2-[(E)-3-(6-chloroimidazo[2,1-b]thiazol-5-yl)prop-2-enyl]-5,6-dimethoxy-3-methyl-1,4-benzoquinone: a new inhibitor of NADH dehydrogenase with antitumor activity[1]

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Dedicated to Prof. Vincenzo Tortorella on the occasion of his "Fuori Ruolo" status

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Abstract

In this work we describe the synthesis and the biological activity of 2-[(E)-3-(6-chloroimidazo[2,1-b]thiazol-5-yl)prop-2-enyl]-5,6-dimethoxy-3-methyl-1,4-benzoquinone i.e. an imidazothiazole derivative connected to the benzoquinone ring of Q_0. This compound was tested as specific inhibitor of the NADH:ubiquinone (UBQ) reductase activity of NADH dehydrogenase in mitochondrial membranes. Binding of the imidazothiazole moiety to the quinone site normally occupied by the isoprenoid lateral side chain increases the inhibitory effect (with an IC_{50} for NADH-Q_1 activity of 0.24 μM) whereas the benzoquinone moiety seems to lose the capability as electron acceptor from Complex I. The new compound was also tested as potential antitumor agent at the National Cancer Institute.

Keywords: Imidazothiazole, quinone, coenzyme Q, antitumor activity

Introduction

Mitochondrial NADH-CoQ oxidoreductase, also known as Complex I, is the most complicated and the least understood of the respiratory chain complexes. Despite recent progress in structural studies, there is no structural information about Complex I that is comparable with the crystallographic data available for other respiratory complexes. This lack of information is principally due to its complexity (the mammalian enzyme consists of at least 46 subunits). Another characteristic feature of Complex I is related to the high number of its inhibitors: over sixty different families of compounds are known to inhibit Complex I, most of which are

commonly used as pesticides, especially acaricides and anthelmintics. At the cellular level, Complex I function is particularly crucial in tissues such as neurons and pancreatic islet beta cells that rely heavily upon mitochondrial ATP and NAD-linked pathways.[2,3] The potent cytotoxic effect of inhibitors in cancer cell cultures suggests that Complex I activity is particularly essential also in transformed cells.[4,5] Moreover in the last decades the interest in this NADH-ubiquinone reductase is increasing due to its possible involvement in the pathogenesis of human neurodegenerative diseases such as Alzheimer's, Parkinson's, diabetes and in aging. All physiological quinones present a common moiety: the 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0 see Figure 1) and a long isoprenoid chain (from 6 to 10 isoprenoid units) in the 6 position of the ring.

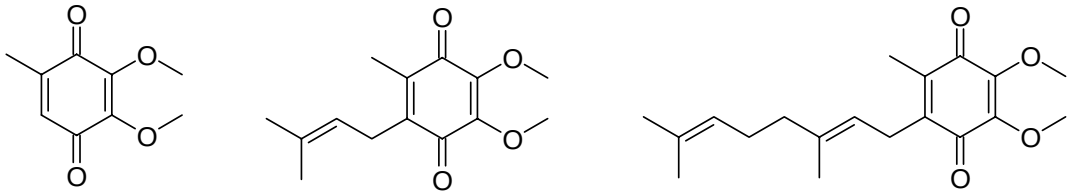
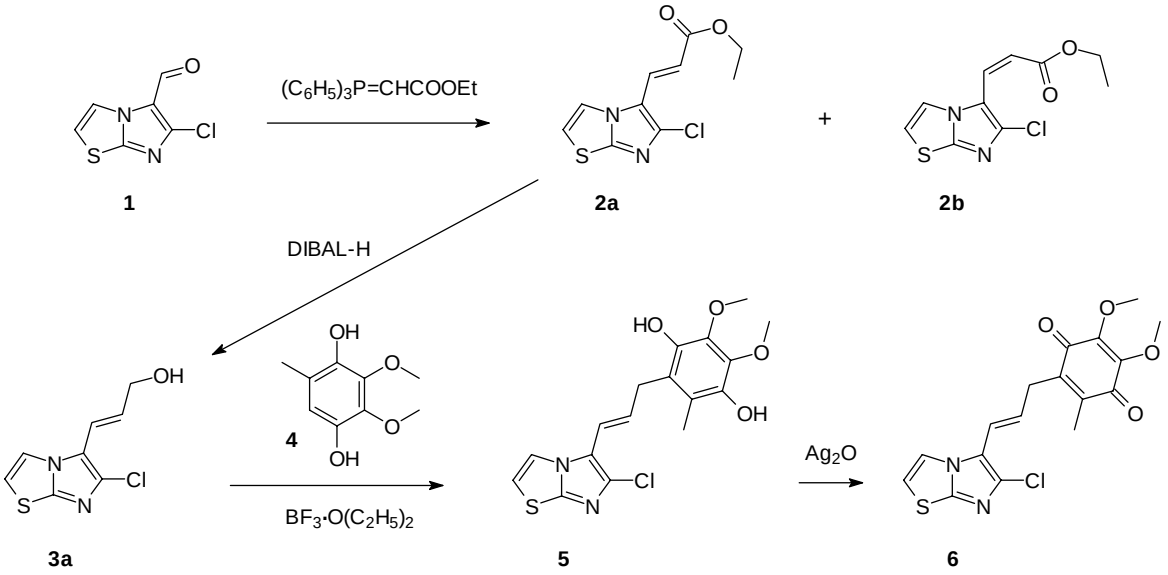


