Cytotoxic and genotoxic effects of some substituted tetrazolo[1,5-c]quinazolines*

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Nine substituted tetrazolo[1,5-*c*]quinazolines have been tested for cytotoxic effects and structure activity relationship on the murine cancer cell line B16 and four bacterial strains. The most cytotoxic activity had non-substituted in the aromatic ring or substituted by bromo- or chloro- goup, and in the pyrimidine ring of quinazoline skeleton by phenyl or morpholine group, respectively. In the bacterium all tested quinazolines had a lower antibacterial effect than ampicillin. 9-bromo-5-morpholino-tetrazolo[1,5-*c*]quinazoline (BMTQ) at the highest concentration tested (30.0 μmol/l) had an acute cytostatic effect manifested by the total inhibition of the cell proliferation. Other concentrations caused a cytotoxicity proportional to the concentation used. The IC_{50} values were found to be less than 4 μg/ml, a limit put forward by the National Cancer Institute (NCI) for clasification of the compound as a potential anticancer drug. BMTQ induced mutations in a dose-related manner, starting from 10 μg/plate in strains TA100 and TA102. Lesser but significant increases in revertant colonies were also obtained in strain TA98. The mutagenity was slighly enhanced by metabolic activation.

Key words: Cytotoxicity, genotoxicity, antibacterial activity, quinazoline derivatives, cancer cell line B16, cell proliferation

Many derivatives of quinazoline (benzopyrimidine; 1,3-benzodiazine) are used in the pharmaceutical industry, in medicine and in agriculture due to their antimicrobial [3, 10, 28], antiinflammatory [9, 24], diuretic [7], anticonvulsant [20], antiallergic [32], antihypertensive [4, 22] and antiparkinsonian effects [33]. Some of the quinazolines induced mutagenic activity [6, 36].

As documented in the literature, many quinazolines act as anticancer active agents [2, 8, 38] and they also act as antimetabolites from the group of folic acid analogues [26]. They are antifolate thymidylate synthase inhibitors [11], inhibitors of the tyrosine kinase activity of the epidermal growth factor [21, 29], inhibitors of DNA repair enzyme poly(ADP-ribose) polymerase [5] and some of these are now in clinical development [1, 18, 37].

With the aim of obtaining new antitumor agents, a series of substituted tetrazolo[1,5-*c*] quinazolines was prepared [34]. These compounds were tested for cytotoxic properties *in vitro* on human tumor cells HeLa. One of them, 9-bromo-5-morpholino-tetrazolo[1,5-*c*] quinazoline (BMTQ) which exhibited a high cytotoxic activity (IC_{50}=3.7 μmol/l) was further screened in standard antitumor systems, including leukemic L1210 cells and colon cancer Caco-2 cells [13]. This drug induced after 24 h of culturing a significant a concentration/dependent and time /dependent cytotoxic effect (IC_{50}=18.5 μmol/l for HeLa cells; IC_{50}=15.1 μmol/l for L1210 cells; IC_{50}=11.8 μmol/l for Caco-2 cells). BMTQ caused in human colon tumor cells Caco-2 and in Chinese hamster lung V79 cells increase of the level of ssDNA breaks [14, 16] in comparison to the control cells. In contrast, to increased level of ssDNA breaks, BMTQ did not induce apoptic DNA fragmentation.

The main aim of this study was to investigate in the primary screening the cytotoxic effect of nine tetrazolo[1,5-*c*]quinazolines in murine melanoma B16 cells and four bacteria strains. Futher, we studied the effect of BMTQ on the proliferation of B16 and genotoxic effects of quinazolines.

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Material and methods

Cultivation of cells. The murine tumor fibroblast cell line B16 were used. These cells grown in minimal Eagle medium enriched with 15% foetal calf serum. The following were added to the medium: penicillin (200 U/ml), streptomycin (100 μg/ml) and kanamycin (100 μg/ml). Cells were cultured in plastic Petri dishes in a CO_2 incubator, at 37 °C. Before a uniform monolayer was formed, B16 cells were freed from the surface of the culture dish by a 0.25% solution of trypsin (Biocom). Viability of the cells was determined by 0.4% trypane blue staining.

