRT2 comparing amination-NH2 at the C-terminus of RT2 show lower toxicity against mammalian cells than the native CRT2 peptide because the amidation to increase net positive charge results to decrease hemolytic activity against humans (RBCs). Moreover, the higher hydrophobic, lower charge, and poor polar angles fact parameters may be to affect hemolytic activities respectively. the selective RT2 and CRT2 involving further analysis.

The designed all peptides YT1, RT1, RT2, and CRT2 was good selectivity and involved MTT assay against RWA-264.7 mouse monocyte-macrophage cell lines. It represents the mammalian immune system. The Cell viability % of RT2 and CRT2 increased to compare then YT1 and RT1 shown in **(figure 3b)** its results indicate that tyrosine replaced arginine shows lower toxicity, which corresponds to hemolytic activity. Comparing the best selective YT1 and RT1, slightly toxic the RT2 and CRT2 peptides are. However, the RT2 and CRT2 amidation of C-terminal due to increasing the cell viability and the resulting high hydrophobicity supports to the effectiveness of peptide against mammalian cells [16] [17].

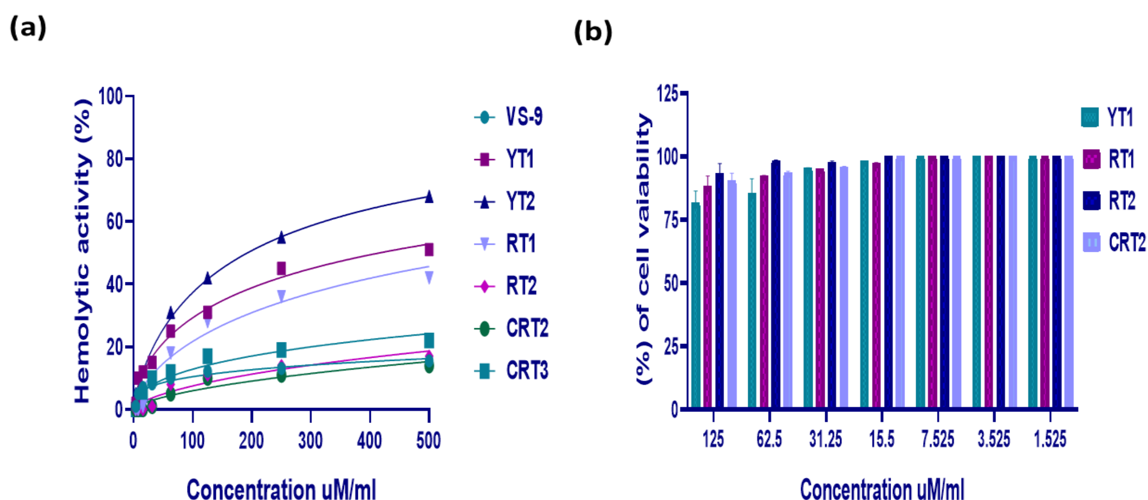

Figure 3: hemolytic activity comparison of all peptides at the concentration 1 to 500µg/ml (a) effect of four selective peptides against RAW 264.7, cell lines (b) all the data were presented as mean ± SD (n=6)

