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Anti-VEGFR-2 Kinase Effects of Cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)- L-lysine] and its Anticancer Activities Against Different Cancer Cell Lines

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The current work aimed at preparing a cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine] from previously established synthetic routs. The derivative was investigated for its potential anticancer activities as well as its possible mechanism of action. The prepared compound showed variable anticancer activities against all tested cell lines. Furthermore, it showed very promising activities in terms of obtained IC50 values compared to known used drugs. The mechanism of action studies showed that the prepared tripeptide may act on cancerous cells through its inhibitory action on tyrosine kinase pathway. Animal model experiments proved the potential of the synthesized tripeptide as an anticancer agent against PC3 cancer cells.

Key words: Cyclic tripeptides, Pyridine derivatives, Anticancer, Animal model, Inhibition of VEGFR-2 tyrosine kinase

Introduction

Carboxamide derivatives have biological and medicinal importance, such as: antimicrobial[1,2], *In vivo* anti-inflammatory and *In vitro* antioxidant[3,4], c-Src kinase inhibitors[5], antiviral[6], anti-atherothrombosis[7], antiemetic[8], anxiolytic[9], antimalarial and antileishmanial[10] activities. In previous publications[11–16], we have shown that pyridine candidates are excellent derivatives for the synthesis of heterocyclic systems very interesting as anti-microbial, potential analgesic and anti-inflammatory, and anti-breast cancer agents. In continuation of our previous work[11–16] in heterocyclic and peptide chemistry, herein we reported the anticancer activities against different cancer cell lines of cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine] derivative.

Materials and Methods

Chemistry

The chemistry and characterization (physical and spectroscopic data) of **4** was reported previously by Amr *et al.*[17]

***Synthesis of methyl 4,14-diisopropyl-2,5,13,16-tetraoxo-3,6,12,15-tetraaza-1(3,5)-pyridinacyclohexadecaphane-7-carboxylate (3) by Mixed anhydride method*[18]:**

A mixture of **2** (1 mmol) and ethyl chloroformate (2 mmol) in CH_2Cl_2 (25 mL) in the presence of TEA (2 mmol) was stirred for 20 min. at -20°C, then L-lysine methyl ester, (1 mmol) in CH_2Cl_2 (5 mL) was added at (-20°C) with stirring for 6 hrs and then overnight at room temperature. The mixture triturated with H_2O, 1N HCl, 1N $NaHCO_3$ and H_2O to give derivative **3**. The obtained product 4 was identified and comparison with authentic sample.[16]

Synthesis of 4,14-diisopropyl-2,5,13,16-tetraoxo-3,6,12,15-tetraaza-1(3,5)-pyridin-acyclohexadecaphane-7-carboxylic acid "cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine]" (4)

To a cold mixture of **3** (1 mmol) in methanol (10 mL), methanolic sodium hydroxide was added at -5°C with stirringfor 3 hrs and for 12 h at room temperature. The mixture was acidified with 1 N HCl to pH ≈ 3. The obtained solid was filtered off, washed with water, dried, and recrystallized from ethanol to afford the derivative**4**. The obtained product **4** was identified and comparison with authentic sample.[16]

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Biological Activities

Anticancer activities

The prepared compound was evaluated for its anticancer activities against 17 different cancerous cell lines representing different cancer tissues and types. The assay depended on crystal violet staining[19] after formalin fixation for the cells exposed to different serial dilutions of the prepared compound. DMSO was used as solvent as well as negative control throughout the experiments, while different known pharmaceutical drugs were used as positive control to compare the expected activities of the compound with known used drugs. Briefly, cells were kept and propagated at standard cell culture conditions. Microtiter plates were seeded with exponentially growing cells at a concentration of 2×10^4/well, and were incubated for 24 h at 37°C. Afterwards, wells received 50 µl of each serial dilution of the prepared compound and were further incubated for another 24 h. Then, the plates were inverted and shake to free cells from plates. 10% formalin was added as a fixative for 10 min, and then was thoroughly washed and aspirated. 0.09% crystal violet was used to stain the cells for 15 min, and then plates were washed with saline twice, examined and counted microscopically for differentiation between dead and living cells. IC_{50} values were determined from the linear regression curves of the obtained data.

VEGFR-2 kinase activity

The inhibitory effect of the synthesized compound (4) against VEGFR−2 tyrosine kinase enzyme was evaluated using ELISA technique as described in Zhang *et al*.[20] The enzyme substrate/PBS was used to label well plates, which were then incubated for 12 h at 37°C. Plates were washed and dried. Samples were added and then VEGFR-2 kinase were added. The reaction processed as normal and the antibody PY99 was used, followed by peroxidase addition. OPD coloring solution was added and plates were incubated for 30 min. The reaction was terminated with 2 M H_2SO_4, and the absorbance was read at 490 nm.