Figure 1

Because of their high hydrophobicity, these long chain quinones are not useful for "in vitro" determination of NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase activity; on the other hand short chain quinones (with 5-10 carbon atoms either in a isoprenoid or in a saturated side chain) are the choice molecules for the enzymatic assay.[6,7] In particular Q_1 (see Figure 1) is the most widely used as electron acceptor from Complex I, whereas Q_2 (see Figure 1) shows an activity that is 25-30% with respect to NADH-Q_1 reductase activity. This difference is due to an inhibition effect of Q_2 on Complex I**.** The identification and characterization of specific quinone features involved on the inhibition of Complex I represent a significant advance in understanding the functional mechanism of the complex and also contribute to the development of drugs specifically acting on mitochondria. In a previous study it was shown that thienylimidazo[2,1-b]thiazoles[8,9] were able to inhibit Complex I acting in a non competitive way with the ubiquinone substrate and interact with a site which is mutually exclusive with that of rotenone but not exclusive with that of piericidin and several inhibitors of NADH dehydrogenase. Founded on these data we planned the synthesis of an imidazothiazole derivative connected to the benzoquinone ring of CoQ_0 by means of a short chain resembling the isoprenoid side chain of Q_1 in order to evaluate the effect on Complex I of the modified CoQ_0 and to elucidate the ambiguous quinone specificity in the interaction with the enzyme. We have also tested the new derivative as potential antitumor agent.

Chemistry

The synthesis of the designed compound is outlined in the Scheme 1.



Scheme 1

The esters **2a** and **2b** were obtained by a Wittig reaction from the appropriate ylide and the aldehyde **1**. The two isomers were purified by flash chromatography and the structures were assigned unambiguously by ^{1}H-NMR. The double bond protons in **2a** (trans) are doublets at 6.50 and 7.67 ppm with a high coupling constant (J=16.2 Hz) whereas the same protons in **2b** (cis) are at 6.12 and 7.07 ppm with a coupling constant of 12 Hz. The esters **2a** and **2b** were reduced to the corresponding vinyl alcohol **3a** and **3b** using diisobutylaluminiumhydride.
2,3-Dimethoxy-5-methyl-1,4-benzoquinone Q_0 was converted to 2,3-dimethoxy-5-methylhydroquinone **4** by a conventional reductive step employing sodium dithionite as the reducing agent. In the subsequent step, compound **4** was reacted with the alcohol, trans isomer, **3a**. The condensation was carried out in dioxane at room temperature and under nitrogen in order to prevent oxidation of the hydroquinone to the corresponding benzoquinone. Boron trifluoride etherate was used as the acidic condensing agent.[10,11] The hydroquinone **5** was not characterized but was subjected as such to mild oxidation with silver(I) oxide to afford the substituted benzoquinone **6**. When the reaction was performed with the alcohol, cis isomer, **3b** did not react with the hydroquinone **4**.

