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Tandem screening of toxic compounds on GFP-labeled bacteria and cancer cells in microtiter plates ☆

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Abstract

A 96-well fluorescence-based assay has been developed for the rapid screening of potential cytotoxic and bacteriocidal compounds. The assay is based on detection of green fluorescent protein (GFP) in HeLa human carcinoma cells as well as gram negative (*Escherichia coli*) and gram positive bacteria (*Mycobacterium avium*). Addition of a toxic compound to the GFP marked cells resulted in the loss of the GFP fluorescence which was readily detected by fluorometry. Thirty-nine distinct naphthoquinone derivatives were screened and several of these compounds were found to be toxic to all cell types. Apart from differences in overall toxicity, two general types of toxic compounds were detected, those that exhibited toxicity to two or all three of the cell types and those that were primarily toxic to the HeLa cells. Our results demonstrate that the parallel screening of both eukaryotic and prokaryotic cells is not only feasible and reproducible but also cost effective.
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As large quantities of novel chemical compounds are generated, companies and researchers are faced with the growing challenge of screening these compounds for their potential therapeutic and/or toxic effects; thus leading to a growing demand for novel cell-based high-throughput screening assays [1–3]. A significant proportion of potential anti-cancer drugs exhibits unacceptable levels of toxicity against normal cells and tissues, and for this reason it is imperative to pre-screen such compounds in vitro [4,5]. We have recently reported a relatively simple fluorescence-based toxicity screening assay [6] that involves the detection of DNA degradation in a human cancer cell line that constitutively expresses GFP in the nuclei [7]. Due to the stable nuclear localization of the histone H2B–GFP-fusion protein, altered apoptotic and/or necrotic nuclei can be readily detected in situ by fluorescence microscopy. Several structurally similar naphthoquinone compounds, including the well-known anti-cancer compound plumbagin, were screened using this assay [6]. From this initial screen of 11 compounds, two novel naphthoquinones were found to be more toxic than plumbagin. Our subsequent assays revealed that the most toxic naphthoquinones, including plumbagin, elicited both apoptotic and necrotic modes of cell death. These results were consistent with the generation of reactive oxygen species (ROS) as demonstrated with

☆ *Abbreviations:* H2B, histone H2B; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; LD50, lethal dose, 50%; ROS, reactive oxygen species; *E. coli*, *Escherichia coli*; *M. avium*, *Mycobacterium avium*.

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plumbagin [8]. In a related analysis, the HeLa-GFP assay was used to evaluate the potential toxicity of a small library of acetophenone compounds with potential antimicrobial properties [9]. Although some of the antibacterial acetophenone compounds exhibited varying levels of cytotoxicity, three of the most promising compounds were determined to exert their activity selectively against *M. avium* [9] .

Unfortunately, the HeLa-GFP assay we previously reported cannot be applied to high-throughput analyses as it relies on the visual detection of cell death through fluorescence microscopy. In this report, we describe a modification of the aforementioned assay to facilitate the simultaneous screening of multiple compounds in a 96-well format using an automated fluorescence plate reader. This assay can theoretically be used as a biosensor for genotoxic compounds on a large variety of cell types and organisms that express the GFP protein [10].

Although mycobacterial infections are on the increase worldwide, particularly in immunocompromised populations as is the case of *M. avium* in AIDS patients, there have been no new drugs developed against these organisms since the 1960s ([9] and references within). The emergence of drug-resistant bacteria has also led to an increased interest in the generation of novel antibiotics. Due to the ease of screening multiple compounds simultaneously, the HeLa assay was expanded to include GFP-expressing bacteria (*E. coli* and *M. avium*) to screen for compounds that might be preferentially toxic to bacteria but not mammalian cells. Our results demonstrate that the parallel screening of both eukaryotic and prokaryotic cells is not only feasible and reproducible but also cost effective.

Materials and methods

Cell culture. HeLa-GFP cells were cultured in DMEM supplemented with 10% heat-inactivated newborn calf serum as previously described [6]. Stock cultures were prewashed twice with PBS before the treatment of trypsin for cell resuspension and subsequently re-plated at the concentration of 4000 cells per well. *E. coli*-GFP cells were grown in LB broth media while the MAC104 *M. avium* strain was grown in 7H9-ADC media.

