CYTOTOXICITY AND DETECTION OF DAMAGE TO DNA BY 3-(5-NITRO-2-THIENYL)-9-CHLORO-5-MORPHOLIN-4-YL[1,2,4]TRIAZOLO[4,3-C]QUINAZOLINE ON HUMAN CANCER CELL LINE HeLa

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Quinazolines – 1,3-benzodiazines are biological active compounds, which are used in the pharmaceutical industry, in agriculture and in the medicine. As documented in the literature, many derivatives demonstrated anticancer activity and they act as multitarget agents. 3-(5-Nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c] quinazoline (NTCHMTQ) – a new synthetically prepared quinazoline derivative was the most effective derivative in our primary cytotoxic screening. In this study, we evaluated cytotoxic/antiproliferative activity of NTCHMTQ using human tumor cell line HeLa. Possible interaction of 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c] quinazoline with calf thymus DNA was tested by the DNA – modified screen – printed electrode. Quinazoline derivative acted cytotoxically on tumor cell line HeLa. The IC₁₀₀ value was 10 µg/ml. The IC₅₀ values was found to be less than 4 µg/ml, a limit put forward by the National Cancer Institute (NCI) for classification of the compound as a potential anticancer drug. Quinazoline at micromolar concentrations induced morphological changes and necrosis of HeLa cells. Using the DNA based electrochemical biosensor, we have not found damage to DNA under in vitro conditions at an incubation of the biosensor in mixture with quinazoline.

INTRODUCTION

Quinazoline derivatives are used in the pharmaceutical industry, in medicine and in agriculture because of their antimicrobial, anti-inflammatory, diuretic, anticonvulsant, antiallergic, antihypertensive, antiparkinsonian effects and other properties¹⁻⁴.

As documented in the literature, many derivatives act as anticancer active compounds and they act as multitarget agents. They are antifolate thymidylate synthase inhibitors; epidermal growth factor receptor tyrosine kinase inhibitors; inhibitors of enzymes dihydrofolate reductase and tyrosine kinase, aldose reductase and cyclic GMP phosphodiesterase. Some quinazolines interact with cytoskeleton, they induce apoptosis and affect DNA topoisomerase⁵⁻⁹.

3-(5-Nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c] quinazoline (NTCHMTQ) is new synthetically prepared derivative, which in our previous study showed cytotoxic effects on cancer cell line B16. Quinazoline at micromolar concentrations induced morphological changes and necrosis of B16 cells, at nanomolar concentrations it demonstrated changes of F-actin cytoskeleton. It did not cause changes in cell cycle, did

not induce apoptotic cell death in B16 cells, did not have mutagenic effect.

The interactions of some anticancer agents with DNA have been investigated by a variety of techniques and there is a growing interest in the electrochemical methods for the determination of anticancer agents.

In this study, we investigated the cytotoxic effect of 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c] quinazoline on further cancer cell line HeLa and the electrochemical interaction of quinazoline with dsDNA at carbon paste electrode.

MATERIAL AND METHODS

We used the human tumor cell line HeLa obtained from the American Type Culture Collection (Rockville, MD, USA) which was adherently grown and propagated in supplemented minimal Eagle medium (Biocom Bratislava, Slovak Republic). Quinazoline prepared by Špírková et al.^{10,11} was dissolved in dimethyl sulfoxide (DMSO). All other chemicals were obtained from Sigma Chemicals (St Louis, MD).

Antiproliferative activity *in vitro* was measured by the cell growth inhibition assay. After 24 h of incubation, the

Fig. 1. The growth curves of HeLa cells treated for up to 72 h with NTCHMTQ.

Concentration of derivative ($\mu\text{g/ml}$): + = control, ◆ = 100, ■ = 75, ▲ = 50, × = 10, * = 1, ● = 0.1. Data represent means $\pm$ S.D. of three independent experiments. S. D. < 10 %.