Biological results

(a) Evaluation of activity on mitochondrial NADH dehydrogenase

The biological activity of the new compound **6** was evaluated on mitochondrial NADH dehydrogenase. It was initially tested to evaluate whether it was an electron acceptor itself. The electron acceptors commonly employed as substitutes of the physiological ubiquinones are short

chain CoQ homologues (the most commonly used acceptor has been Q_1) and analogues (such as decylbenzoquinone). The experiments were conducted using submitochondrial particles from beef heart (SMP). For comparison purpose Q_0, Q_1 and **3a** were also tested. As shown in **Table 1,** neither **3a** nor **6** are electron acceptors; thus we tested if they inhibited the NADH-Q_1 reductase of Complex I.

Table 1. Activity on mitochondrial NADH dehydrogenase

Coenzyme Q analogues	IC_{50} (μM) for NADH-Q_1 activity	IC_{50} (μM) for NADH-O_2 activity	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
Q_0	--	--	65	0.18
Q_1	--	--	24	0.66
3a	57.04	49.66	Not electron acceptor	
6	0.25	0.12	Not electron acceptor	

We assayed the effect of **3a** and **6** on the NADH-O_2 activity to measure their inhibition of the overall electronic flux and their effect on the NADH-Q_1 activity (NADH Coenzyme Q reductase) using Q_1 as electron acceptor and blocking Complex III (ubiquinol cytochrome c reductase) by antimycin and Complex IV by KCN. Compound **6** (where quinone and imidazothiazole moieties are connected by a propylene chain) shows a good potency as Complex I inhibitor with an IC_{50} = 0.25 μM for NADH-Q_1, IC_{50} = 0.12 μM for NADH-O_2. It has an IC_{50} for NADH-O_2 lower than IC_{50} for NADH-Q_1, this induces to think that it is a competitive inhibitor compared to Q_1. Moreover the derivative **3a** lacking the quinone head, is a very weak inhibitor: IC_{50} = 57.04 μM for NADH-Q_1, IC_{50} = 49.66 μM for NADH-O_2. These last experimental results suggest that the hydrophilic benzoquinone ring is fundamental to inhibit the NADH-ubiquinone reductase activity of Complex I and probably it locates the imidazothiazole derivative in the hydrophobic core of Complex I.

(b) In vitro growth inhibition and cytotoxicity[12]

The test was performed by the National Cancer Institute (NCI, Bethesda, MD). According to a primary screening, compound **6** was evaluated for its cytotoxic activity on three human cell lines, such as NCI-H460 lung cancer, MCF7 breast cancer and SF-268 glioma. A compound is considered active when it reduces the growth of any of the cell lines to 32% or less. Compound **6** was active and it was passed on for evaluation in the full panel of sixty cell lines. This panel is organized into subpanels representing leukemia, melanoma and cancers of lung, colon, kidney, ovary, breast, prostate and central nervous system. The tested compound was evaluated using five concentrations at ten-fold dilutions (the highest being 10^{-4}M and the others

10^{-5},10^{-6},10^{-7},10^{-8}M) taking into consideration the growth inhibitory power (GI_{50}), the cytostatic effect (TGI) and the cytotoxic effect (LC_{50}). The values of GI_{50} (expressed as $\log_{10}$) range from –4.65 to –6.40, $\log_{10}$TGI from –4.07 to –5.27 and $\log_{10}LC_{50}$ from –4.00 to –4.57.

Figure 2 reports only the tumor lines where compound **6** showed GI_{50} values < -5.10. In conclusion compound **6** was particularly selective against two subpanels (leukemia, melanoma) and two cell lines: NCI-H23 (non-small-cell lung cancer) and OVCAR-4 (ovarian cancer).

