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Bacterial Cytological Profiling Reveals the Mechanism of Action of Anticancer Metal Complexes

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ABSTRACT: Target identification and mechanistic studies of cytotoxic agents are challenging processes that are both time consuming and costly. Here we describe an approach to mechanism of action studies for potential anticancer compounds by utilizing the simple prokaryotic system, *E. coli*, and demonstrate its utility with the characterization of a ruthenium polypyridyl complex [Ru(bpy)₂dmbpy²⁺]. Expression of the photoconvertible fluorescent protein Dendra2 facilitated both high throughput studies and single cell imaging. This allowed for simultaneous ratiometric analysis of inhibition of protein production and phenotypic investigations. The profile of protein production, filament size and population, and nucleoid morphology revealed important differences between inorganic agents that damage DNA vs. more selective inhibitors of transcription and translation. Trace metal analysis demonstrated that DNA is the preferred nucleic acid target of the ruthenium complex, but further studies in human cancer cells revealed altered cell signaling pathways compared to the commonly administered anticancer agent cisplatin. This study demonstrates *E. coli* can be used to rapidly distinguish between compounds with disparate mechanisms of action, but more subtle distinctions within in studies in mammalian cells.

KEYWORDS: Cancer, drug discovery, cisplatin, ruthenium, bacterial cytological profiling

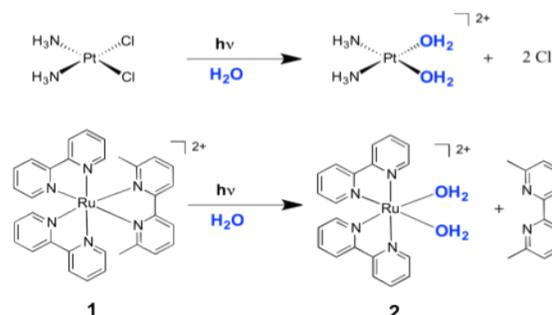
INTRODUCTION

The fortuitous observation of filamentous growth of *E. coli* by Barnett Rosenberg led to the discovery of cisplatin, one of the most important and widely used chemotherapeutic agents.¹⁻³ Cisplatin, and its later generation analogues, are essential components in clinical treatments of ovarian, testicular, small cell lung, and head and neck cancers.⁴⁻⁶ The administration of platinum drugs, however, is limited by adverse side-effects, including nephrotoxicity, neurotoxicity, ototoxicity and other complications.⁷⁻⁸ Drug resistance (either intrinsic or acquired) compromises the efficacy of platinum drugs as well.⁹⁻¹¹ These deficiencies have necessitated the development of new chemotherapeutic agents to overcome such obstacles.

Significant efforts have been applied in the field of medicinal inorganic chemistry to identify cytotoxic agents that replicate the efficacy of cisplatin, with the hope of adding to our current arsenal of chemotherapeutic drugs.¹²⁻¹⁴ While many of the new chemical entities show promising efficacy, the understanding of their biological activities is often incomplete. The very nature of inorganic agents (with variable charge states, geometries, and coordination numbers, all of which can be altered by speciation) adds to the challenge, and can result in polypharmacology.^{13,15} As a result, elucidation of the biological effects of potential medicinal inorganic agents has lagged far behind chemical innovation. For example, oxaliplatin, which has been in clinical use for over twenty years, was recently reported to induce ribosome biogenesis stress,¹⁶ rather than the previously accepted mechanism involving DNA damage similar to cisplatin. Organic or inorganic agents developed through target-based drug discovery avoid some of these pitfalls, but undesired off-target effects are prevalent for these systems as well. Thus, mechanistic studies are necessary even for compounds designed to inhibit single, well-validated targets.¹⁷⁻¹⁹

Despite multiple technological advances, the identification of the mechanism of action for cytotoxic compounds remains a time consuming and challenging process. While simple *in vitro* systems such as purified enzymes and nucleic acids can provide key insights, there are undeniable advantages to working in living cells. Bacteria are intrinsically simpler systems than eukaryotic cells, with *E. coli* containing only 4,288 genes,²⁰⁻²¹ as opposed to the approximately 30,000 genes found in the human genome.²²⁻²³ Essential processes are homologues between bacteria and eukaryote, including DNA replication, transcription, and translation. It is well known that many agents that are toxic to eukaryotic systems also have antibacterial activities, such as classical antitumor antibiotics, though many orthogonal variations do exist between the two.²⁴⁻²⁵

Rosenberg's classical experiment illustrated that a simple prokaryotic system could be employed to discover anticancer agents. Recently, other groups, including those of Lippard and Brabec, have utilized *E. coli* phenotypic assays as qualitative means to characterize potential anticancer agents, and as with



Scheme 1. Thermal hydrolysis of A) cisplatin and B) the photochemical hydrolysis of compound 1.