HeLa-GFP fluorometric cytotoxicity assay. All cell lines were seeded in clear-bottomed 96-well assay plates (3603, Costar, Corning, Corning, NY) in order to minimize background fluorescence. HeLa cells were grown in a total volume of 0.2 ml medium and incubated overnight for proper cell attachment. The quinone compounds were then added to each well (in triplicate) and then incubated for a period of 18 h. For the determination of fluorescence, the assay plates were then read using the Fluoroskan Ascent F1 fluorometer (Thermo Electron Corp.) set at the excitation and emission parameters of 485 and 518 nm, respectively. Since the HeLa-GFP cell line is adherent, the fluorometer was set to read from the bottom of the plate for closer proximity to the cells. Before the readings were performed, all supernatant media was removed to avoid any background interference and thus enhancing the sensitivity of the optical readings. For calibration purposes, three untreated control wells were used to determine the maximum level of fluorescence (100%). It should be noted that 1 μl DMSO was added to these control since this was the quinone solvent and previously shown to exhibit little, if any, cytotoxicity [6]. As a cytolysis control (complete loss of fluorescence), three wells containing cells were exposed to 0.2 ml of deionized water. These controls were then used to calculate the percent loss of GFP signal after compound exposure as determined by comparison with control wells containing cells that were not exposed to the chemical agent (see data analysis below).

Toxicity assays with GFP-expressing bacteria. Escherichia coli was transformed with a GFP-expression plasmid that also confers tetracycline resistance and was kindly provided by Dr. David Schneider [11]. *M. avium* strain MAC104 was transformed with p996A461 plasmid that confers hydromycin resistance and carries the GFP cassette under the control of the strong constitutive ribosomal promoter, *rpsL* (for additional details see [12]). Both bacterial strains were grown and selected based on antibiotic resistance and GFP expression. All toxicity assays were performed using the plates and fluorometric parameters described in the previous section. Bacterial cell suspensions were seeded in a final volume of 100 μl at an optical density of 0.05 and 0.25 at 650 nm, for *E. coli*-GFP and *M. avium*-GFP, respectively. After exposure to the test compounds, assay plates were incubated at 37 °C in a humid environment to prevent dehydration in the wells. Unlike the HeLa-GFP assays, reading was performed without removing the supernatants to avoid loss of cells. Several untreated wells containing bacteria and media were used to determine the maximum level of fluorescence. Several wells containing media alone (no bacteria) were included in each assay to remove background fluorescence.

Data analysis. All compounds, including controls, were screened in triplicate. The average of the three wells that were used for the removal of the background fluorescence (wells containing water for the HeLa-GFP assays and wells containing media alone for bacteria) was subtracted from each individual well. The mean of these triplicate values for each compound was then obtained. These mean values were then divided by the average obtained for maximum fluorescence (cells not treated with compound) and then multiplied by 100 to obtain the percentage values as shown in Table 1. It should be noted that a few data points were derived from duplicate samples if the third reading was significantly different from the other two. Calculation of compound physical parameters, $\log P$ and MW, was performed with ChemOffice Ultra (Cambridge Soft) and these data are presented in the Supplementary materials section. Note that $\log P$ values were not calculated for two of the compounds, Q30–31. Correlation of physical properties with compound toxicity was performed via the Spearman rank correlation using SAS for Windows, version 7 (SAS Institute Inc.).

Fluorescent microscopy cytotoxicity assays. HeLa-GFP cells were plated on 24-well plates and left overnight to adhere. Different dilutions of the quinones were then added to each well (indicated in the legend) using the same concentrations of the positive and negative controls as described in the previous assay. Cell counts were done under fluorescent microscopy to determine cytotoxicity. The status of the fluorescent GFP nuclei and the typical morphological characteristics of cells undergoing cell death were taken into account when differentiating between dying or viable cells as previously described [6].