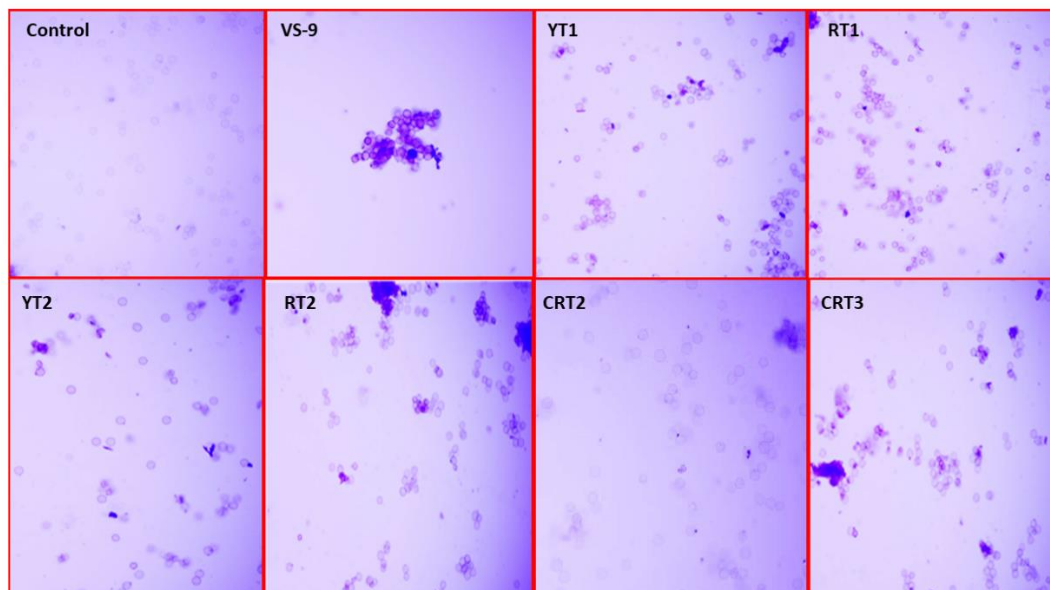

Figure 4: Morphological images of all peptides treated (hRBC) at the concentration range of 25µg/ml

Morphological analysis for good selective RT2 and CRT2 with four human colon cancer cell lines HT-29, HT-116, SW-620, and SW-480 by using phase contrast microscopy. The live viable cells were exposed in green fluorescence, early apoptotic cells showed slight orange-yellow, and late apoptosis or DNA fragmented cells showed red color, respectively [18]. Meanwhile, RT2 and CRT2-treated colorectal cancer cells increase in apoptosis HT-29, HT-116, sw-620, and SW-480 cells shown in **(figure 5,6).** Among them, CRT2-treated cells showed a higher rate of apoptosis, and the staining images of each group were analyzed. apoptosis is clearly shown in depicted, cell damages or cell death in compression to control grouped cells. The percentage of apoptosis rate indicated that CRT2 showed 1.5-fold more apoptotic and necrotic cells compared to control cells, respectively. Data were coordinated with MTT assay.