1 cisplatin, showed a good correlation between activity in the
2 prokaryotic system and cancer cells.²⁶⁻²⁸ We also have an
3 interest in simple biological systems, but our motivation is
4 instead to utilize them as a tool to investigate mechanistic
5 details of anticancer agents. Our premise is that compounds
6 which are found to be active in mammalian cells but not *E. coli*
7 can be expected to affect processes or targets absent in the
8 simpler biological system. Alternatively, compounds which
9 show similar activities in the two cell types can be deduced to
10 inhibit processes common to both. Thus, it should be possible
11 to use *E. coli* as a first-pass screen to radically reduce the
12 number of likely biological entities or processes targeted by
13 cytotoxic agents. Furthermore, *E. coli* is readily amenable to
14 incorporation of genetically encoded reporter systems, allowing
15 for additional phenotypic analysis to be used to rapidly parse
16 mechanistic features of active compounds.^{18, 29} This approach
17 could greatly expedite mechanism of action studies.

18 Here we describe studies that demonstrate that *E. coli* is an
19 excellent model for mammalian systems to investigating the
20 effect of metal complex inhibition of cell growth, and
21 phenotypic changes consistent with DNA damage.³⁰ A
22 promising light-activated ruthenium complex developed in our
23 laboratory³¹ (compound **1**, Scheme 1) was compared to cisplatin,
24 along with three organic antibiotics. Noteworthy differences
25 were observed between the inorganic compounds and organic
26 compounds in the bacterial system; these differences directly
27 correlate with their different mechanisms of action. Moreover,
28 differences between compound **1** and cisplatin in mammalian
29 cells suggest more subtle disparities in their mechanistic
30 features, which offers the possibility to maintain anticancer
31 efficacy without experiencing the same resistance profile by
32 altering the metal center from platinum to ruthenium.

30 METHODS

31 *E. coli* culture maintenance

32 The Dendra2 gene was cloned into a pCW-ori plasmid modified
33 to contain an N-terminal 6x histidine tag with multiple
34 restriction enzyme cloning sites. *Escherichia coli* BL21(DE3)
35 competent cells transformed with pCWori plasmid containing
36 Dendra2 gene (pCWori-Dendra2) were cultured in Luria Broth
37 (LB) at 37 °C with 180 rpm shaking.

39 Mammalian cell maintenance

40 Human promyelocytic leukemia HL60 cells were purchased
41 from ATCC. Dulbecco's modified eagle medium (DMEM),
42 Iscove's modified Dulbecco's medium (IMDM), Opti-MEM I
43 reduced serum medium, heat inactivated fetal bovine serum
44 (FBS), Penicillin/Streptomycin (5,000U/mL), Trypsin-EDTA
45 (0.5%), Dulbecco's phosphate buffered saline (DPBS), Trypan
46 Blue Solution (0.4%) were purchased from Life Technologies.
47 HL60 cells were maintained in IMDM supplemented with 10%
48 FBS and 50 U/mL of Penicillin/Streptomycin. A549 cells were
49 maintained in DMEM with the same supplements. Cells were
50 maintained at 37°C with 5% CO₂.

52 Cytotoxicity determination

53 *E. coli* BL21(DE3) cells transformed with pCWori-Dendra2
54 plasmid were plated in M63 minimal medium at 4 x 10⁶ cells
55 per well in 96 well flat bottom transparent tissue culture treated
56 plates (Greiner Bio One). Compounds were dosed from 0 – 300

μM, followed by 3 min of light irradiation (7 J/cm² blue light (>
400 nm)). The cells were then incubated for 16 hours with the
compounds, and cell growth was determined by measurement
of the optical density at 600 nm using a SpectraMax Multiwell
Plate Reader (Molecular Devices).

HL60 cells were plated in Opti-MEM supplemented with 1%
FBS and 50 U/mL of Penicillin/Streptomycin at 30,000 cells per
well in 96 well flat bottom transparent tissue culture treated
plates (Greiner Bio One). Compounds were dosed from 0 – 300
μM, and incubated for 16 hrs, followed by light irradiation with
7 J/cm² blue light (> 400 nm) in 30 second pulses for a total
light exposure of 3 minutes. The cells were then incubated for
72 hours, and cell viability determined by conversion of
resazurin to resorufin. Dark controls were run in parallel. The
emission of resorufin was measured on a SpectraFluor Plus
Plate Reader (Tecan).

