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Scientific Paper

CYTOTOXIC AND GENOTOXIC EFFECTS OF THE QUERCETIN/LANTHANUM COMPLEX ON HUMAN CERVICAL CARCINOMA CELLS *IN VITRO*

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Quercetin is the main flavonoid in diet with a potential in the treatment of cancer, cardiovascular, and neurodegenerative diseases. Due to its specific planar chemical structure, quercetin readily forms chelates with metal ions. Complexes of bioactive compounds and metal ions such as lanthanum often show strong cytotoxic and antitumour properties. The aim of this study was to compare the genotoxic effects of the quercetin/lanthanum complex on human cervical carcinoma cells with compare it to the effects of free ligands, quercetin, and lanthanum alone. The quercetin/lanthanum complex showed considerable cytotoxicity in the concentration range of (100 to 1000) mmol mL^{-1} and exposure time of three hours. The complex also induced a dose-dependent pro-oxidative effects and the formation of single-strand and double-strand DNA breaks. Although we obtained promising results on the cell level, future experiments should answer whether the quercetin/lanthanum complex is cancer-specific and stable enough in physiological conditions to make a potential new antitumour drug.

KEY WORDS: *chelates, DNA damage, flavonoid/metal complexes, oxidative damage*

Evidence abounds about antitumour activity of small-molecule compounds, including metal complexes with flavonoids, which directly interact with DNA (1). Recently, much attention has also been paid to how cancer cell growth and apoptosis are affected by rare earth metals and compounds such as lanthanum and cerium salts (2, 3).

Lanthanum salts can arrest the cell cycle in the G1/S phase in leukemic cell lines (2) and inhibit telomerase activity in the lymphoblastoid tumour cell line (3). Both induce apoptotic events. Contrary to this effect observed in tumour cells, no effect could be determined in normal bone marrow haematopoietic cells (2). Lanthanum complexed with bioactive compounds from plants has shown even stronger cytotoxic effect than when administered alone (4), suggesting that electron transfer and oxidative stress are responsible for the cytotoxic effect. Kostova et al. (5) found that lanthanum complexes with bis-coumarins caused apoptotic cell death of several leukaemia and lymphoma cell lines, causing DNA fragmentation and morphological changes. The authors believe that this cytotoxic effect depends on the metal-ligand structure. Wang et al. (1) analysed a lanthanum-naringenin Schiff-base complex and found that the ligands were coordinated to the lanthanum ion through deprotonated hydroxyl groups. This complex bound directly to calf-thymus DNA, probably through intercalation, while the same concentrations caused a strong cytotoxic effect on human leukaemia and lung adenocarcinoma cells. Intercalation is the main cytotoxic mechanism of numerous antitumour agents such as cisplatin, but their use is limited by drug resistance. Determination and synthesis of new agents

with similar mechanism of action could improve the chances for successful cancer treatment, even though drug resistance has already occurred (6). In the Wang et al. study (1), lanthanum-coumarin complexes caused a significant cytotoxic effect on drug-resistant cell lines and clearly confirmed their potential as antitumour agents.

Studies of flavonoids have also provided ample evidence of their bioactive properties *in vitro* and *in vivo*; quercetin as one of the most abundant flavonoids, present in all plant parts, possesses anti-inflammatory, anti-proliferative, and anti-atherosclerotic properties (7). It acts as an antioxidant and a free-radical scavenger (8, 9). There is also evidence of its prooxidative action (8, 10). Van der Houde et al. (11) have found that quercetin biphasically stimulates cell division at concentrations ranging from 0 mmol mL^{-1} to 60 mmol mL^{-1}, probably because it binds to oestrogen receptors, and can arrest the cell cycle at concentrations above 60 mmol mL^{-1}. According to them, what inhibits cell division is DNA breakage, induction of apoptosis, and inhibition of kinases involved in cell-cycle regulation. Marinić et al. (12) found that quercetin intercalated into double-strand (ds) DNA, dsRNA, and single-strand RNA.