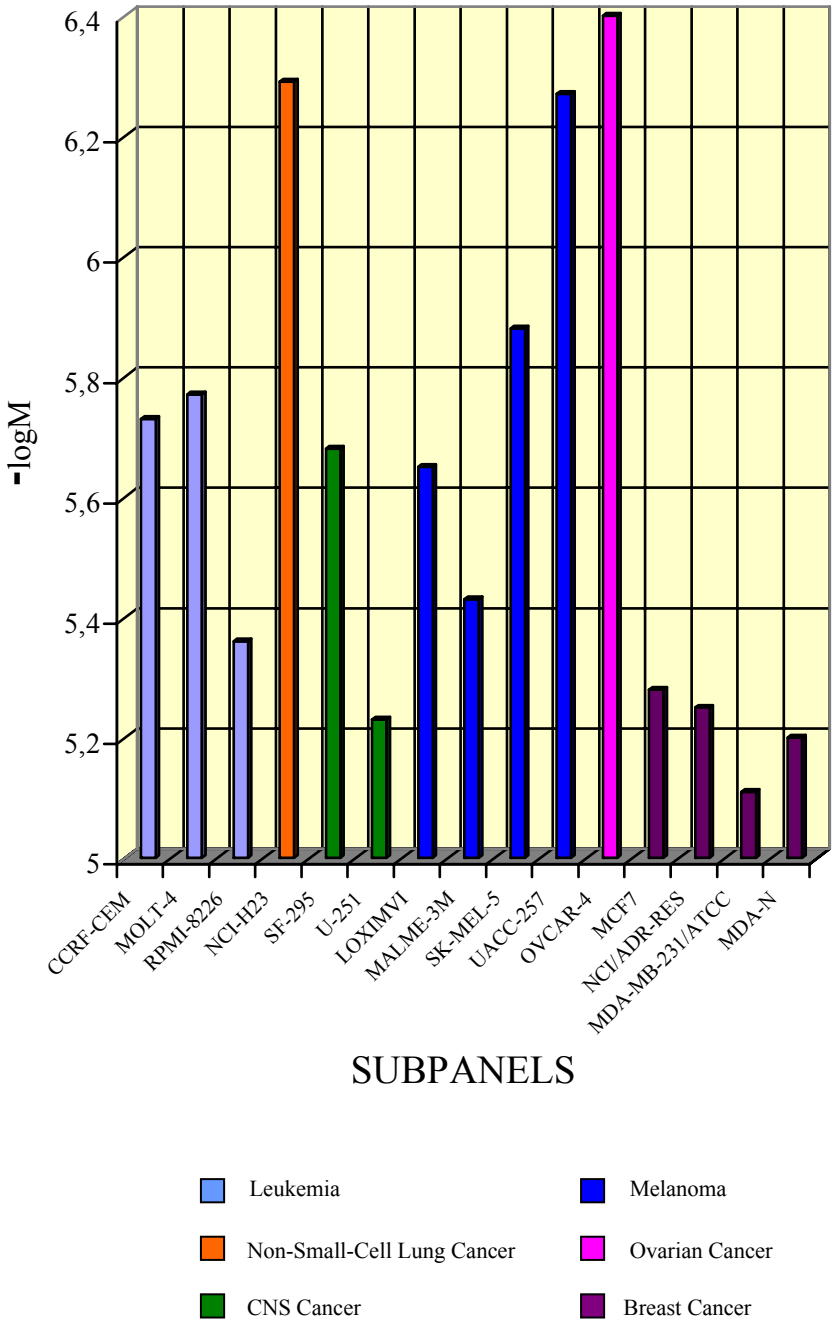


Figure 2. Antitumor activity of compound **6**. GI_{50} expressed as -log of the molar concentration.

Experimental Section

(a) Chemistry

The melting points are uncorrected. Analyses (C, H, N) were within ±0.4% of the theoretical values. Bakerflex plates (silica gel IB2-F) were used for TLC. Kieselgel 60 (Merck) was used for column chromatography and Kieselgel 200-400 mesh (Merck) for flash chromatography. The IR spectra were recorded in nujol on a Nicolet Avatar 320 ESP; ν_{max} is expressed in cm^{-1}. The ^{1}H-NMR spectra were recorded in $(CD_3)_2SO$ on a Varian Gemini (300 MHz) spectrometer; the chemical shifts (referenced to solvent signal) are expressed in δ (ppm) and J in Hz. In this data the abbreviation th = thiazole. Petroleum ether refers to the fraction boiling at 60-80 °C. Compound **1** was synthesized according to the literature.[13] All reagents, unless otherwise stated, were supplied by Aldrich Chemical Company Ltd and were used as supplied.