Compound synthesis. Naphthoquinone derivatives, Q2–Q39 (see Supplementary materials), were synthesized on solid support utilizing the Dotz reaction with solid supported Fischer carbene complexes as recently described [13]. Plumbagin was purchased from Sigma. Test compounds were dissolved in DMSO at 20 mg/ml and stored at −20 °C until used.

Results and discussion

In order to rapidly screen novel compounds for their toxic properties, a relatively simple GFP-based assay

Table 1
Percent GFP-fluorescence after incubation with test compounds

Compound	HeLa-GFP[a] (20 μg/ml)[b]	HeLa-GFP (2 μg/ml)	*E. coli*-GFP[c] (20 μg/ml)	*M. avium*-GFP (20 μg/ml)
Q1	9.0	13.0	1.0	7.0
Q2	29.0	14.0	111.0	72.0
Q3	4.0	0	41.0	38.0
Q4	3.0	70.0	56.0	26.0
Q5	22.0	13.0	58.0	69.0
Q6	20.0	78.0	105.0	55.0
Q7	25.0	13.0	89.0	35.0
Q8	16.0	89.0	134.0	102.0
Q9	31.0	90.0	115.0	85.0
Q10	52.0	84.0	110.0	81.0
Q11	14.0	84.0	98.0	69.0
Q12	14.0	81.0	82.0	66.0
Q13	21.0	89.0	93.0	72.0
Q14	29.0	83.0	90.0	58.0
Q15	53.0	85.0	80.0	40.0
Q16	10.0	78.0	127.0	105.0
Q17	18.0	80.0	115.0	100.0
Q18	22.0	88.0	112.0	127.0
Q19	38.0	78.0	99.0	73.0
Q20	29.0	28.0	86.0	77.0
Q21	23.0	57.0	122.0	57.0
Q22	14.0	75.0	110.0	91.0
Q23	3.0	14.0	56.0	66.0
Q24	23.0	5.0	87.0	202.0
Q25	18.0	0	118.0	133.0
Q26	27.0	26.0	110.0	97.0
Q27	21.0	21.0	117.0	133.0
Q28	4.0	17.0	115.0	97.0
Q29	19.0	80.0	100.0	105.0
Q30	47.0	84.0	105.0	96.0
Q31	82.0	88.0	107.0	94.0
Q32	57.0	88.0	99.0	80.0
Q33	62.0	88.0	102.0	74.0
Q34	49.0	86.0	87.0	64.0
Q35	58.0	85.0	104.0	108.0
Q36	59.0	89.0	100.0	93.0
Q37	62.0	90.0	105.0	92.0
Q38	61.0	81.0	103.0	119.0
Q39	62.0	88.0	108.0	96.0

[a] HeLa and *M. avium*-GFP cells were incubated with compound for 18 h.
[b] Compounds were tested at concentrations of 20 or 2 μg/ml as indicated. See Materials and methods for assay details.
[c] *E. coli*-GFP was incubated with compound for 10 h.

was recently utilized to simultaneously screen several compounds without having to perform other elaborate assays [6]. Although easy to implement, this assay could not be applied to high-throughput analysis as it relied on the visualization of cell death [6]. Given this obvious limitation, the assay was modified to facilitate the simultaneous screening of multiple compounds in a 96-well format using an automated fluorescence plate reader. Since small quantities of compounds are required, this assay is particularly well suited for the screening of combinatorial chemical libraries. Another advantage of these microplate assays is the ability to perform all assays in duplicate or triplicate to derive more consistent results, and for this reason, all experiments were performed in triplicate.

As proof of concept, we tested 39 naphthoquinone compounds (Fig. 1) on human HeLa-GFP cells in 96-well plates. As potentially important anti-microbial agents would not be detected with the HeLa-GFP assay alone, this approach was used to test the same compounds on GFP-expressing *E. coli* and *M. avium* strains. As can be seen in Table 1, our results show that both mammalian cells and bacteria can be analyzed in tandem to rapidly determine which compounds are specifically toxic to one of these cell types. It should be noted that this assay can only detect reduction of GFP signal (presumably due to cytolysis) but cannot differentiate the pathways or modes of death, which can subsequently be analyzed with well-established assays. In these assays, plumbagin (Q1) was clearly the most toxic of the test compounds on both the mammalian cells and the two bacterial strains. In addition, two similar compounds, Q3 (naphthazarin) and Q5 (menadione-see

