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Photocytotoxicity of a 5-nitrofuran-ethenyl-quinoline antiseptic (Quinifuryl) to P388 mouse leukemia cells

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Abstract

Quinifuryl (MW 449.52), 2-(5'-nitro-2'-furanyl)ethenyl-4-{N-[4'-(N,N-diethylamino)-1'-methylbutyl]carbamoyl} quinoline, is a water soluble representative of a family of 5-nitrofuran-ethenyl-quinoline drugs which has been shown to be highly toxic to various lines of transformed cells in the dark. In the present study, the toxicity of Quinifuryl to P388 mouse leukemia cells was compared in the dark and under illumination with visible light (390-500 nm). Illumination of water solutions of Quinifuryl (at concentrations ranging from 0.09 to 9.0 μg/ml) in the presence of P388 cells resulted in its photodecomposition and was accompanied by elevated cytotoxicity. A significant capacity to kill P388 cells was detected at a drug concentration as low as 0.09 μg/ml. The toxic effect detected at this drug concentration under illumination exceeded the effect observed in the dark by more than three times. Moreover, the general toxic effect of Quinifuryl, which included cell proliferation arrest, was nearly 100%. Both dose- and time-dependent toxic effects were measured under illumination. The LC_{50} value of Quinifuryl during incubation with P388 cells was ~0.45 μg/ml under illumination for 60 min and >12 μg/ml in the dark. We have demonstrated that the final products of the Quinifuryl photolysis are not toxic, which means that the short-lived intermediates of Quinifuryl photodecomposition are responsible for the phototoxicity of this compound. The data obtained in the present study are the first to indicate photocytotoxicity of a nitroheterocyclic compound and demonstrate the possibility of its application as a photosensitizer drug for photochemotherapy.

Key words
- Phototoxicity
- Nitrofurane derivatives
- Quinifuryl
- Oxygen and nitrogen active species

Introduction

Photochemotherapy, and photodynamic therapy in particular (1,2), are relatively new approaches to the treatment of various diseases, including cancer. An extensive search for new photochemotherapy agents continues (3-7) and studies on the mechanism of their phototoxicity are being developed (8,9).

Quinifuryl (Figure 1) is a representative of the family of 5-nitrofuran-ethenyl-quinolines, which were synthesized in the early 70's by Dr. N.M. Sukhova at the Institute of Organic Synthesis, Latvian Academy of Sci-

ences, Riga, Latvia, with the aim of developing new antitumor agents. These compounds have shown significant toxicity to various lines of cancer cells (10,11), with Quinifuryl possessing the highest cytotoxic activity (10) and showing radiosensitizing activity *in vitro* (12).

Quinifuryl absorbs light in the visible spectral region owing to the presence of a developed system of π-conjugation that makes it photolabile under irradiation in this spectral region. The excited singlet state is the first product of photoexcitation and may either decompose or form the excited triplet state (13). The latter reacts with either an electron donor, including another molecule of Quinifuryl in the ground state, or transfers its excitation energy to molecular oxygen (13). Illumination of a system containing the drug should therefore result in a decrease of Quinifuryl concentration and the formation of new compounds resulting from its phototransformation. Thus, the effect of illumination on Quinifuryl cytotoxicity is unpredictable because the products of phototransformation may be more or less toxic to transformed cells than Quinifuryl itself.

In the present study, the cytotoxicity of Quinifuryl to mouse leukemia P388 cells was compared in the dark and under continuous illumination with light in the spectral region from 390 to 500 nm.

Material and Methods

Cells

A mouse macrophage monocyte line, $P388D_1$, that grows in semi-suspension culture was obtained from the American Type Culture Collection (No. CCL46). Cells were grown in Fisher's medium supplemented with 10% heat-inactivated horse serum in 175-cm^3 culture flasks at 37ºC with 5% CO_2. On the day before the experiment, cells were seeded at 5 x 10^5 cells/ml. The number of cells in suspension was calculated using a Neubauer chamber (0.0025 mm^2). Slow magnetic stirring was used to maintain the suspension in a homogeneous state. All manipulations were carried out under low intensity red light (λ >550 nm) at room temperature (22ºC).