Synthesis of (E) and (Z) ethyl 3-(6-chloroimidazo[2,1-b]thiazol-5-yl)prop-2-enoate (2a) and (2b). A stirred solution of absolute ethanol (100 mL) containing sodium (0.37 g, 16 mmol) was treated under nitrogen with (carbethoxyethylidene)-triphenylphosphorane (5.57 g, 16 mmol) at room temperature. After 30 min a solution of **1** (2.99 g, 16 mmol) in absolute ethanol (80 mL) was added dropwise to the stirred reaction mixture. Stirring was continued for 6-8 h until TLC (petroleum ether/diethyl ether 8/2) indicated loss of starting material, the solution was acidified to pH 5 (2N HCl) and the solvent was removed by evaporation *in vacuo*. The residue was suspended in water (60 mL) and the resultant yellow suspension extracted with $CHCl_3$ (3 x 30mL). The combined organic phase was dried (Na_2SO_4) and evaporated *in vacuo* to afford the crude products **2a** and **2b** which were separated by flash chromatography using petroleum ether/diethyl ether 8/2 as the eluent. Compounds **2a** (68%) and **2b** (22%) were obtained as pale yellow solids.

(2a). $C_{10}H_9ClN_2O_2S$ (256.71); IR(Nujol): 3146, 3111, 1703, 1630, 1176; ^{1}H NMR (300 MHz; $(CD_3)_2SO$): 1.28 (3H, t, CH_2CH_3, J=7), 4.22 (2H, q, CH_2CH_3, J=7), 6.50 (1H, d, CH=, J=16.2), 7.58 (1H, d, th, J=4.5), 7.67 (1H, d, CH=, J=16.2), 8.50 (1H, d, th, J=4.5); mp 133-135°C

(2b). $C_{10}H_9ClN_2O_2S$ (256.71); IR(Nujol): 3147, 3125, 1700, 1622, 1174; ^{1}H NMR (300 MHz; $(CD_3)_2SO$): 1.18 (3H, t, CH_2CH_3, J=7), 4.13 (2H, q, CH_2CH_3, J=7), 6.12 (1H, d, CH=, J=12), 7.07 (1H, d, CH=, J=12), 7.45 (1H, d, th, J=4.5), 7.66 (1H, d, th, J=4.5); mp 105-106°C.

Synthesis of (E) and (Z)-3-(6-chloroimidazo[2,1-b]thiazol-5-yl)prop-2-en-1-ol (3a and 3b). A solution of 2 mL of DIBAL-H (diisobutylaluminium hydride as 1 M solution in hexane) was added dropwise over a period of 15 min. to a stirred solution of **2a** or **2b** (0.26 g, 1 mmol) in anhydrous toluene under dry N_2 at –13 °C. The mixture was stirred for 2 h at 0°C and for 18 h at room temperature. The excess reagent was decomposed by careful addition of methanol (30 mL) followed by 5% HCl (50mL). The mixture was adjusted to pH 7 with saturated $NaHCO_3$ solution and extracted with ethyl acetate (4x30mL). The organic layers were combined, washed with brine (2x20mL) and dried (Na_2SO_4). The solvent was evaporated *in vacuo* to afford the crude alcohol (**3a** or **3b**). Purification by column chromatography (petroleum ether/acetone 9/1) gave pure **3a** as a white solid (66%) or **3b** as a white solid (60%).

3a: $C_8H_7ClN_2OS$ (214.67); IR(Nujol): 3355-3193, 1021, 958, 722; ^{1}H NMR (300 MHz; $(CD_3)_2SO$): 4.19 (2H, m, *CH*$_2$OH), 5.03 (1H, t, CH$_2$*OH*), 6.43 (1H, dt, =*CH*CH$_2$, J=16.4, J=5.3), 6.68 (1H, d, CH=, J=16.4), 7.47 (1H, d, th, J=4.5), 8.23 (1H, d, th, J=4.5); mp 96-98°C

3b: $C_8H_7ClN_2OS$ (214.67); IR(Nujol): 3342-3095, 1025, 708, 656; ^{1}H NMR (300 MHz; $(CD_3)_2SO$): 4.01 (2H, m, *CH*$_2$OH), 4.93 (1H, t, CH$_2$*OH*), 5.94 (1H, dt, =*CH*CH$_2$, J=11.3, J=5.6), 6.37 (1H, d, CH=, J=11.3), 7.42 (1H, d, th, J=4.4), 7.88 (1H, d, th, J=4.4); mp 150-151°C.