Having in mind these biological effects of flavonoids, lanthanum, and lanthanum complexes, we wanted to see how the lanthanum-quercetin complex behaved in physiological conditions. In addition, we wanted to see if the lanthanum-quercetin complex had a stronger cytotoxic, prooxidative, and genotoxic effects on human cervical carcinoma cell line (HeLa) than lanthanum and quercetin alone. In case it did, lower doses of the complex would have the same biological effect on the tumour cells, without the side effects associated with high drug concentrations.

MATERIALS AND METHODS

Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), malondyaldehyde (MDA), nicotine adenine dinucleotide (NADH), sodium-pyruvate, neutral red (NR), thiobarbituric acid (TBA), quercetin (Q) and lanthanum (La) were obtained from Sigma Chemical Co. (St. Louis, USA).

Stock solutions of quercetin and lanthanum were prepared in 96 % ethanol (Kemika, Zagreb, Croatia) in the concentration of 5.84 mol mL^{-1} for quercetin and 1.22 mol mL^{-1} for lanthanum. The Q/La complex was prepared before HeLa cell treatment. Dilutions were prepared in a physiological solution (0.9 % NaCl; Imunološki zavod, Zagreb, Croatia) because La complexes are formed in physiological solution at 37 °C. The final concentration of ethanol in the samples after dilution did not exceed 1 %. This concentration was not toxic for the cells (data not shown). As the stability of the La complexes in growth medium drops significantly in the presence of phosphate ions, HeLa cells were incubated without the growth medium. The time of incubation was set to maximum three hours, during which time cells can survive without the growth medium.

Human cell line

Human cervix carcinoma cell line HeLa was grown as a monolayer culture in 75 cm^2 T-flasks (Falcon, USA) in Dulbecco's Modified Eagle Medium (DMEM; Imunološki zavod, Zagreb, Croatia) supplemented with 10 % of foetal bovine serum (Gibco), 4,500 mg L^{-1} glucose, pyridoxine, and 1 % penicillin/streptomycin solution (Gibco).

Cell survival

Briefly, the HeLa cell line was seeded in 96-well plates ($1.8x10^4$ cells/0.18 mL per well). After attachment, the cells were treated with Q, La, and the Q/La complex for three hours, using a wide range of concentrations (from 100 mmol mL^{-1} to 1000 mmol mL^{-1}) in order to determine their nontoxic, subtoxic, and toxic concentrations.

After the treatment, the cells were washed and treated either with a 10 % MTT solution or with a 1 % neutral red solution to determine cell survival (13, 14). The absorbance of formazan (540 nm) or neutral red (492 nm) imported into cellular lysosome was measured using a microtitre reader (Cecil Instruments Ltd., Technical Centre Cambridge, UK) and compared with the absorbance measured in untreated (control) cells. Cell survival was calculated relative to control. The experiments were repeated three times (13, 14).

Oxidative damage of cellular lipids

$1.5x10^6$ cells were seeded in 10-cm Petri dishes, attached, and treated with previously determined subtoxic concentrations of Q, La, and the Q/La complex (300 mmol mL^{-1}, 400 mmol mL^{-1}, and 500 mmol mL^{-1}, for each) for three hours. The cells

were then scraped, washed, and lysed, and the cell supernatant incubated with trichloroacetic acid, cooled, and mixed with thiobarbituric acid. The absorbance was read at 532 nm and 600 nm. The concentration of malondyaldehyde-thiobarbituric acid (MDA-TBA) complex was determined from the standard curve (15, 16) and expressed in mg of cellular protein (17). Each experiment was repeated three times.

Alkaline comet assay

The comet assay followed the procedure described by Singh et al. (18). Three replicate slides per sample were prepared as previously described by Rusak et al. and Durgo et al. (19, 20). If not specified otherwise, chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, USA).