The data were normalized to the untreated control and fitted
to a sigmoidal dose response model using Prism 6.02 to
determine IC₅₀ values. Minimal inhibitory concentration (MIC)
was fitted to the model published by Lambert et al using Prism
6.02.³²

Protein synthesis inhibition

E. coli BL21DE3 cells transformed with pCWori-Dendra2 were
cultured in LB medium to an OD₆₀₀ of 0.8. Cells were then
resuspended in M63 minimal media and induced with 0.5 mM
IPTG for 3 hours at 37 °C with 180 rpm shaking. Photoconversion
of Dendra2 was carried out with a 405 nm LED flood array
(Loctite) with a total light exposure time of 2 minutes. Cells
were then plated in 96 well plates at 6 x 10⁷ cells per well.
Green and red emission was measured directly after
photoconversion using a SpectraMax Multiwell Plate Reader
(Molecular Devices) for a baseline evaluation of Dendra2
protein (t = 0 hour). For green emission, an excitation
wavelength of 491 nm and emission wavelength of 538 nm was
used; for red emission, the excitation wavelength was 544 nm
and emission wavelength was 590 nm. Compounds were then
dosed from 0 μM to 300 μM, and compound **1** was activated
with light as described above. The cells were incubated for 16
hours before the green and red emission was measured again for
an evaluation of protein synthesis with compound treatment (t =
16 hours).

The average fluorescence ratio of green/red at t = 0 hour and
t = 16 hours was calculated, the values were normalized, and
the data fitted to a sigmoidal dose response.

E. coli filamentous growth

E. coli were cultured as above and plated at 3 x 10⁸ cells per
well in 24 well flat bottom transparent tissue culture treated
plates (Greiner Bio One). IPTG was added at a concentration of
0.5 mM for induction of Dendra2 production. Compound
treatment was then carried out, with cells dosed at the MIC or
10x MIC for each compound and cultured at 37 °C with 180
rpm of shaking for 6 and 16 hours before imaging.

E. coli cell imaging

After compound treatment, *E. coli* cells were centrifuged at
8000 rpm for 2 minutes, washed twice with PBS, and 3 x 10⁷
cells were resuspended in 1 mL of PBS. The fluorescent dyes
FM4-64 and Hoechst 33342 were added to a final concentration

of 5 µg/mL and 10 µg/mL respectively. The cells were protected from light for 20 minutes, and then 2 µL of cell suspension was placed on a slide and a cover glass was applied before imaging. Imaging was carried out on an Olympus IX2-RFAEVA-2 microscope with the following filter settings:

Dendra2 (green), excitation filter: 473/10 nm BrightLine® single-band bandpass filter, FF01-473/10-25 (Semrock, Rochester, NY, USA); emission filter: 525/50 nm BrightLine® single-band bandpass filter, FF03-525/50-25 (Semrock, Rochester, NY, USA). Dendra2 (red) and FM4-64, excitation filter: HQ 550/30 (Chroma, Bellows Falls, VT, USA); emission filter: 664 nm EdgeBasic long-pass edge filter, BLP01-664R-25 (Semrock, Rochester, NY, USA). Hoechst 33342, excitation filter: BP 360-390 (Chroma, Bellows Falls, VT, USA); emission filter, HQ470/30 M (Chroma, Bellows Falls, VT, USA).

Imaging data was processed and analyzed with ImageJ.

Metal uptake in bacterial cells

E. coli were cultured in M63 minimal medium as discussed above and dosed with 20 µM compound **1** or cisplatin. Cells treated with compound **1** were irradiated with 7 J/cm² blue filtered light (> 400 nm) for a total of 3 minutes, or were protected from light. Cells were collected 24 hours after compound addition by centrifugation at 8000 rpm for 5 minutes. The culture medium was separated for analysis, and cells were washed twice with PBS and pelleted. Both cell content and medium were heated at 110 °C for 3 hours with 20% (v/v) HNO₃. Total RNA and genomic DNA were isolated using Qiagen kits. RNA and DNA samples were digested in HNO₃ as described above. Following sample digestion, the metal content was analyzed using a Varian AAS with a replicate reading and a spiked reading.