Synthesis of 2-[(E)-3-(6-chloroimidazo[2,1-b]thiazol-5-yl)prop-2-enyl]-5,6-dimethoxy-3-methyl-1,4-benzoquinone (6). 2,3-Dimethoxy-6-methyl-1,4-benzoquinone (Q_0) (0.26 g, 1.4 mmol) was dissolved in 100 mL of diethyl ether and shaken with sodium dithionite (1.74 g, 10 mmol) in 50 mL of water until the solution become colorless. The mixture was extracted with diethyl ether. The organic phase was dried over anhydrous sodium sulphate, filtered and evaporated. The hydroquinone **4**, obtained as colorless crystals, was dissolved in 20 mL of dry dioxane. This solution was added to **3a** (0.30 g, 1.4 mmol) dissolved in 20 mL of dry dioxane and stirred in a nitrogen atmosphere at room temperature. The resulting solution was treated with 0.18 mL of boron trifluoride etherate (1.4 mmol) in 10 mL of dry dioxane over 20 min. The mixture was stirred for 12 h at room temperature, treated with 80 mL of water and extracted with diethyl ether (4x20mL). The organic phase containing the crude compound **5** was dried over anhydrous sodium sulfate, treated with silver(I) oxide (0.32 g, 1.4 mmol) and stirred for 6h. The solution was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (petroleum ether/diethyl ether 70/30). Compound **6** was obtained by precipitation using a mixture acetone/petroleum ether as an orange solid (19%).

6. $C_{17}H_{15}ClN_2O_4S$ (378.84); IR(Nujol): 1651, 1611, 1264, 873, 723; ^{1}H NMR (300 MHz; $(CD_3)_2SO$): 2.00 (3H, s, CH_3), 3.44 (2H, m, CH_2), 3.88 (6H, s, OCH_3), 6.15 (1H, dt, =*CH*CH$_2$, J=16.3, J=6.5), 6.54 (1H, d, CH=, J=16.3), 7.43 (1H, d, th, J=4.5), 8.16 (1H, d, th, J=4.5); mp 156-158°C.

(b) Biochemistry

NADH (b-form), CoQ_0, CoQ_1 and Antimycin A were purchased from Sigma Co. St. Louis, MO. Submitochondrial particles (SMP) were prepared from Beef Heart Mitochondria (BHM) by sonic irradiation of the frozen and thawed BHM,[14] the particles were essentially broken membrane fragments.[15] Protein was evaluated by the Biuret method of Gornall et al.[16] with addition of 10% Sodium Deoxicholate and using Bovine Serum Albumin (BSA) as standard. NADH-CoQ reductase was assayed essentially as described by Yagi[17] and modified by Degli Esposti et al.[18] in presence of 2 mM KCN and 2 μM Antimycin A to block Complex IV and III respectively. Determination of the kinetic constants were accomplished following the decrease in absorbance in a double wavelength spectrophotometer at 340 minus 380 nm (extinction coefficient 3.5 $mM^{-1}cm^{-1}$) due to the oxidation of NADH (75μM) in presence of different ubiquinone concentrations. NADH-O_2 reductase activity was assayed essentially in the same conditions but omitting KCN and Antimycin A from the assaying mixture.

(c) Antitumor activity

Three cell panel

Each cell line was inoculated and preincubated on a microtitre plate. Test agents were then added at a single concentration and the culture incubated for 48 h. End-point determinations were made with sulforhodamine B (SRB), a protein-binding dye. Results for each test compound are reported as the percent of growth of the treated cells when compared to the untreated control cells.

Sixty cell panel

Each cell line was inoculated onto microtitre plates, then preincubated for 24-28 h at 37°C for stabilization. Subsequently, test agents were added in five 10-fold dilutions and the culture was incubated for an additional 48 h in 5% CO_2 atmosphere and 100% humidity. For each test agent, a dose-response profile was generated. End-point determinations of cell growth were performed by in situ fixation of cells, followed by staining with SRB which binds to the basic amino acids of cellular macromolecules. The solubilized stain was measured spectrophotometrically in order to determine relative cell growth in treated and untreated cells.

A plate reader was used to read the optical densities, and a microcomputer processed the optical densities in the special concentration parameters: GI_{50}, TGI and LC_{50}. The optical density of the test well after 48 h period of exposure to the test drugs is T, the optical density at time zero is T_0 and the control optical density is C.

GI_{50} is the concentration of test drug where $100x(T-T_0)/(C-T_0)=50$

TGI is the concentration of test drug where $100x(T-T_0)/(C-T_0)=0$

LC_{50} is the concentration of test drug where $100x(T-T_0)/T_0= -50$ (the control optical density is not used in the calculation of LC_{50}).

Acknowledgments

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