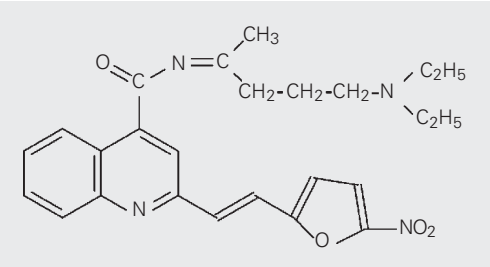

Figure 1. Structure of Quinifuryl, 2-(5′-nitro-2′-furanyl)ethenyl-4-{N-[4′-(N,N-diethylamino)-1′-methylbutyl]carbamoyl} quinoline.

Drug

A stock solution of 43.0 mM Quinifuryl, 2-(5'-nitro-2'-furanyl)ethenyl-4-{N-[4'-(N,N-diethylamino)-1'-methylbutyl]carbamoyl} quinoline (Figure 1), was prepared in Milli-Q quality water (Millipore).

Tests

The cell survival test (test 1) was based on the method of intravitral staining (14) using Trypan blue (TB) as a dye to which the living cell is impermeable (15). The number of dead P388 cells was counted using a Neubauer chamber (0.0025 mm^2) with 10 µl of TB added to 90-µl aliquots of a cell suspension withdrawn consecutively from the sample or control mixtures after appropriate time intervals. This test was employed for the comparative examination of the cytotoxicity of Quinifuryl in the dark and of the final products of its photodecomposition to P388 cells.

The toxicity test was based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) assay and was performed in two ways as described previously (11) and designated here as tests 2 and 3. MTT is reduced only by live cells to yield a colored product that may be interpreted as a measure of viability (16). For the

MTT assay, cells were seeded in DMEM containing 5% FBS into 96-well ELISA-type plates and exposed to Quinifuryl concentrations ranging from 0.2 to 20 µM for time intervals of 60 min in the dark or of 10 to 90 min in the presence of illumination. At the end of the drug exposure periods, plates were centrifuged to pellet the cells and the supernatants were displaced with either MTT dissolved in PBS (test 2) or with fresh medium (test 3). In the latter case, the cells were incubated for an additional 52 h (2 cell population-doubling times) with daily medium changes, followed by the MTT assay. Plates with MTT were incubated in the dark for 4 h, after which the water-insoluble MTT-formazan crystals were dissolved in DMSO, and the absorbance was determined with an ELISA plate reader Dynex MRX spectrophotometer (Dynex Technologies Inc., Chantilli, VA, USA) at 570 nm. The initial seeding densities ranged from 2 x 10^4 to 6 x 10^4 cells/well. Cell viability was assessed by TB dye exclusion at the beginning of each experiment and was always greater than 96%.

Irradiation

Samples and controls were irradiated in the spectral range from 390 to 500 nm using a standard tungsten lamp (150 W) through a colored glass filter 5-57 KOPP (Dieburg, Germany). Irradiation was performed either in a standard quartz 1-cm cuvette (test 1) or directly in ELISA-type plates (tests 2 and 3). The illumination intensity was 22 mW/cm^2, as measured with a Spectra-Physics 407A radiometer. A standard 1-cm quartz cuvette with water was used as a thermal filter to prevent collateral heating effects. Absorbance was monitored using a DU 650 Beckman spectrophotometer (Fullerton, CA, USA).

The protocol for the Quinifuryl photocytotoxicity studies is summarized in Table 1 and is described as follows: a) sample (S): Quinifuryl was added to a cell suspension and the mixture was immediately irradiated for time intervals up to 60 min; b) control 1 (C1) - the cell suspension was maintained in phosphate buffer for ≥60 min in the dark and in the absence of Quinifuryl; c) control 2 (C2) - the cell suspension was incubated for ≥60 min in the presence of Quinifuryl in the dark; d) control 3 (C3) - the cell suspension was irradiated for ≥40 min in the absence of Quinifuryl. This series of experiments was repeated six times.