After 1-hour lysis in ice-cold alkaline lysis solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} Na_2EDTA, 10 mmol L^{-1} Tris-HCl, 1 % Na-sarcosinate, pH 10) with 1 % Triton X-100 and 10 % dimethyl sulphoxide (Kemika, Zagreb, Croatia), DNA was denatured in alkaline conditions (300 mmol L^{-1} NaOH, 1 mmol L^{-1} Na_2EDTA, pH 13.0; 4 °C for 20 min) and electrophoresised at 25 V for 20 min. After electrophoresis, the slides were neutralised with Tris-HCl buffer (pH 7.5), stained with ethidium bromide and analysed using a 250x epifluorescence microscope (Leitz, Germany) and the Comet Assay II image analysis system (Perceptive Instruments Ltd., Suffolk, UK). To measure DNA damage, we selected tail DNA % as a reliable parameter. A total of 150 comets per sample (50 from each replicate slide) were scored and data pooled.

Statistical analysis

For statistical analysis we used the commercial software SPSS version 8.0 (SPSS Inc., Chicago, IL, USA). We calculated the standard parameters of descriptive statistics. Inter-group comparisons were performed using the one-way analysis of variance (ANOVA) with post-hoc Dunnett's test. The probability level of $P<0.05$ was considered significant.

RESULTS

The results of the MTT assay (Figure 1a) show that treatment with La and Q alone did not reduce cell survival below 80 %. Lower concentrations of Q/La complex (100 mmol mL^{-1} to 600 mmol mL^{-1}) decreased cell survival to 70 %, while higher concentrations restored it back to 80 % (Figure 1a).

Results obtained with the NR assay showed stronger differences in the cytotoxic effect of Q and Q/La complex; Q decreased cell survival gradually, in a dose-dependent manner, and the concentrations higher than 400 mmol mL^{-1} decreased cell survival below 80 %. The Q/La complex showed the strongest cytotoxic effect; we calculated its EC_{50} to be 771.83 mmol mL^{-1} (Figure 1b), while with the MTT assay the EC_{50} value for the Q/La complex could not be calculated, because cell survival never dropped below 60 % (Figure 1a).

The prooxidative effects of Q, La, and the Q/La complex were examined at nontoxic and subtoxic concentrations, which were chosen from Figure 1b. The prooxidative effect of the Q/La complex was recorded at concentrations higher than 400 mmol mL^{-1} (Figure

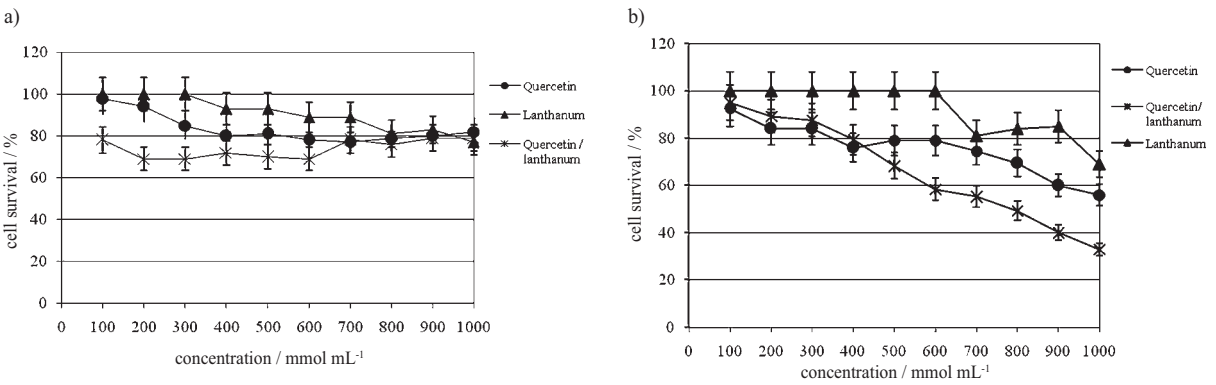

Figure 1 *Cell survival after a 3-hour treatment with quercetin, lanthanum, and the lanthanum/quercetin complex determined with the a) MTT assay and b) the NR assay. Results are shown as means ±SD of three experiments. Estimated cytotoxicity (EC_{50}) of the lanthanum/quercetin complex determined with the NR assay was 771.83 mmol mL^{-1}.*

2), which coincided with its cytotoxic effect (Figure 1b). At the nontoxic concentration of 300 mmol mL^{-1}, it seems to have acted as a free-radical scavenger and lipid protector, as the TBA-MDA levels were lower than in control cells (Figure 2). A similar effect was noticed for Q at 300 mmol mL^{-1}. Higher concentrations however, did not increase lipid peroxidation above control levels. This is also true for La; moreover, at the highest concentration (500 mmol mL^{-1}) it was the most effective free-radical scavenger (Figure 2).