Bacterial strains. The bacteria *Escherichia coli* CCM 5172, *Pseudomonas aeruginosa* CCM 3955, *Staphylococcus aureus* CCM 3953 and *Enterococcus faecalis* CCM 4224 were used for the primary antibacterial test. The bacteria were kept on Müller-Hinton agar or Nutrient Broth agar (*E. faecalis*) at 4 °C and were subcultured every 7 day. Two days before the test the bacterial strains were transfered to fresh Müller-Hinton or Nutrient Broth agars.

Histidine-dependent strains of *Salmonella typhimurium* TA98, TA100 and TA102 were received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic). They were stored at –80 °C and regularly checked for their genetic markers.

Preparation of drugs. Substituted quinazolines were prepared by Dr. STANKOVSKÝ from the Department of Organic Chemistry, Faculty of Chemical and Food technology, Slovak University of Technology [34]. Chromatographically pure quinazoline derivatives dissolved immediately before use in dimethyl sulfoxide (DMSO, Sigma); its final concentration in the medium never exceeded 1% (V/V) in either control a treated cells. Murine B16 cells were treated with different concentrations of quinazolines ranging from 300.0 to 0.015 μmol/l (primary screening) and from 30.0 to 0.15 μmol/l (growth inhibition assay). In the bacterial tests were used quinazolines in the range from 500 to 0.1 μg/ml. Genotoxic activity of 9-bromo-5-morpholino-tetrazolo[1,5-*c*]quinazoline was tested at concentrations in the range from 1000 μg/plate to 10 μg/plate. Ampicillin obtained from Sigma was dissolved in water and used as a positive control in antibacterial test.

Primary screening. A three-day culture of B16 cells was trypsinized (0.25% trypsine) and used to prepare a suspension with a concentration of $2x10^4$ B16 cells/ml. The experiments were carried out in Leighton flasks into which 2 ml of the above-mentioned suspension were pipetted. After 24 h of culturing at 37 °C in a humidified incubator, 20 μl of one of the solutions of quinazoline derivatives, at seven concentrations in the range of 300.0–0.015 μmol/l, was added to the cells. First, the effect of the substances on cell morphology was microscopically evaluated after 48 h of incubation at 37 °C, then the intensity of cell growth was assessed using the LOWRY method [23] for determination of the total cell protein content with bovine serum albumin as the standard. The cytotoxic activity of the tested derivatives was determinated from the inhibitory concentrations IC_{50} (i.e. such concentration of a derivative which, in comparison to the control, inhibited the contents of the total cell proteins by 50 per cent, respectively) which were read out from the toxicity curves.

The growth inhibition assay. A starting inoculum $2x10^4$ B16 cells/ml in exponential phase of growth was used. Five ml of the suspension were added into plastic Petri dishes (diameter 60 mm) each. After 24 h of culturing, 50 μl of BMTQ at the indicated concentrations were added to the cells. Control cells were treated with the same amount of DMSO. After 24, 48 and 72 h, the treated and control B16 cells were washed once with phosphate buffered saline (PBS) to remove fragments and dead cells. The dishes were harvestes in triplicate with 0.25% trypsin, washed once with PBS and resuspended in the latter. The cells were recognized by their ability to exclude trypan blue stain (0.4%), unstained cells were counted in a counting chamber. Cytotoxic effects were evaluated in terms of inhibition of cell proliferation. Relative inhibition was calculated using the formula:

$$\% \text{ inhibition} = \frac{(K - E_x)}{K - K_o} \cdot 100$$

where K_o are cell counts at the time of addition of the respective compound; K are cell counts of control cells, E_x are cell counts of treated cells.

Antibacterial assay. The antibacterial effect was assayed by a microdilution method in 96-well microtitration plates [12]. The bacteria were cultured on Müller-Hinton medium at 30 °C. An overnight inoculum was prepared 12–16 h before the test. The growing inoculum was filtred and a 1.5% suspension of bacteria was prepared for the experiments. This suspension (180 μl) was added to 20 μl of the tested complex solution and cultured for 8 h on a reciprocal shaker in a termostat at 30 °C. The time course of absorbance (A_{630}) was then determined in three parallels. To compare the antibacterial activity, ampicillin at concentrations 100, 10, 1, 0.1 and 0.01 mg/l was used as positive control. After an 8 h cultivation with quinazolines the bacteria were inoculated to a solid culture medium and cultured statically for 1 day at 37 °C. The microbistatic (MBS) or microbicide (MBC) concentrations were determined.