Cellular uptake was calculated as follows:

$$\text{Cellular uptake} = \frac{\text{Metal measured in cell sample } (\mu\text{mol})}{\text{Metal in cell sample} + \text{media } (\mu\text{mol})} \times 100\%$$

Genomic DNA and total RNA were quantified by measuring their absorbance at 260 nm. Mass to DNA nucleotide pair conversion was calculated using the average molecular weight of DNA nucleotide pairs. The number of DNA nucleotide bases per metal center was calculated as following.

$$\text{DNA nt./mc.} = \frac{2 \times \text{DNA nucleotide base pairs } (\mu\text{mol})}{\text{Metal measured with DNA samples } (\mu\text{mol})}$$

Mass to RNA nucleotide base conversion was calculated using the average molecular weight of RNA nucleotide bases. Number of RNA nucleotide bases per metal center was calculated as following.

$$\text{RNA nt./mc.} = \frac{\text{RNA nucleotide bases } (\mu\text{mol})}{\text{Metal measured with RNA samples } (\mu\text{mol})}$$

Metal uptake in HL60 cells

HL60 cells were plated in Opti-MEM supplemented with 1% FBS and 50 U/mL Penicillin/Streptomycin at a density of 1x 10⁶ cells/mL in 25 cm² cell culture flasks, and dosed with 20 µM compound **1** or cisplatin. Cells treated with compound **1** were incubated 12 hours protected from light before irradiating with 7 J/cm² blue filtered light (> 400 nm) in 30 second pulses for a total of 3 minutes, or protected from light. Cells were collected 24 hours after compound addition by centrifugation at 124 x g for 5 minutes. The culture media was separated for

analysis, and cells were washed twice with PBS. Total RNA and genomic DNA were also isolated, and the nucleic acids, cell content, and media were prepared for analysis as described above.

Immunoblotting

HL60 cells were harvested 0, 1, 3, 6, 12, 24, 30 and 48 hours after treatment, pelleted by centrifugation at 124 x g for 5 minutes, washed twice with DPBS. A549 cells were plated at 2 x 10⁵ cells per well in 6 well flat bottom transparent tissue culture treated multiwell plates and in the same treatment condition detailed for HL60 cells. Cells were harvested at 0, 6, 12, 24 and 48 hours after treatment.

All cells were lysed in RIPA buffer supplemented with 5 mM sodium pyrophosphate (2 x 10⁶ cells/100 µL) for 15 minutes on ice. The insoluble fraction was removed by centrifugation at 20,817 x g for 10 minutes at 4 °C. The supernatant was collected and the protein concentration was determined by BCA assay. 20 µg of protein was loaded onto 4-12% bis-tris gels and followed by transfer to nitrocellulose membranes. After blocking with 2.5% BSA in DPBS with 0.1% Tween20 (PBST) for 1 hour at room temperature, the membrane was immunoblotted with the following primary antibodies and correspondence dilutions.

Cleaved caspase 3, cleaved PARP, p-p53, p21, p-Chk1, p-JNK and γ-H2AX at 1:1000 dilutions; p53 and p-ERK at 1:500 dilutions; and GAPDH at a 1:2000 dilution in 2.5% BSA overnight at 4 °C. Immunoblots were washed with PBST for 10 minutes for four times and incubated for 1 hour with secondary antibodies at a 1:10000 dilution for GAPDH and 1:5000 dilutions for all other antibodies. Detection was carried out with Clarity Western ECL Substrate and imaged with a ChemiDoc MP System (Bio-Rad).

DNA fragmentation

HL60 cells were cultured, and treated as described above. Cells were harvested at 0, 3, 8, 12, 24 and 30 hours after treatment, pelleted by centrifugation at 124 x g for 5 minutes, washed twice with DPBS, and prepared with an apoptotic DNA-ladder kit as per manufacturer instructions (Rosch). Gel electrophoresis was carried out using a 1 % agarose gel containing 0.5 µg/mL ethidium bromide for 90 minutes at 75 V. Gel imaging was performed with the ChemiDoc MP.

Flow cytometry

HL60 cells were cultured, and treated as detailed previously. Cells were harvested at 24 hours after treatment, pelleted by centrifugation at 124 x g for 5 minutes, washed twice with DPBS. For cell death mechanism analysis, cells were stained 15 minutes with FITC-Annexin V and PI; for cell cycle analysis, cells were stained 15 minutes with PI only. Cells were analyzed with a FACSCalibur (Becton-Dickenson). A minimum of 20000 events were measured for each sample.