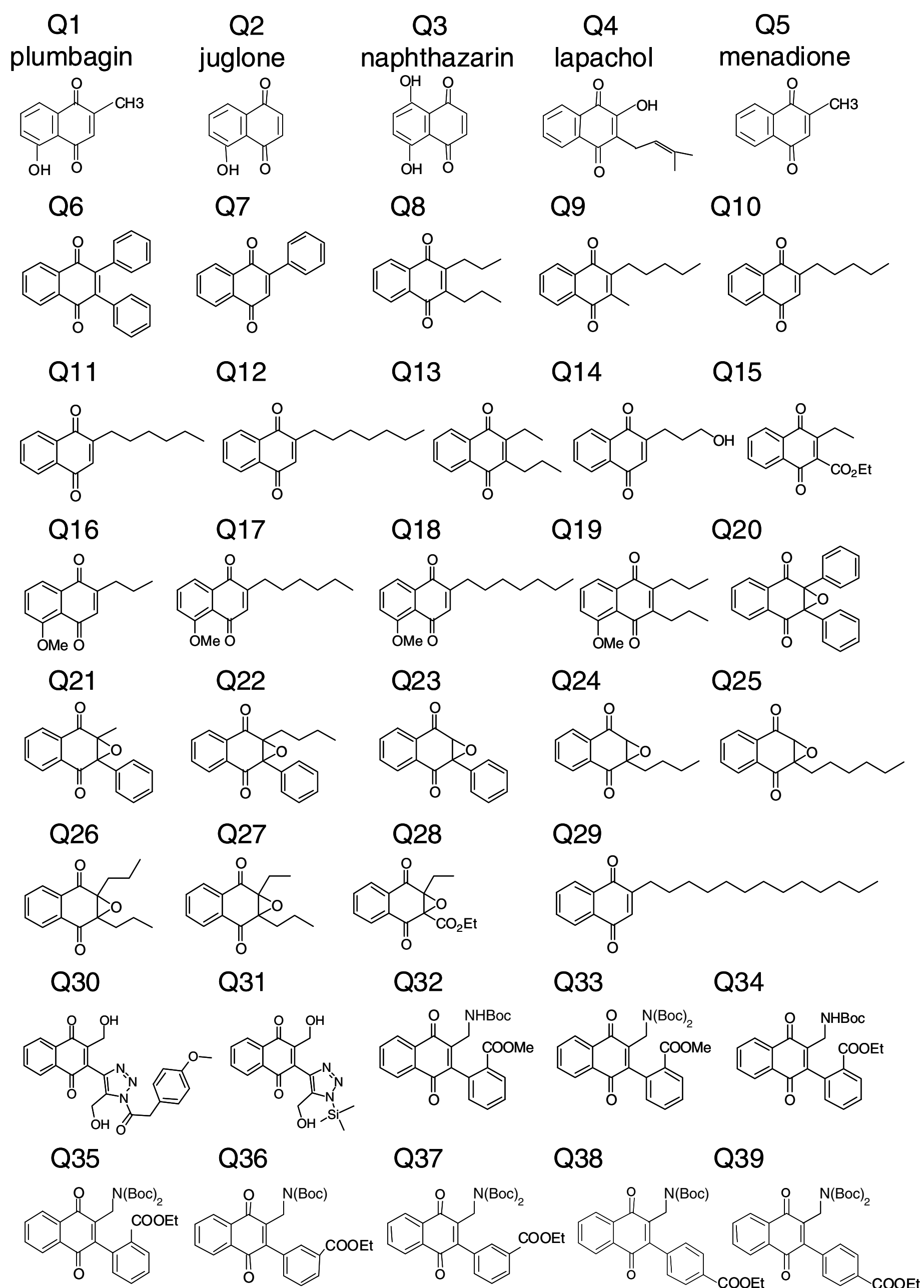

Fig. 1. Chemical structure of the naphthoquinone compounds that were synthesized for this study (see Materials and methods and supplementary materials for additional details).