HeLa cells (the starting inoculum of 3×10^4 cells/ml) were treated with quinazoline (concentrations of 100, 75, 50, 10, 1, 0.1 $\mu\text{g/ml}$). Control cells were treated with DMSO, its final concentration never exceeded 1%. After 24, 48 and 72 h of quinazoline treatment, HeLa cells were harvested in triplicate, first washed with PBS, then harvested by 0.25 % trypsin, and latter resuspended. Cell growth and viability were assessed by direct counting of 0.4% trypan blue dye-excluding cells.

DNA-modified screen-printed carbon electrode was applied to the study of interaction between quinazoline and DNA at the electrode surface¹². A computerized voltammetric analyzer ECA pol. fitted with a screen-printed three-electrode assembly including a carbon working electrode (SPE, 25 mm² geometric surface area), a silver/silver chloride reference electrode (Ag/AgCl/SPE with potential of 0.284 V vs conventional Ag/AgCl/sat. KCl electrode) and a carbon counter electrode was used for voltammetric measurements. The working electrode without any electrochemical preconditioning was chemically modified *ex situ* by covering with 5 μl of the dsDNA stock solution and leaving the electrode to dry overnight.

Calf thymus dsDNA was obtained from Merck. Its stock solution (0.1 mg/ml) was prepared in 1×10^{-2} mol/l

Fig. 2. The cytolytic effect of NTCHMTQ on morphology of HeLa cells during 72 h of influence. The control and treated cells grown on the cover slips. Then HeLa cells were taken out of the culture medium and photographed under a light microscope. Concentration of derivative ($\mu\text{g/ml}$): a = control cells, b = 100, c = 75, d = 50, magnification 10×8

Fig. 3. DP voltammograms of the $[\text{Co}(\text{phen})_3]^{3+}$ indicator at DNA/SPE before (1) and after (2) incubation in solution of 100 (b), 50 (c), 10 (d), 1 (e), 0.1 (f) $\mu\text{g/ml}$ quinazoline. (0) is blank. Control (a) is cis-Pt (5 $\mu\text{g/ml}$). Conditions: 120 s accumulation from 5×10^{-7} mol/l $[\text{Co}(\text{phen})_3]^{3+}$ in 5 mmol/l phosphate buffer solution of pH 7.0.

Table 1. Effect of quinazoline on DNA at 10 min incubation of DNA/SPE sensor in mixtures with quinazoline at 22 °C. Conditions: 5 mmol/l phosphate buffer solution pH 7.0 under stirring. S.D. is < 10 %.

Concentration of quinazoline (mg/l)	DNA marker signal (I/I_0)
100	0.91 ± 0.03
50	0.92 ± 0.04
10	0.96 ± 0.05
1	0.97 ± 0.04
0.1	0.99 ± 0.03
No quinazoline	0.99 ± 0.05
cis-Pt (5 µg/ml)	0.72 ± 0.02

Tris-HCl and 1×10^{-3} mol/l EDTA solution of pH 8.0 and stored at -4 °C. As a dsDNA redox indicator the tris(1,10-phenanthroline)cobalt (III) $[\text{Co}(\text{phen})_3]^{3+}$ complex was used. Concentrations of tested quinazoline were 100, 50, 10, 1, 0.1 µg/ml.

The DNA/SPE sensor was pre-treated by immersing to 5 mmol/l phosphate buffer solution (PBS) pH 7.0 under stirring for 5 min, and then rinsed with water. The $[\text{Co}(\text{phen})_3]^{3+}$ marker was accumulated from 5 ml of its 5×10^{-7} mol/l solution in 5 mmol/l PBS under stirring for 120 s at an open circuit. The differential pulse voltammogram (DPV) was recorded immediately from +0.300 to -0.500 V at the pulse amplitude of 100 mV, 2 mV scan step and the scan rate of 10 mV/s. The marker DPV peak current (I_0) was obtained using the evaluation against a base-line by standard software and the correction substantiating the mean marker peak current measured at the unmodified SPE under the same conditions. Then, the DNA/SPE sensor was regenerated by a removal of the electrostatically accumulated $[\text{Co}(\text{phen})_3]^{3+}$ ions from the DNA layer at treating in the buffer medium of higher ionic strength (1×10^{-1} mol/l PBS pH 7.0) under stirring during 60 s. To detect the damage to DNA, the same DNA sensor was incubated in a separate cell in the quinazoline mixture in 5 mmol/l PBS for 10 min under stirring and then rinsed with water. The marker peak current (I) was obtained again in duplicate using the DPV measurement/biosensor regeneration scheme and the normalized (relative) signal I/I_0 was calculated.