Calculation and statistical analysis

The toxic effect (TE) of Quinifuryl was calculated as TE = [DC]/[Cell]^{control}, where [Cell]^{control} is the cell concentration in control wells (cells incubated for the same period of time without the drug) and [DC] is the dead cell concentration. [DC] is calculated as [Cell]^{control} - [Cell]^{final}, where [Cell]^{final} is the live cell concentration in wells exposed to the drug (5). The cell concentration was measured using a calibration curve constructed for P388 cells by the MTT-staining method.

The LC_{50} of Quinifuryl was estimated as 100 x (T^0 - T)/T^0 = 50, where T^0 and T are the absorbances of the test well at time zero (when the drug is added) and after exposure to the test compound (17), respectively. The data are presented as the mean ± SD of 6 measurements, and statistical analyses were

Table 1. Protocol for the study of Quinifuryl photocytotoxicity.

Group	Irradiation time (min)	Incubation time (min)*	Post-incubation time (h)	Quinifuryl (µM)
S1 Sample, test 1	0 to 60	0 to 60	No	20
S2 Sample, test 2	0 to 90	0 to 90	No	0.2; 2; 20
S3 Sample, test 3	60	60	52	0.2; 2; 20
S4 Sample, test 3	0	60	52	0.2; 2; 20
C1 Control	0	0 to 60	No	No
C2 Control	0 to 60	0	No	0.2; 2; 20
C3 Control	0 to 40	0	No	No

For details of S1-S4 samples and C1-C3 controls, see Material and Methods.
*Time of cell incubation with Quinifuryl.

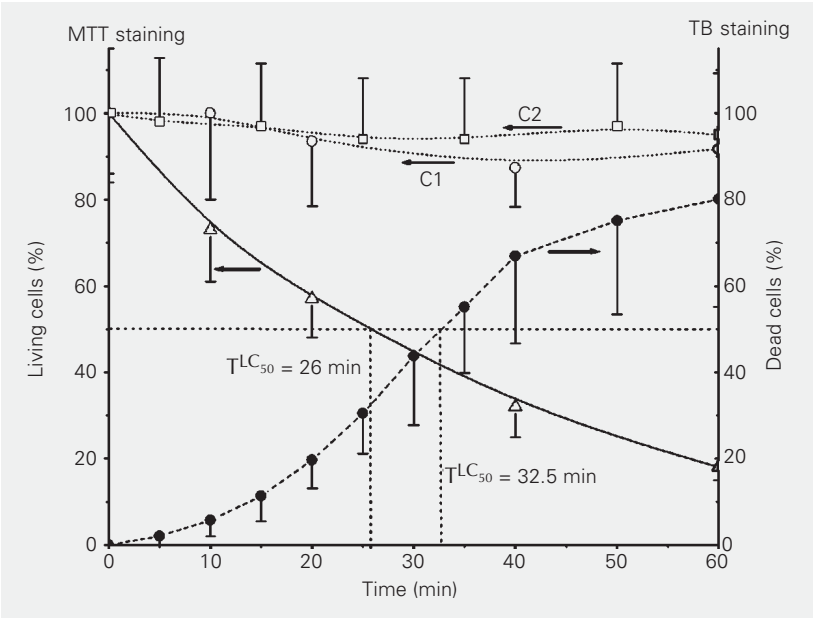

Figure 2. Photocytotoxicity of Quinifuryl (20 µM) to mouse leukemia P388 cells measured by methods of dye exclusion (Trypan blue (TB), dead cells; open circles) or dye inclusion (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT), living cells, triangles). Controls: C1 - cells incubated with Quinifuryl in the dark (closed circles) and C2 - cells illuminated for the time intervals indicated in the absence of Quinifuryl (squares). Cell concentration was ~1 x 10^6/ml in sodium phosphate buffer, pH 7.4. $T^{LC_{50}}$ is the irradiation time that was necessary to kill 50% of cells at 20 µM Quinifuryl. For the other experimental conditions, see Material and Methods.