Fig. 1), were also found to be highly toxic to all cell types. Apart from plumbagin, lapachol (Q4) was found to be the most toxic to *M. avium* as was previously determined with other assays [12]. Interestingly, lapachol and similar quinones have recently been shown to exhibit strong inhibitory properties against two species of *Leishmania* associated to tegumentar leishmaniasis [14]. Lapachol has also been used as an anti-cancer drug but found unsuitable for clinical use due to its toxic side effects [15]. This compound has recently been demonstrated to have anti-metastatic effects by inhibiting the invasiveness of cancer cells [16].

Although most of the compounds tested exhibited the highest level of toxicity at the higher concentration tested, several compounds were just as toxic at a 10-fold dilution (see Q1–3 and Q23–25 at 0.2 μg, Table 1) against the HeLa-GFP cell line. Apart from differences in overall toxicity, two general types of toxic compounds were detected in these assays, those that exhibited toxicity to two or all three of the cell types (Q1–7) and those that were primarily toxic to the HeLa cells (Q24, Q26–28). As these two sets of compounds target different cell types, it is likely that these compounds will have different modes of action. Future experiments should help

elucidate if these compounds have different molecular targets or if it is just a question of membrane permeability (see below). It is, however, important to point out that the observed differences would not have been detected if the compounds had been tested on a single cell type.

Several of the most toxic compounds were tested at various concentrations to determine the approximate LD50. As shown in Fig. 2, compounds Q1 and Q3 exhibited very similar LD50s of ~3.0 μM while those of compounds Q2, Q5, Q23, and Q24 were between 3 and 8 μM. It is interesting to note that although compounds Q27 and Q28 were not as toxic as the other naphthoquinones at the highest concentrations, they had similar LD50 values as the other compounds (between 3 and 8 μM). In previous work, a subset of the naphthoquinone compounds (Q1–4 and Q8) was tested in a microbroth assay for bacteriocidal and bacteriostatic effects, and found to have significant antimycobacterial activity against *M. smegmatis*, *M. avium*, and *M. tuberculosis* [12].

Activities against the three different types of cells and the molecular weight and compound solubility were analyzed for possible correlations. This analysis revealed that compound molecular weight significantly correlated with toxicity to HeLa cells ($r = 0.455$, $P = 0.0019$), with a weaker correlation to activity against *M. avium* ($r = 0.287$, $P = 0.0592$), and did not correlate with killing of *E. coli* ($r = 0.173$, $P = 0.262$). Solubility (quantified as $\log P$) followed the same trend (HeLa $r = 0.622$, $P < 0.0001$; *M. avium* $r = 0.314$, $P = 0.0586$; *E. coli* $r = 0.222$, $P = 0.1866$). This would be expected, as much of the mass added to the larger compounds were alkyl or phenyl groups, which increase hydrophobicity. Interestingly, toxicity against the two prokaryotic organisms strongly and significantly correlated with each other ($r = 0.648$, $P < 0.0001$), but not with the eukaryotic cell line (*E. coli* to HeLa $r = 0.228$, $P = 0.1361$, *M. avium* to HeLa $r = 0.221$, $P = 0.1488$). This suggests that the mechanism of action of quinones is different against prokaryotic and eukaryotic cells, which has been observed in other studies as well [12]. In addition, *M. avium* (mean viability after exposure 83.2 ± 34.1) and *E. coli* (96.6 ± 24.1) were more tolerant to the quinones than the HeLa cells (44.0 ± 33.1). This is likely because of the great permeability barrier of mycobacteria [17] and the fact that *E. coli* often uses efflux pumps against quinones [18]. Compounds containing a 2,3-epoxide were highly toxic to eukaryotic cells (compounds Q20–Q28, mean viability post-exposure of HeLa 7.0 ± 9.3), but exhibited no killing of prokaryotic cells (*E. coli* 102 ± 22, *M. avium* 106 ± 44). As mentioned earlier, the differences between the mammalian and prokaryotic cells could be attributed to the activation of different pathways and/or modes of death.