The antibacterial effect was characterized by IC_{50} values, i.e. the minimal inhibition concentration of a substance which inhibits bacterial growth by 50% relative to the control, and MIC, i.e. the minimal inhibitory concentration of a substance which inhibits the bacterial growth by 100%. The IC_{50} and MIC values were determined from toxicity curves.

Bacterial mutagenicity test. The plate-incorporation

method according to MARON and AMES [25] was used. To 2 ml of melted top agar containing 50 μmol/l of L-histidine-biotin, 0.1 ml of acell suspension (cultivation for 16 hours at 37 °C, approximate cell density 2–5x10^8 cells/ml) and 0.1 ml of a solution of the tested compound were added. In the test with metabolic activation 0.5 ml of freshly prepared S9 mix was added. The mixture was poured onto a minimal glucose agar plate and incubated for 48 h at 37 °C and then the number of histidine-independent revertants was counted. Data points represent at least three separate experiments, each run in triplicate. A positive response was defined as a reproducible, two-fold increase of revertants with dose-response relationship and statistical evaluation using t-test.

Preparation of the S9 fraction. Liver S9 homogenate was prepared from male Wistar rats induced by Delor 103 (500 mg/kg) five days before sacrifice as described by MARON and AMES [25].

Statistics. The values IC_{50}, MIC in Tables 1, 2, 3 and the growth curve in Figure 1 represent the results obtained from the three separate experiments (for each concentration of BMTQ three separate cultivation dishes were used). The individual data points are presented as arithmetic means $\pm$S.D. (standard deviation). The statistical significance of the results was evaluated by Student's t-test, with probability values of 0.05 being considered as significant. The results from the Ames test are from three experiments (with three parallel samples) and were statistically evaluated using the Student's t-test.

Table 1. Cytotoxic activity[a] of substituted tetrazolo[1,5-c]quinazolines (formula 1) on B16 cells

Compound	X	R	IC_{50}	IC_{100}
1	H	Dpha	95.0 $\pm$ 4.2	>295.0
2	9-Cl	Mo	6.0 $\pm$ 0.25	34.4 $\pm$ 2.5
3	9-Br	Mo	1.5 $\pm$ 0.08	29.8 $\pm$ 1.1
4	9-Cl	Pi	21.3 $\pm$ 1.8	346.0 $\pm$ 21.0
5	H	Mo	7.8 $\pm$ 0.5	390.0 $\pm$ 20.5
6	H	Ph	90.0 $\pm$ 2.1	>400.0
7	9-Cl	Ph	6.0 $\pm$ 0.3	117.0 $\pm$ 10.3
8	9-Br	Ph	15.3 $\pm$ 1.0	>306.0
9	7-CH_3	Ph	19.0 $\pm$ 0.9	>380.0

[a]values IC_{50} and IC_{100} are in μmol/l and were obtained from the determination of total cell protein content after 48 h of treatment; Dpha – diphenylamino, Mo – morpholinyl, Pi – piperidinyl, Ph – phenyl.

Table 2. Cytotoxic effect[a] of 72 h 9-bromo-5-morpholino-tetrazolo[1,5-c]quinazoline treated of B16, L1210[b], HeLa[b] and Caco-2[b] cells

Time (h)	B16	L1210	HeLa	Caco-2
24	2.45 $\pm$ 0.01	15.1 $\pm$ 0.9	18.5 $\pm$ 0.8	11.8 $\pm$ 0.7
48	0.15 $\pm$ 0.005	24.6 $\pm$ 0.8	12.2 $\pm$ 0.5	20.7 $\pm$ 1.0
72	0.15 $\pm$ 0.004	22.4 $\pm$ 0.7	8.4 $\pm$ 0.2	32.2 $\pm$ 1.4

[a]values IC_{50} are in μM; [b]values are from the article JANTOVÁ et al [16].