Table 1 shows the DNA damage measured by the comet assay. Tail intensity in treated cells did not significantly differ from control, save for the highest Q/La complex concentration ($P<0.05$, Dunnett's post-hoc test), which also resulted in significant difference from the other two concentrations and from Q and La alone.

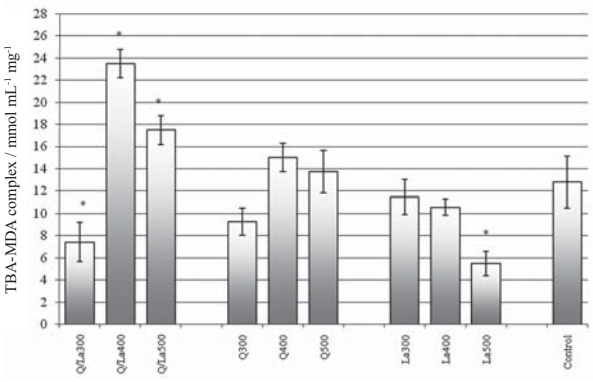

Figure 2 *Concentration of TBA-MDA complex as a measure of lipid peroxidation in the HeLa cells measured after a 3-hour treatment with the lanthanum-quercetin complex (QL) and quercetin (Q) and lanthanum (L) alone (300 mmol mL^{-1}, 400 mmol mL^{-1}, and 500 mmol mL^{-1}). Control was incubated in the growth medium. Results are shown as means ±SD of three experiments. * statistically significant difference between treated and control cells ($p<0.05$).*

DISCUSSION

We used two methods to determine cell survival; the MTT and the NR assay (Figures 1a and 1b), and the latter showed better sensitivity, while MTT gave false negative results. Microscopy confirmed that Q and the Q/La complex decreased cell survival and changed cellular morphology, starting from the concentration of 400 mmol mL^{-1} up (Figure 1b), but this effect was not detected by the MTT assay (Figure 1a). It is quite possible that small, invisible crystals were formed on the cells incubated with polyphenols, which later reacted with MTT to form a purple, formazan-like complex and yield false-negative results (21), even though the stock solution was prepared well. Van der Houde et al. (11) suggest that this effect can be prevented by adding ascorbic acid and 10 % of foetal bovine serum into growth medium. In that case, Q will not precipitate at concentrations lower than 250 mmol mL^{-1} (11). However, we could not introduce a new compound such as ascorbic acid because it would significantly compromise our results with its antioxidative/prooxidative action. This is why we eventually opted for the NR assay as an adequate test system for determining cell survival (22).

Table 1 *Alkaline comet assay results in the HeLa cell line after three hours of treatment with the lanthanum-quercetin complex and quercetin and lanthanum alone*

Concentration / mmol mL^{-1}	HeLa cell line								
	Tail intensity / % DNA								
	Quercetin / Lanthanum (Q/L)			Quercetin (Q)			Lanthanum (L)		
	mean±SD	median	range	mean±SD	median	range	mean±SD	median	range
300	1.18±0.65	1.01	0.69 to 3.18	1.1±0.43	0.91	0.69 to 3.21	0.88±0.25	0.8	0.69 to 2.6
400	1.18±0.66	0.88	0.69 to 3.5	0.97±0.28	0.88	0.28 to 1.96	0.9±026	0.8	0.69 to 1.93
500	1.64±0.88[ab]	1.5	0.69 to 4.17	1.05±0.54	0.81	0.69 to 3.22	1.01±0.41	0.84	0.69 to 2.5
Control	1.1±0.43	1	0.69 to 2.74	1.1±0.43	1	0.69 to 2.74	1.1±0.43	1	0.69 to 2.74

SD - standard deviation
150 comets per compound were measured. Inter-group comparisons were performed using ANOVA with Dunnett's post-hoc test.
[a] significantly different ($P<0.05$) from control
[b] significantly different from the treated cells

The Q/La complex showed the strongest cytotoxic effect in our study (EC_{50}=771.83 mmol mL^{-1}). Rusak et al. (19) have shown that the Q/La complex intercalates even better to dsDNA and RNA and is more stable than Q alone. Strong intercalation and the arrest of cell division is probably the mechanism of cytotoxic activity of the Q/La complex.