RESULTS AND DISCUSSION

Figure 1 represents the growth curves of HeLa cells treated for up to 72 h with NTCHMTQ at concentrations ranging from 100 µg/ml to 0.1 µg/ml. After 24 h, the three highest concentrations tested (100, 75 and 50 µg/ml)

had an acute cytolytic effect manifested by degeneration – lysis (necrosis) of certain parts of the HeLa cells. In the next time intervals the degeneration of cell population increased. The concentration of 10 mg/l induced a delayed cytotoxic effect. After 24 h of culturing 39.1% of the cell population proliferated but after 48 h and 72 h 100% degeneration of cell population was found. The concentration 1.0 mg/l of NTCHMTQ induced a cytotoxicity that was time-dependent. The HeLa cells treated for 72 h with the lowest concentration (0.1 µg/ml) grew as well as the control cells. These changes in viable cell number were also observed when aliquots of the cultures were examined by light microscopy.

The cytolytic effect of NTCHMTQ observed by light microscopy at concentrations 100, 75, 50 µg/ml for HeLa cells during 72 h of culturing is illustrated in Fig. 2. The control cells (Fig. 2a) grew on the surface of cultivation slide and during 72 h of incubating a monolayer was formed. Figures 2 b, c, d show significant morphology changes which occurred within 72 h in different stages of dying cell detachment from the covering slip. As seen from figures NTCHMTQ induces integrity damage of cytoplasmic membrane and lysis – necrosis of cells. The dying cells were stained by trypan blue dye.

The redox marker can be accumulated effectively within the DNA double helix from the $[\text{Co}(\text{phen})_3]^{3+}$ solution at both a polarization of the modified electrode by a positive potential and an open circuit. Depending on an ionic strength of the medium, intercalation (predominantly at high ionic strength) and electrostatic forces (predominantly at low ionic strength) take part in binding of the marker particles. The interaction as well as electrostatic binding are equilibrium processes which can be utilized for a removal of $[\text{Co}(\text{phen})_3]^{3+}$ from the DNA layer in solution without $[\text{Co}(\text{phen})_3]^{3+}$.

Damage to DNA was expressed by the relative signal I/I_0 , where I and I_0 are the corrected marker DPV peak currents in experiments with and without NTCHMTQ (Table 1). Calf thymus DNA on biosensor was exposed to different concentrations (100, 50, 10, 1, 0.1 µg/ml) of NTCHMTQ. Typical DP voltammograms of the $[\text{Co}(\text{phen})_3]^{3+}$ complex obtained at the DNA/SPE without and with quinazoline are shown in Fig. 3.

The treatment of the sensor in solution of quinazoline did not lead to a change in the signal. We can see (Fig. 3, Table 1) that NTCHMTQ did not cause change in calf thymus dsDNA. The little damage of dsDNA (only 9.3 %) was discovered only at concentration of 100 mg/l. A potential shift of the guest molecule is usually taken as a confirmation of its binding mode.

Based on results obtained here we can conclude that 3-(5-nitro-2-tienyl)-9-chloro-5-morpholine-4-yl-[1,2,4]triazolo[4,3-c] quinazoline manifested the significant *in vitro* cytotoxic/antiproliferative effect on human cancer cell line HeLa. Using the DNA based electrochemical biosensor, we have not found damage to DNA under *in vitro* conditions at an incubation of the biosensor in

mixture with quinazoline. Derivative at micromolar concentrations induced morphological changes and necrosis of HeLa cells.

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