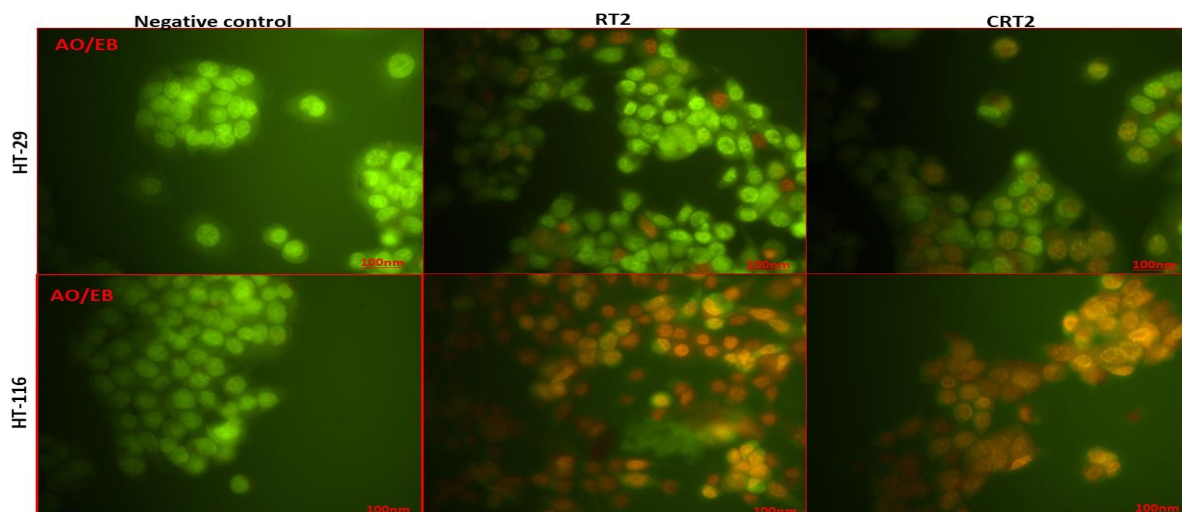

Figure 5: Morphological analyses to identify live, apoptosis, and necrotic cells by using AO/EB staining assay via green and red fluorescence, respectively HT-29 and HT-116 cell lines treated with RT2 and CRT2 peptide.

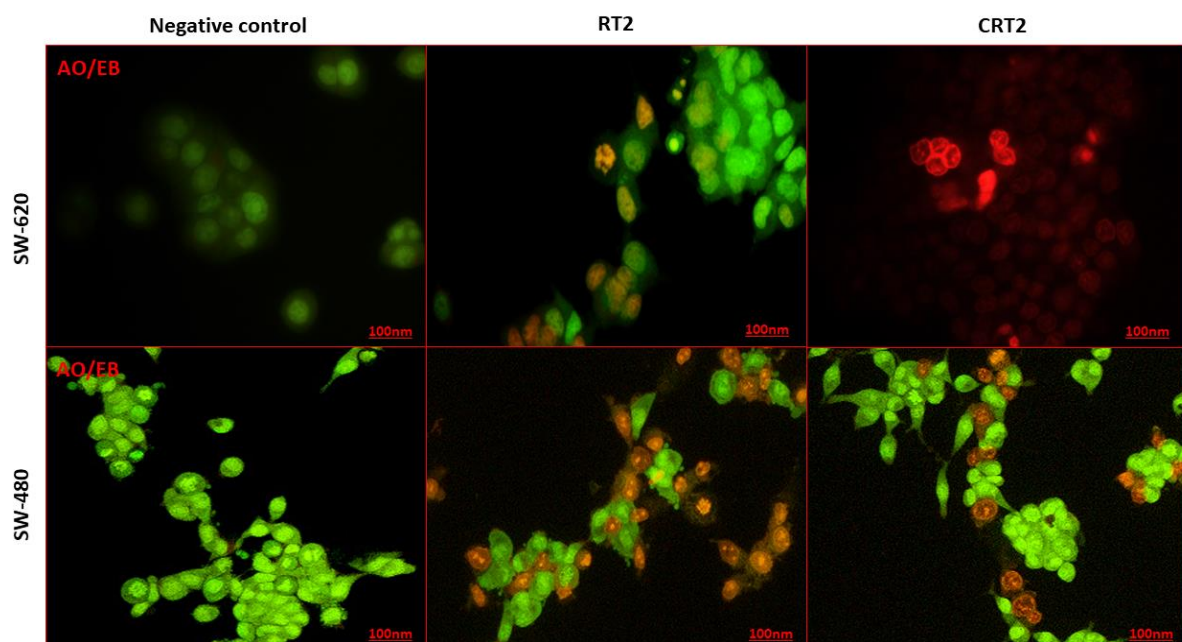

Figure 6: Morphological analyses to identify live, apoptosis, and necrotic cells by using AO/EB staining assay via green and red fluorescence, respectively SW-620 and SW-480 cell lines treated with RT2 and CRT2 peptide

CONCLUSION

Fmoc solid-phase synthesis developed an innovative therapeutic peptide, RT2, and CRT2, which was evaluated for anti-cancer properties on the HT-29, HT-116, SW-620, and SW-480 cell lines. The findings revealed their interaction with apoptotic proteins, as well as their potential role in cell proliferation decrease and capacity to trigger apoptosis in colon cancer cell lines. To the best of our knowledge, this is the first reveal of RT2 and CRT2 anti-cancer properties.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study. The authors are responsible for the content and writing of the papers.

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