Table 3. Antibacterial activity[a] of substituted tetrazolo[1,5-c]quinazolines

Compound	*E. coli*		*P. aeruginosa*		*S. aureus*		*E. faecalis*	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
1	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
2	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
3	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
4	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
5	>1000	>1000	>1000	>1000	>1000	>1000	940.0	>1000
6	290	>1000	900	>1000	110	250[b]	<100	250[b]
7	450	1000[b]	450	>1000	105	1000[b]	300	1000[b]
8	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
9	700	>1000	500	1000[b]	560	1000[b]	200	>1000
Amp	0.28	1[c]	>500	>500	0.015	0.04[c]	<1	1

[a]values IC50 and MIC are in μg/ml; [b]microbistatic effect; [c] microbicide effect; Amp – ampicillin.

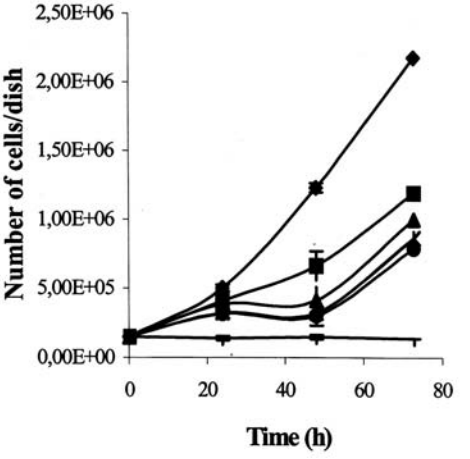

Figure 1. Effect of 9-bromo-5-morpholino-tetrazolo[1,5-c]quinazoline on B16 cell division. Concentrations of quinazoline μmol/l: ◆ = control; ■ = 0.15; = ■ 0.29; x = 0.75; * = 1.5; = ● 3.0; + = 30.0.

Results

The results obtained in the primary screening of cytotoxicity of the nine substituted tetrazolo[1,5-*c*]quinazolines are shown in Table 1. The values of inhibitory concentrations IC_{50} were obtained from the total cell protein content. The highest cytotoxic effect on the B16 cells was induced by 9-bromo-5-morpholino-tetrazolo[1,5-*c*]quinazoline (derivative 3; BMTQ) and by 9-chloro-5-morpholino-tetrazolo[1,5-*c*]quinazoline (derivative 2; IC_{50}<5 μmol/l, IC_{100}<50 μmol/l). Certain cytotoxicity was observed in 9-chloro-5-phenyl-tetrazolo[1,5-*c*]quinazoline (derivative 7) and in 5-morpholino-tetrazolo[1,5-*c*]quinazoline (derivative 5). The weakest activity was found with 2-phenyl- tetrazolo[1,5-*c*]quinazoline (derivative 6) and with 2-diphenyl-amino- tetrazolo[1,5-*c*]quinazoline (derivative 1). The same results were found on the HeLa cells [13].The results of the primary screening led to further characterization on the biological effect of BMTQ on cell division.

Table 4. Mutagenic effect of 9-bromo-5-morpholino-tetrazolo[1,5-c]quinazoline with (+S9) and without (-S9) the metabolic activation

Dose	TA 98				TA 100				TA 102			
μg/plate	-S9		+S9		-S9		+S9		-S9		+S9	
	M[a]	±SD[b]	M	±SD	M	±SD	M	±SD	M	±SD	M	±SD
0	27	2.8	29	3.0	154	16.1	144	5.7	295	20.5	287	23.5
10	24	3.4	27	2.6	215**	21.6	229**	19.9	380**	36.3	401**	20.3
100	29	3.8	33	3.1	233**	22.5	262**	21.4	420**	48.2	447**	30.9
500	47*	9.7	59**	5.27	327**	24.5	351**	14.3	430**	30.7	461**	32.0
1000	58**	7.4	60**	4.9	409**	23.2	431**	40.5	520**	35.8	556**	28.4
PC[c]	279**	23.2	300**	15.3	1179**	55.9	1219**	33.3	892**	77.6	1146**	42.9

[a]mean number of revertants/plate (M); [b]standard deviation (SD); [c]positive control without metabolic activation: 5-nitro-2-furylacrylic acid (sodium salt) 5 μg/plate; positive control with metabolic activation: 2-aminoanthracene 5 μg/plate. *$p<0.01$, **$p<0.001$.