RESULTS

Comparison of Compound Efficacies in *E. coli* and Mammalian Cancer Cells.

The capacity of *E. coli* to serve as a model system for cancer cells was first evaluated by comparing the relative cytotoxicities of the metal complexes in the two cell types. The ruthenium

Table 1. Cytotoxicity values and inhibition of protein production for various compounds in *E. coli* and HL60 cells.

	<i>E. coli</i>			HL60
	MIC (μM)	Growth Inhibition IC_{50} (μM)	Dendra2 Production Inhibition IC_{50} (μM)	Cytotoxicity IC_{50} (μM)
1 light	6.1 \pm 0.8	2.6 \pm 0.4	77 \pm 3	3.4 \pm 0.3
1 dark	> 300	> 300	> 300	> 300
Cisplatin	4.4 \pm 0.5	2.0 \pm 0.1	85 \pm 11	2.6 \pm 0.4
Rifampicin	0.6 \pm 0.3	0.3 \pm 0.1	2.8 \pm 0.3	n.d.
Tetracycline	10 \pm 1.1	4.8 \pm 0.7	1.3 \pm 0.2	n.d.
Nalidixic Acid	5.2 \pm 2.0	2.6 \pm 0.4	4.6 \pm 0.1	n.d.

Table 2. Cellular metal uptake and metal content with different nucleic acids measured by AAS.

	<i>E. coli</i>			HL60		
	Cellular uptake ^a	DNA nt / mc ^b	RNA nt / mc ^c	Cellular uptake ^a	DNA nt / mc ^b	RNA nt / mc ^c
1 light	10%	2000 \pm 200	3800 \pm 600	0.64%	4800 \pm 400	5000 \pm 700
1 dark	2%	- ^d	- ^d	0.11%	- ^d	- ^d
Cisplatin	6%	3000 \pm 200	4700 \pm 900	0.72%	7000 \pm 200	7800 \pm 700

^aCellular uptake was calculated as metal content measured in cells divided by total metal content in both cell samples and cell culture media samples. ^bDNA nt / mc was calculated as DNA nucleotide bases (μmol) divided by metal content measured in DNA sample (μmol). ^cRNA nt / mc was calculated as RNA nucleotide bases (μmol) divided by metal content measured in DNA sample (μmol).

^dRuthenium levels in DNA and RNA samples were under the detection limit (< 2 ppb).

complex prodrug, compound **1**, and cisplatin were tested in dose response, along with the antibiotics rifampicin, tetracycline and nalidixic acid. Optical density was used to quantify the response in *E. coli*. The activity of **1** was evaluated both in the absence of light and after light activation (described as “dark” and “light”; irradiation results in the formation of compound **2**; Scheme 1). The half maximal inhibitory concentration (IC_{50}) value was compared with the minimum inhibitory concentration (MIC),³³ an important clinical standard parameter³²⁻³⁵ that effectively defines the lowest concentration to achieve a complete inhibition effect. As shown in Table 1, IC_{50} values of 2.6 and 2.0 μM for light-activated **1** and cisplatin were obtained in *E. coli*, with MIC values that were 2–3-fold higher.

The biological activity of cisplatin and compound **1** was also studied in human promyelocytic leukemia HL60 cells. This relatively fast growing suspension cell line and was chosen over adherent cell lines to more closely resemble bacterial growth conditions. Upon light irradiation, **1** exhibited an IC_{50} of 3.4 μM , similar to the IC_{50} of 2.6 μM for cisplatin. No cytotoxic effect was seen for compound **1** at 300 μM in the dark, resulting in a phototoxicity index (PI) of > 88 . As expected, the cytotoxicity of cisplatin was not affected by treatment with light. These experiments demonstrated that light irradiated **1**, like cisplatin, is cytotoxic in both prokaryotic and eukaryotic cells, and with very similar potencies, suggesting the mechanism of action is through general cellular targets or biological processes present in both cell types.

Cellular Uptake and Nucleic Acid Metallation.

Cellular uptake of the metals in *E. coli* was measured by atomic absorption spectroscopy (AAS; Table 2). Light irradiation of compound **1** resulted in a 5-fold increase in cellular uptake, with

a total of 10% of the dosed compound localized in *E. coli* cells. Only 6% of the dosed cisplatin was found in the cells.