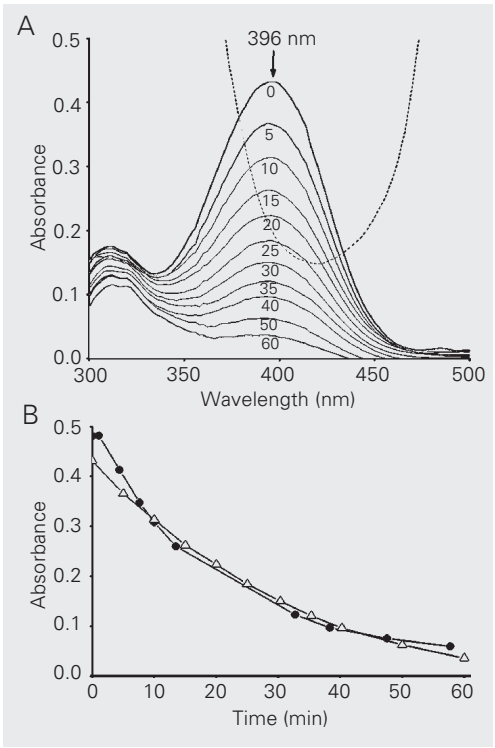

Figure 3. Photodecomposition of Quinifuryl under illumination with filtered light in the visible region. *A*, Spectral changes showing photobleaching of Quinifuryl (20 µM) in the presence of P388 cells. The arrow shows the direction of spectral changes. Numbers show the time of illumination in min. The dashed line shows the spectrum of glass filter absorption. *B*, Dynamics of Quinifuryl photobleaching at λ_{max} = 396 nm (arrow, A) in the absence (circles) and presence (triangles) of P388 cells. Conditions of illumination and measurements of spectra were the same as described in the legend to Figure 2.

performed by the Student *t*-test. The Tukey test with a 95% confidence interval was applied to compare the means. The statistical analyses were done using the InStat software program for Windows (GraphPads software, San Diego, CA, USA).

Results and Discussion

The results presented in Figure 2 show the effect of illumination on Quinifuryl toxicity to P388 cells which was measured by both dye (TB) exclusion and dye (MTT) inclusion methods. A significantly elevated toxic effect of Quinifuryl on P388 cells was observed under illumination of the incubation mixture with visible light (390-500 nm). A 20 µM Quinifuryl concentration caused death of 50% of cells after 26 min (dye inclusion method) or 32.5 min (dye exclusion method) of illumination, and >80% of cells died after 60 min of illumination. The toxicity observed for 60-min illumination in the absence of Quinifuryl (Figure 2, curve C1) or in the presence of Quinifuryl in the dark (Figure 2, curve C2) was <10% of that observed under illumination in the presence of Quinifuryl.

The photocytotoxic effect of Quinifuryl under irradiation was accompanied by photodecomposition of Quinifuryl (Figure 3A) that was monitored by a decrease of the specific absorption of the compound at 396 nm (ε_{396} = 2.47 x 10^4 M^{-1} cm^{-1}; Ref. 18). The presence of P388 cells did not alter the dynamics of Quinifuryl photobleaching (Figure 3B).

The photocytotoxic effect of Quinifuryl was further investigated in a series of experiments with different Quinifuryl concentrations performed using the MTT inclusion method (test 2). The time dependence of the toxic effect of Quinifuryl on P388 cells shows that the drug cytotoxicity in the dark was significantly reduced compared to the photocytotoxicity over the range of drug concentrations from 0.2 to 20 µM (Figure 4). According to these data, the TE_L/TE_D ratios

(toxic effects under illumination and in the dark, respectively) after 60 min of drug incubation with cells were 6.6 ± 1.4, 2.4 ± 0.7, and 9.9 ± 2.4 at initial Quinifuryl concentrations of 0.2, 2.0, and 20.0 μM, respectively.