We did not determine the EC_{50} of Q and La alone, because they did not decrease cell survival below 60 % even at the highest concentrations used. Q and La (in all investigated concentrations) did not act as oxidants (Figure 2). This is in line with the findings of Sergediene et al. (23), showing that flavonoid cytotoxicity correlates with prooxidative activity. The same authors proved that flavonoid prooxidative properties were dose-dependent; Q was cytotoxic to most cell lines at concentrations higher than 300 mmol mL^{-1} (23).

Transition metal complexes which are stable, inert, and could serve as probes for different biological systems (1) have become interesting as potential anticancer drugs not only because of these chemical properties, but also because of their ability to bind to DNA and induce cell death through intercalation (1, 6, 12). The degree of intercalation corresponds to the planarity of the ligand in a complex; the more planar a ligand the stronger the intercalation effect. Intercalation also depends on coordination geometry and the type of ligand donor atom (1). Beside intercalation, two other mechanisms are responsible for the cell death; groove binding and external static charge influence (1).

Our results suggest that the prooxidative action of the Q/La complex grows with its cytotoxic effect. The associated DNA intercalation presumes that this complex can cause single-strand breaks, since Q alone inhibits topoisomerases II and IV (24, 25). The consequences of intercalative and prooxidative effects are shown in Table 1; the highest complex concentration caused significant DNA breakage, while Q and La did not. It is important to mention that it takes a long incubation time for the dose-dependent effect of quercetin on DNA to show (19), but long incubation seriously compromises Q's stability (11). In contrast, the time needed for the Q/La complex to cause significant biological damage was short (3 hours) at concentrations which are relatively low. These effects could not be obtained with Q or La alone. The antiproliferative character of the Q/La complex is owed to its planar structure and its intercalator affinity, resulting in DNA damage and oxidative stress on the cell level. Although we obtained promising results on the cell level, future experiments should answer whether the Q/La complex is cancer-specific and stable enough in physiological conditions to make a potential new antitumour drug.

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Sažetak

GENOTOKSIČNI UČINAK KOMPLEKSA KVERCETINA I LANTANA NA LJUDSKE STANICE KARCINOMA GRLA MATERNICE

Kvercetin je jedan od najzastupljenijih flavonoida u prehrani. Zbog specifične kemijske strukture i bioloških svojstava jedan je od najproučavanijih flavonoida kao potencijalni lijek koji bi se rabio za liječenje kardiovaskularnih, neurodegenerativnih i tumorskih bolesti. Kvercetin zbog svoje planarne kemijske strukture vrlo lako stupa u interakciju s metalnim ionima te tvori kelate. Cilj ove studije bio je utvrditi potencijalni genotoksični učinak kompleksa kvercetina i lantana na ljudskoj staničnoj liniji karcinoma grla maternice i usporediti taj učinak s genotoksičnim učincima pojedinačnih spojeva, kvercetina i lantana. Istraživani kompleks izazvao je značajnu toksičnost u rasponu koncentracija od 100 mmol mL^{-1} do 1000 mmol mL^{-1} i u vremenu inkubacije od 3 sata. Kompleks je pokazao prooksidativnu aktivnost u ovisnosti o koncentraciji te je pri najvišim istraživanim koncentracijama izazvao lomove DNA. Ako bi u daljnjim istraživanjima kompleks kvercetina i lantana pokazao selektivno djelovanje prema stanicama raka i stabilnost u fiziološkim uvjetima, mogao bi se promatrati u svjetlu potencijalnog antitumorskog lijeka, što bi trebala rasvijetliti daljnja istraživanja.

KLJUČNE RIJEČI: *flavonoid/metal kompleksi, kelati, oksidacijsko oštećenje, oštećenje DNA*

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