Genomic DNA and total RNA isolation was performed after 24 hours of treatment, followed by AAS analysis for ruthenium or platinum. While no ruthenium was found with either of the nucleic acids for compound **1** in the dark, 1.3% of the ruthenium was found with the DNA when the compound had been exposed to light. This corresponds to a ratio of 2000 nucleotide bases per metal center (nt / mc). Only 0.5% of ruthenium was found with the RNA, providing a ratio of 3800 nt / mc. As a result, the active compound **2** appears to be slightly more reactive with DNA than RNA, with about a 1.5–2-fold difference between the metal levels in the two nucleic acids. A similar trend of increased reactivity with DNA over RNA was observed for cisplatin, with 3000 nc / mc in DNA and 4700 nt / mc in RNA, to give a 1.6-fold difference in apparent reactivity.

The uptake of the compounds was also assessed in mammalian cells. After 24 hour treatment with 20 μM compound **1**, 0.64% of dosed ruthenium was found in HL60 cells with light irradiation, in contrast to only 0.11% present when the cells were kept in the dark. These results indicate that the prodrug form is taken up much less effectively than the active species. The metal content of the active compound in cells is comparable to the 0.72% of cisplatin that accumulated under the same conditions. Isolation of DNA and RNA and metal content analysis revealed that no nucleic acid-bound ruthenium was observed for **1** in the dark, but treatment of **1** and irradiation resulted in 4800 nt / mc in DNA, and 5000 nt / mc in RNA. This corresponds to 1.3% of the cellular ruthenium found with the DNA and 2.0% in the RNA. Quantification of the metal binding of cisplatin gave 7000 nt / mc in DNA and 7800 nt / mc in RNA (1.1 and 1.5%). The nucleotide base to metal center ratios were