The LC_{50} values estimated from the drug concentration dependence of the toxic effect (Figure 5) confirmed the above conclusion that the Quinifuryl toxicity was much higher under illumination than in the dark. Indeed, for 60 min of cell incubation with Quinifuryl under illumination, the death of 50% of the cells was observed with the drug concentration of 10 μM, while cell incubation with up

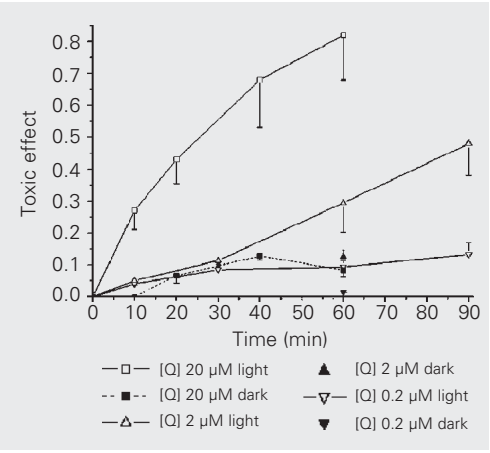

Figure 4. Time dependence of the toxic effect of Quinifuryl [Q] on P388 cells measured in the dark (filled symbols) and under illumination with visible light (390-500 nm) (open symbols). Initial drug concentrations were: 0.2 μM (inverted triangles), 2 μM (triangles), and 20 μM (squares). Experiments were performed in 96-well ELISA-type plates (2 x 10^4 cells/well) by dye (MTT) inclusion methods (for further details, see test 2 in Material and Methods). TE = $[DC]/[Cell]^{control}$, where TE is toxic effect, $[Cell]^{control}$ is cell concentration in control wells (cells incubated for the same period of time with no compound) and [DC] is the dead cell concentration. Each point indicates the mean ± SD of 6 measurements.

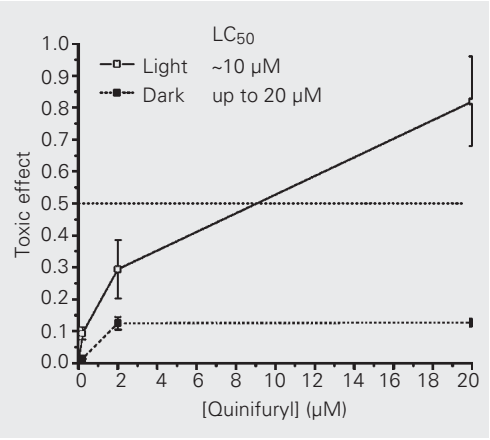

Figure 5. LC_{50} estimation: the concentration dependence of the toxic effect of Quinifuryl on P388 cells measured in the dark (dashed line, filled squares) and under illumination with visible light (390-500 nm; solid line, open squares). The time of drug incubation with cells was 60 min. Experiments were performed and toxic effect values calculated as described in the legend to Figure 4. The LC_{50} was estimated as 100 x (T^0 - T)/T^0 = 50, where T^0 and T are the absorbances of the test well at time zero (when the compound is added) and after exposure to Quinifuryl. Conditions of illumination were the same as described in the legend to Figure 2. Each point represents the mean ± SD of 6 measurements.

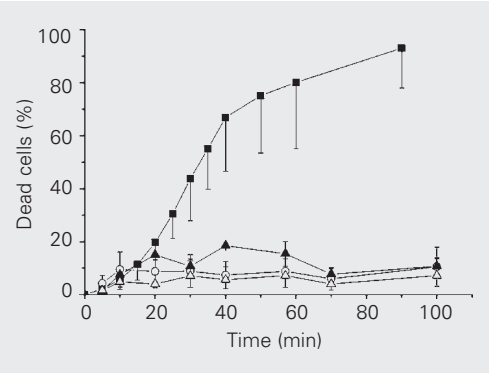

Figure 6. Cytotoxicity of Quinifuryl (filled symbols) and final products of its photodecomposition (open symbols) to mouse leukemia P388 cells in the dark (open circles, filled triangles) and under illumination (filled squares, open triangles) as measured by the dye exclusion method (Trypan blue; test 1, Material and Methods). Cell concentration was ~5 x 10^4 in sodium phosphate buffer, and Quinifuryl concentration was 2 μM, pH 7.4. Each point represents the mean ± SD of 3 measurements.

to 20 μM Quinifuryl for the same period of time in the dark caused the death of no more than 15% of cells.