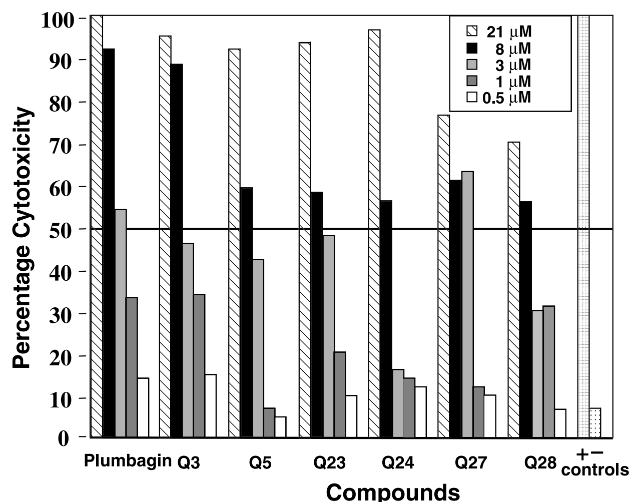

Fig. 2. Determination of the lethal dose (LD) of cytotoxic naphthoquinone compounds. Several of the most toxic compounds detected with our initial screening assay were re-tested at various concentrations to determine the LD50 in HeLa-GFP. The concentrations of the test compounds (micromolar) are shown within the figure. Horizontal bar indicates the 50% toxicity range. H_2O_2 (+ control, 1 mM) was used as a positive control (+; 100% cytolysis) while cells treated with DMSO (1 μl; solvent for all quinones) was used as the negative (−) control.

Plumbagin, which was the most toxic compound to *E. coli*, is also the most toxic to *M. avium*, and is the third-most toxic to HeLa cells. Plumbagin and other similar quinones have been shown to mediate cell death by two distinct mechanisms involving redox cycling and reaction with reduced glutathione [8]. Redox cycling results in the formation of semiquinone radicals which then lead to the generation of superoxide anions and H_2O_2 [8]. Furthermore, oxidative cycling induced by plumbagin appears to be the primary mechanism by which this compound kills mycobacteria (T. Primm, unpublished results). It is well known that reactive oxygen species (ROS) are involved in the cell death process and many apoptosis-inducing agents generate free radicals [8]. In a recent report, plumbagin was demonstrated to induce apoptosis of human cervical cancer cells (ME-180 cell line) through the generation of ROS and a caspase-dependent pathway [19]. Using cell-cytometry and known apoptotic markers, we have shown that plumbagin and other naphthoquinones induce apoptosis in a lymphocyte cell line [6]. Interestingly, a significant level of necrosis was induced by the naphthoquinones and the positive control H_2O_2 [6]. Since oxidative stress has been shown to lead to necrosis [20–22], the detection of necrosis is consistent with the generation of ROS by these compounds as has been previously described [8,19]. It is therefore very likely that both mechanisms are occurring simultaneously leading to the potent cytotoxic effects detected with the compounds that we have tested thus far.

There are few effective antibiotics against *Mycobacterium* sp. and novel drugs are desperately needed

against these pathogenic bacteria. A recent screen of acetophenone and naphthoquinone derivatives revealed that some of these compounds have significant antibacterial properties [9,12]. Acetophenone derivatives with the strongest antibiotic properties were subsequently tested in the HeLa-GFP cytotoxicity assay to determine if any of these compounds exhibited cytotoxic effects against mammalian cells [9]. This analysis revealed that two of the most promising compounds were also highly toxic to mammalian cells. Several of the antibacterial acetophenone compounds exhibited mild cytotoxicity but most importantly three of these compounds were determined to exert their activity selectively against *M. avium* [9]. Although our current analysis of naphthoquinone derivatives on *M. avium* did not yield a compound with selective toxicity against this organism, it is anticipated that through extensive screening such a compound(s) will eventually be discovered.

With the use of existing and new synthesis methods, hundreds, if not thousands, of novel compounds can be readily generated but methods for determining the activity of these compounds have not been fully developed. With the use of the simple screening methods that we have described, we hope that we will be able to identify agents with preferential anti-cancer and/or anti-bacterial properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.07.086.

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