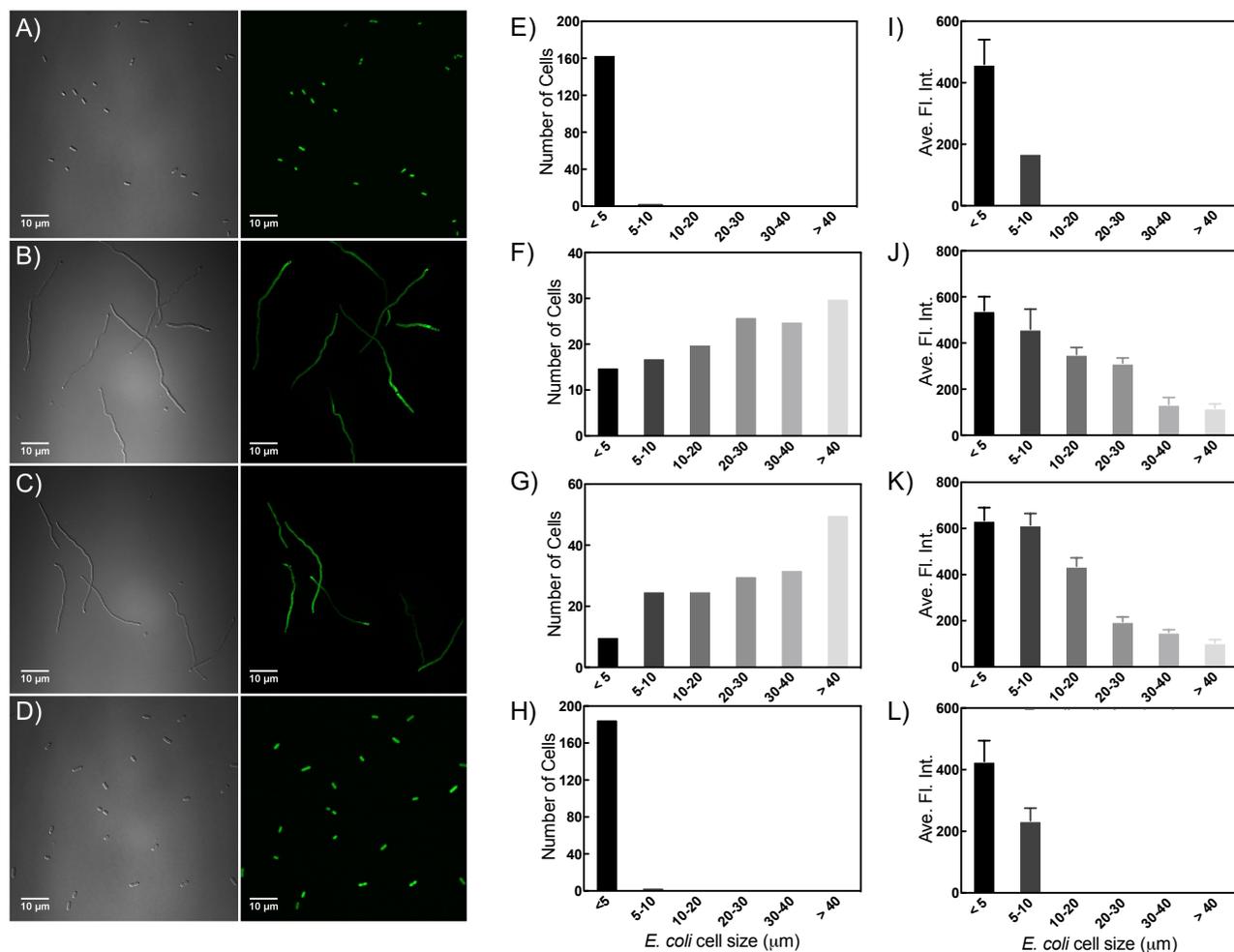


Figure 1. Complex 1 induces filamentous growth and decreased protein production in *E. coli*. Bright field and fluorescent imaging of *E. coli* cells. A) N. C. control, B) cisplatin, C) compound 1 with light, D) compound 1 in the dark. Size distribution histograms of *E. coli* cells associated with the conditions for A) - D): E) N. C. control, F) cisplatin, G) compound 1 with light, H) compound 1 in the dark. Histograms of average fluorescence intensity correlated to cell size with the different treatments: I) N. C. control, J) cisplatin, K) compound 1 with light, L) compound 1 in the dark. Cells were treated with 100 μM of each compound for 6 hours before imaging.

It has been reported by DeRose *et al.* that platinum accumulates more in the cellular RNA than DNA.³⁶ This is partly due to the higher abundance of RNA in the cell (10–50-fold). Despite this difference in abundance for the different nucleic acids, DeRose demonstrated that there is a 3.8-fold preference for cisplatin to react with DNA vs. RNA in *S. cerevisiae*, with 1661 nt / mc in DNA and 6369 nt / mc in RNA after 12 hours of treatment at 100 μM.³⁶ While our study used 20 μM of cisplatin treatment for 24 hours, we observed the same preferential metal binding with DNA over RNA in both *E. coli* and mammalian cancer cells, though we observed a closer nt / mc ratio between DNA and RNA. This similar binding trend across different cell types reveals once more that cisplatin exhibits a general DNA damaging ability in both eukaryotic and prokaryotic systems. The similar biological accumulation characteristics of compound 1 and cisplatin in bacterial, yeast, and mammalian cells suggest a mechanism of action through common biological targets or processes present in both prokaryotic and eukaryotic cell types. In combination with extensive *in vitro* DNA damaging assays,^{31,37} this supports a DNA-based mechanism of

action, though multiple subsequent events may be involved that induce the cytotoxic effects.

Phenotypic Analysis of *E. coli* Following Compound Treatment

1) Filament Size. For imaging studies, the MIC was used in order to more closely mimic physiological treatment conditions; data was also taken at 10x MIC. The cytological characteristics of the *E. coli* were assessed, and elongated cells were observed after treatment with cisplatin and compound 1 with irradiation (Figure 1). Treatment with 1 in the absence of light didn't induce *E. coli* filamentous growth, and the cells were characterized by the same short rod shaped morphology as the untreated control.

To gain a more quantitative understanding of filament formation in populations, cells in multiple views (~200 per condition) were chosen for size analysis. Treatment at 10x MIC with cisplatin and compound 1 with irradiation caused a shift in population distribution, where 68 and 73% of cells were filamentous for cisplatin and 1, respectively. The major population group at 100 μM 1 were cells over 40 μm long (29%),