In vivo antiprostate cancer animal model

Male Wistar rats were used to evaluate the antiprostate cancer activity in vivo according to Kinoyama *et al*.[21] The used experiments depends on treating animals with androgen (testosterone propionate, 0.5 $mg \cdot kg^{-1}$, s.c.) for 5 days alone or in combination with our synthesized compound (10–30 $mg \cdot kg^{-1}$, p.o.). The method followed the described specific protocol. The antiprostate cancer activities activity was determined as the fraction of inhibited testosterone propionate effect.

Results and Discussion

Chemistry

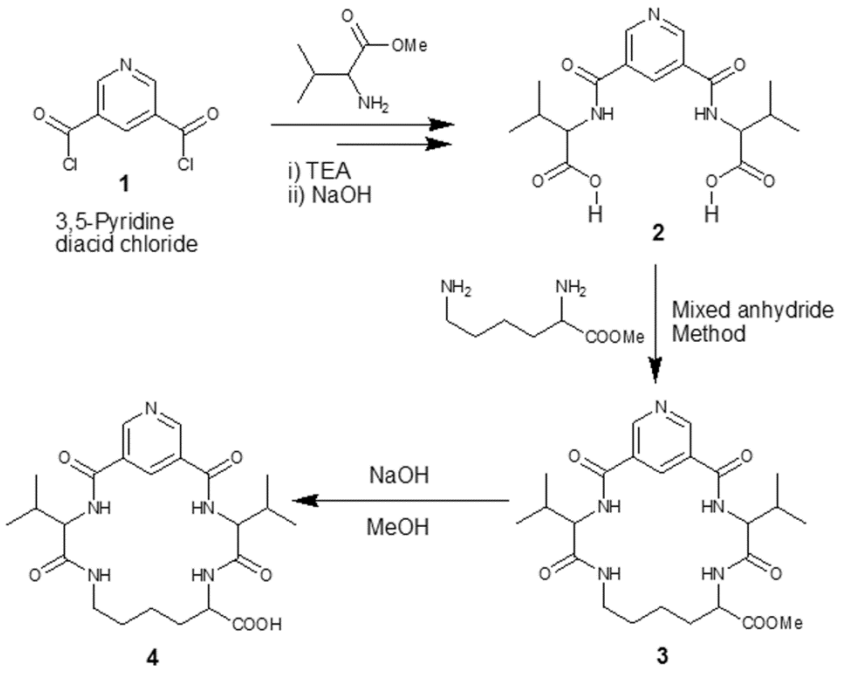
In our previous work[17], 4,14-diisopropyl-2,5,13,16-tetraoxo-3,6,12,15-tetraaza-1(3,5)-pyridin-acyclohexadecaphane-7-carboxylic acid "cyclo (Nα-dinicotinoyl)-bis-[(L-valinyl)- L-lysine]" **(4)** was synthesized by treating of compound **3** with methanolic sodium hydroxide, which was obtained from 3,5-pyridine dicarboxylic acid with L-valine methyl ester to give compound **2**. Compound **2** was cyclized by reacting with L-lysine methyl ester (Mixed anhydride method) to give macrocyclic tripeptide **3** according to reported procedure[1] (Scheme 1). In the current study, we report the evaluation and activities of compound **4** as possible anticancer agent.

Biological Screening

Anticancer activities

This section of the work was designed to evaluate the potential anticancer activities of compound 4 against different cancerous cell lines. Results obtained and presented (Fig 1) clearly show that Compound 4 has potential and promising anticancer activities against most of the tested cell lines. It can be seen that the recorded IC_{50} values varied according to cell type used. This is in great accordance with our previously reported work on anticancer activities of natural extracts and synthesized compounds.[16,22–24] This can be mainly explained due to the variation in cell membrane structures between various cancerous cell types.

Furthermore, in terms of IC_{50} values, it can be seen that the synthesized cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine has higher anticancer activities against HeLa (cervical), HT1080 (fibrosarcoma), K561 (leukemia), RKOP27 (colon), HepG2 (liver) and PC3 (prostate) cell lines. The activities increased by about 13.2-, 5.2-, 1.94-, 1.51-, 1.1-, 1.04-folds from their corresponding positive control drugs. The obtained IC_{50} values for these cell lines recorded 1.56, 2.56, 3.43, 2.87, 3.17 and 3.44 nM, respectively, while their positive controls showed much higher IC_{50}



Scheme 1 — Chemical structure for synthesized compounds[20]