In contrast to test 2 that indicated only the killing effect of the drug, test 3 revealed the complete cytotoxicity that included cell proliferation arrest. The results obtained using this method are presented in Table 2, along with the data obtained using test 2 (the killing effect of Quinifuryl). These data show that Quinifuryl not only caused death, but also attenuated the proliferation of the surviving P388 cells. Both effects were significantly enhanced under illumination. A further conclusion from these data is that the toxic effect of Quinifuryl on P388 cells is due to cell proliferation arrest rather than to cell death.

The cell survival test (test 1, Material and Methods) was employed to determine whether the final products of Quinifuryl photodecomposition might be responsible for the elevated toxicity of this drug under illumination. For this reason, P388 cells (5 x 10^4 cells/ml) were incubated for up to 100 min with either Quinifuryl (at a final concentration of 2 μM) or final products of photodecomposition of the drug. In the latter case, the 18 μM Quinifuryl solution was illuminated until the major Quinifuryl peak at 396 nm disappeared (see Figure 3A). Then, cells were added to a post-photolysis mixture and incubated in the dark. The results presented in Figure 6 show that the final products of Quinifuryl photodecomposition as well as Quinifuryl itself had low toxicity in the dark, causing death of less than 10% cells at 100 min of incubation. The final products of Quinifuryl were also non-toxic under 60-min illumination, while Quinifuryl itself caused death of nearly 90% of the cells. These results clearly show that the final products of Quinifuryl photodecomposition are not responsible for the cytotoxicity of this drug.

Table 2. Toxic effects (test 2) and post-effects (test 3) of Quinifuryl on P388 cells after 60 min of incubation with cells either in the dark or under illumination with visible light (390-500 nm).

Quinifuryl (μM)	Toxic effect			
	Dark		Illumination	
	Test 2	Test 3	Test 2	Test 3
0.2	0.014 ± 0.002	0.62 ± 0.11	0.09 ± 0.02	0.99 ± 0.005
2	0.12 ± 0.02	0.91 ± 0.08*	0.29 ± 0.09	0.96 ± 0.008*

Post-effects were measured 52 h after drug removal from the cell culture. Toxic effects (TE) were measured using the MTT assay and calculated as: TE = [DC]/[Cell]^{control}, where [Cell]^{control} is the cell concentration in control wells (cells incubated for the same period of time without the drug) and [DC] is the dead cell concentration. [DC] is calculated as [Cell]^{control} - [Cell]^{final}, where [Cell]^{final} is the live cell concentration in wells exposed to drug. [DC] was measured either immediately after drug removal (test 2) or after cell incubation with fresh medium for 52 h (test 3). For further details, see Material and Methods.

*P < 0.01 for comparison between the appropriate data obtained in the dark and under illumination (Tukey test).

Based on the data presented above, it seems that the detailed mechanisms of photocytotoxicity of Quinifuryl deserve special investigation. The observation that the photoactivation of Quinifuryl significantly accelerates cell killing, along with the observation that the final products of Quinifuryl photolysis are not responsible for this acceleration, should be interpreted as evidence that more toxic short-lived intermediates are formed during photolysis. Evidence of the formation of reactive species during Quinifuryl photolysis by visible light was reported in our previous studies (13,18). The formation of the triplet excited state of the drug molecule was observed (13), which is capable of producing singlet oxygen (19,20). We had also detected the reactions of the triplet state with the drug in the ground state and with electron donors (13,21), with the formation of superoxide anion radical (18). The formation of reactive oxygen species in the course of Quinifuryl photolysis may be responsible for the photocytotoxicity of the drug. However, the possible toxicity of short-lived intermediates of Quinifuryl decomposition should be further investigated.

To the best of our knowledge, the data presented here are the first to indicate photocytotoxicity of a nitroheterocyclic compound and demonstrate the possibility of its application as a photosensitizer drug for photochemotherapy.

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