Figure 1 represents the growth curves of B16 cells treated for up to 72 h with BMTQ at concentrations ranging from 30.0 to 0.15 μmol/l. After 24 h, the highest concentration tested (30.0 μmol/l) had an acute cytostatic effect manifested by total inhibition of the cell proliferation. The other concentrations tested caused a cytotoxicity proportional to the concentration used. In the next 24 h interval, the cells treated by the all concentrations tested (besides the lowest concentration) were not divided. On the other hand, after 72 h the cell proliferation was found.

Table 2 shows the values of growth inhibitory concentrations IC_{50} of BMTQ. The values were obtained from the growth curve of B16, L1210, HeLa and Caco-2 cells treated for 72 h with BMTQ. The highest sensitivity of B16 cells was observed (the values for B16 cells were 4.8–214.6-times lower than values for other cell lines).

The results obtained in the antibacterial test are shown in Table 3. The sensitivity of G^+ bacteria to the quinazolines was higher than that of G^- bacteria. In the bacterium all tested quinazolines had a lower antibacterial effect than ampicillin. The most effective were compounds 6, 7 and 9. BMTQ was inactive against tested bacterium, the MIC values were higher than 1000 μg/ml.

Assessment of the mutagenic activity of 9-bromo-5-morpholino-tetrazolo[1,5-*c*] quinazoline was performed using *Salmonella typhimurium* TA98, TA100 and TA102 with and without the metabolic fraction S9 (Tab. 4). This derivate induced mutations in a dose-related manner, starting from 10 μg/plate in strains TA100 and TA102. Lesser but significant increases in revertant colonies were also obtained in strain TA 98. The mutagenity was slightly enhanced by metabolic activation.

Discussion

When we searched for new anticancer drugs, in our primary screening we studied the cytotoxic effect of nine substituted tetrazolo[1,5-*c*]quinazolines on HeLa cells [13]. The highest cytotoxic effect was induced by 9-bromo-5-morpholino-tetrazolo[1,5-*c*] quinazoline (BMTQ). Furthermore, the induction of cytotoxicity and ssDNA breaks by BMTQ in L1210 and Caco-2 cells and antiproliferation activity and effect on cell cycle in HeLa and V79B cells were monitored [16]. Our results showed BMTQ to be the most potent antiproliferation and cytotoxic agent which activity depended on its concentration and on the time of treatment. The cytolytic concentration of BMTQ induced integrity damage of cytoplasmic membrane. The S-phase specific effect of BMTQ on the cell cycle of synchronous population of non cancer cell line V79B was found. On the other hand, we found that BMTQ had no effect on the cell cycle profile of asynchronous population of L1210 and Caco-2 cells. Further, BMTQ exhibited a significant increase of the level of ssDNA in V79 cells and single-strand DNA breaks in the Caco-2 cells. On the other hand, BMTQ did not induce apoptosis.

For confirming *in vitro* cytotoxic effects we studied the exposure of BMTQ on other model cancer cell line B16. Therefore, a lot of clinical used cytostatics show antibacterial effects too, further we followed antibacterial activity of tetrazolo [1,5-*c*]quinazolines at four bacterial strains from collection. We also studied their genotoxic activity on the histidine dependent strains of *Salmonella typhimurium* TA98 and TA100.

The comparison of the tetrazole structure and their cytotoxic effect on the B16 cells (Tab. 1) showed that the most active derivatives were non-substituted in the aromatic ring or substituted by bromo- or chloro- group, and in the pyrimidine ring of quinazoline skeleton by phenyl or morpholino group, respectively (derivatives 3, 2, 7 and 5). We obtained the same results with HeLa cells [13].

As can be seen from Figure 1, the highest concentration tested (30 μmol/l) immediately stopped (after 24 h of culturing) the growth of the B16 cells, and a certain part of the cell population degenerated. This effect was proportionally increased with the time of exposure. While 24 h and 72 h exposing of the cells with other concentrations caused increase in the cell growth, in the 48 h time interval B16 cells did not proliferate.