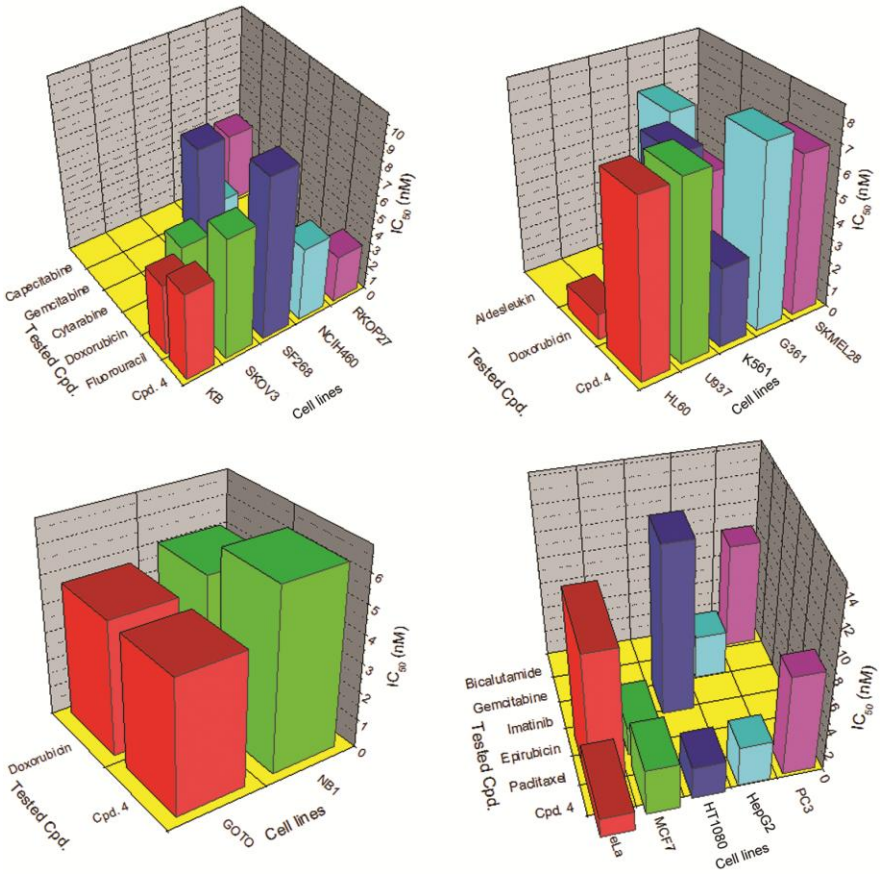


Fig. 1 — Cytotoxic activities of synthesized compound 4 against various tested cell lines

values of 11.8, 13.24, 6.66, 4.33, 3.44 and 8.22 nM for Paclitaxel, Imantinib, Doxorubicin, Capecitabine, Gemcitabine and Bicalutamide, respectively. On the other hand, the synthesized compound ineffective against KB (cervical), SKOV3 (ovarian), SF268 (brain), NCIH460 (lung), HL60 (leukemia), U937 (leukemia), G361 (melanoma), SKMEL28 (melanoma), NB1 (neuroblastoma) and MCF7 (breast) cell lines, when compared to their corresponding IC_{50} values obtained by the positive control drugs. For GOTO neuroblastoma cells, the synthesized compounds showed an IC_{50} value similar to that recorded for the positive control (4.67 and 4.73 nM for Cpd. 4 and Doxorubicin, respectively.

Evaluation of possible mechanism of action

The anti-VEGFR-2 activity assay of the tested compound was performed to gain an insight about its possible mechanism of action in comparison to the known Sorafenib control drug. Results showed that our newly synthesized cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine inhibited the VEGFR-2 enzyme at an IC_{50} of 1.76 nM, which was about 18% higher than the IC_{50} obtained for the control Sorafenib (2.0 nM). These results suggest that the prepared compound exhibited its anticancer activity probably through the inhibition of the VEGFR-2 enzymatic action.

Antiprostate cancer animal model

The *in vivo* antiprostate cancer model was tested in male Wistar rats to evaluate the potential of the synthesized cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine derivative to exhibit antitumorigenic effects in animal models. Results showed that the tested derivative had an increased *in vivo* antitumorigenic activity, which was about 3.28-folds higher than the effect obtained for the positive control. The obtained ED50 values were 3.54±0.023 and 11.60±0.09 for compound 4 and Flutamide, respectively.

Conclusions

The current work was designed to investigate the possibility of synthesizing a new cyclo (N^{α}-dinicotinoyl)-bis-[(L-valinyl)-L-lysine peptide and to evaluate its *in vitro* as well as *in vivo* anticancer activities. Results showed that the obtained compound has a great anticancer potential against 6 out of 17 tested cancerous cell lines, with variable degrees of cytotoxic effects depending on the cell line itself. Furthermore, the mechanism of action by which the synthesized compound is expected to exert its action is supposed to be through the inhibition of VEGFR-2 enzymatic pathway. Additionally, animal model experiments proved the effectiveness of the newly synthesized peptide.

Abbreviations

IC_{50} Half maximal inhibitory concentration
ED_{50} Median effective dose

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