BMTQ acted cytotoxically on murine leukemia cell line L1210 and human colon carcinoma cells Caco-2 cells, too [16]. We found that the two highest concentrations of BMTQ (149.2 and 74.6 μmol/l) exhibited an acute cytotoxic effect, however other tested concentrations (<74.6 μmol/l) manifested a concentration/dependent and time/dependent cytotoxic effect.

The compared values IC_{50} as determined by the growth inhibition assay (Tab. 2) show that the most sensitive to the BMTQ are B16 cells.

Recently, the National Cancer Institute (NCI) [30] has recommended that if the value is less than 4 μg/ml, the compound can be considered as a having a cytotoxic effect and hence can be classified as a potential anticancer drug. As shown in Tables 1 and 2, the potency of BMTQ corresponds to the borderline of this criterion, with the IC_{50} values for B16 cells lower than 4 μg/ml.

From the results in Table 3 it could be suggested that BMTQ has not antibacterial activity because it was inactive against all tested bacteria.

Mutagenic data provided by Ames test showed that BMTQ exhibit mutagenic effect which was slightly enhanced by metabolic activation.

Mutagenic activity of some quinazoline derivatives was found. JIMENEZ et al [17] established that 2-amino-11*H*-pyrido[2,1-*b*]quinazolin-11-one (2-APQ) – a potential antiasthma drug induced a strong mutagenicity in five strains of *Salmonella typhimurium*. S9mix generally increased the response of 2-APQ in *S. typhimurium* dramatically.

With the aim of obtaining new antitumoral agents, a series of 5,8-quinazolinediones was prepared and examined for their cytotoxic properties on L1210, P388, B16 and sarcoma 180 cells by RENAULT et al [31]. 6,7-bis(1-aziridinyl)-5,8-quinazolinedione exhibited a high cytotoxicity and had a significant mutagenic effect on *Salmonella typhimurium* TA98 and TA 100 strains. These results suggested that DNA damage could be responsible for its cytotoxicity.

Four antitumoral 5,8-quinazolinediones were examined for their ability to induce mutation in *Salmonella typhimurium* by CALLAIS et al [6]. Aziridinylquinones were mutagenic in the four strains with or without activation by S9mix.

Batracylin (NSC-320846, 8-aminoisoindolo[1,2-*b*]quinazolin-12(10*H*)-one) is a quinazoline recently evaluated as a potential antitumor agent. STEVENS and MCQUEEN [35] evaluated the genotoxicity of batracylin. The mutagenicity was tested in *Salmonella typhimurium* (TA98, TA100, TA102) with and without Aroclor-induced rat liver S9. Batracylin induced histidine revertants in all three strains with a higher number of mutants formed in the presence of S9.

The DNA changes may be caused by the ability of chemotherapeutic agents demonstrated to intercalate DNA. In our experiments, BMTQ induced single-strand DNA breaks but did not induce apoptosis. The origin of ssDNA breaks could by explained by the ability of BMTQ to intercalate to the double-strand DNA (dsDNA). A possible intercalat capability of the tested BMTQ was pointed out by LABUDA et al [19]. BMTQ fulfils the conditions of the three conjugated aromatic circles that usually allow a given substance to manifest intercalating properties. In intercalation there is not the question of covalent binding of a compound to a DNA molecule, but there is the problem of intervening to the structure of DNA and binding a component of a biopolymer via hydrogen bridges. Our research on binding cooccurrence of BMTQ with redox indicator indicates that intercalation could occur in a potentially adapted dsDNA.

From our results it can be concluded that 9-bromo-5-morpholino-tetrazolo[1,5-*c*] quinazoline based on the NCI criteria has sufficient cytotoxic potency to be classified as a potential anticancer drug. Anticancer activity of BMTQ may be due to DNA interactions, since many chemotherapeutic agents directly target DNA. As the genotoxicity of BMTQ was observed in bacteria, further studies in mammalian system and *in vivo* studies are needed. This will be the subject of our next study.

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