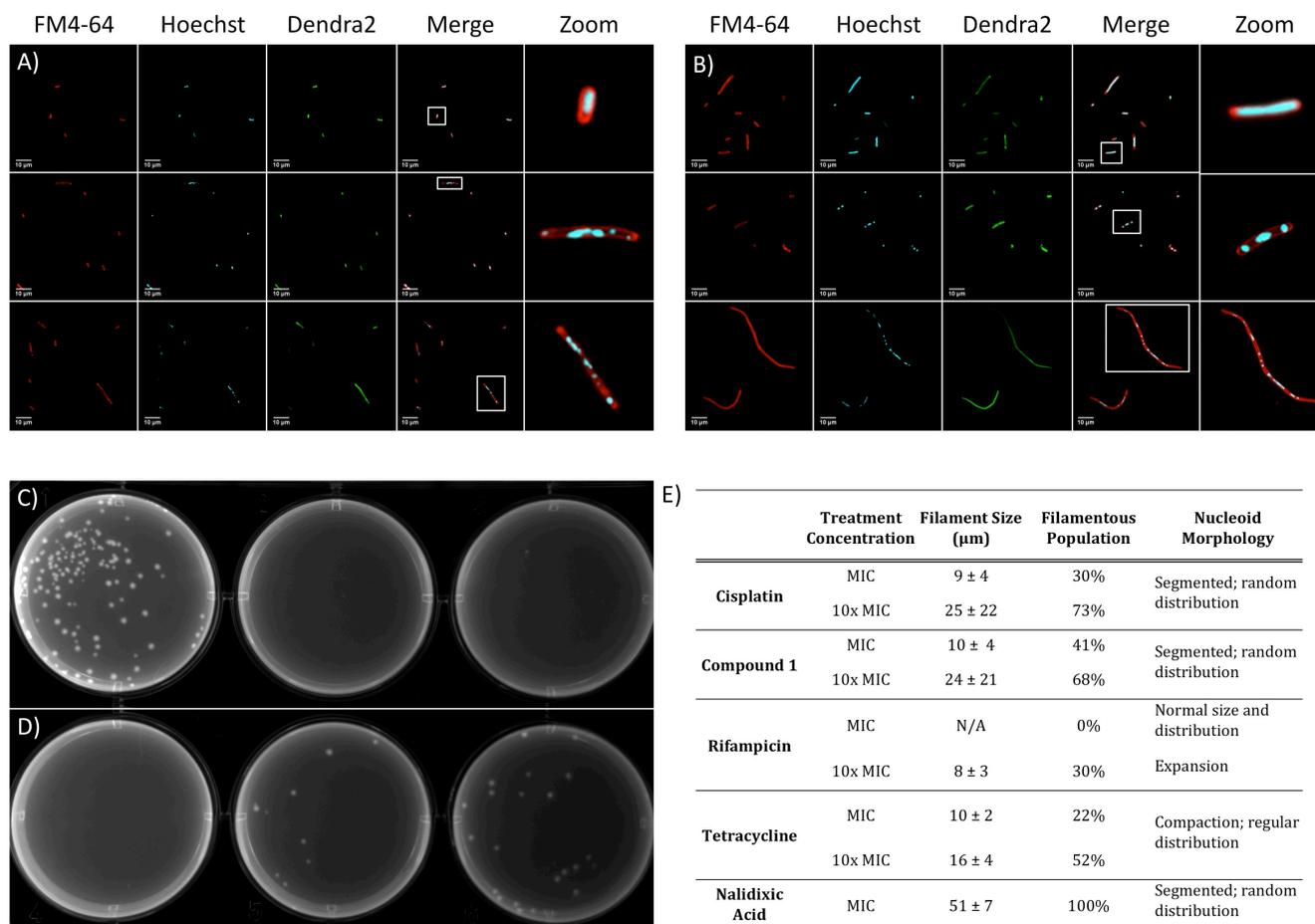


Figure 2. Phenotypic profiles of compounds with different mechanisms of action in *E. coli* cells. Fluorescent imaging: A) Top: N. C. control; Middle: cisplatin; Bottom: compound **1** with light; B) Top: rifampicin; Middle: tetracycline; Bottom: nalidixic acid. The merge is the combination of the Hoechst and FM4-64 membrane stain. Colony forming experiment with various compounds: C) Left: N. C. control; Middle: cisplatin; Right: compound **1** with light; D) Left: nalidixic acid; Middle: rifampicin; Right: tetracycline. Cells were treated with each compound at MIC for 6 hours before imaging or colony forming. E) Quantitative and qualitative analysis of *E. coli* filamentous growth and nucleoid morphology phenotypes in response to compounds treatment.

and only 5% of the cells were in the $\leq 5 \mu\text{m}$ size range. The same trend was seen after cisplatin treatment, and the histograms for the population of filaments are remarkably similar (Figure 1F and G). In contrast, at the MIC, both the filament length and % filamentous population were lower, with only 30% and 41% of cells forming filaments for cisplatin and **1** (Figure 2E and S4). As shown in the histograms in Figure 1E and H, both the no treatment control and dark control for compound **1** exhibited a dominant population (over 97%) of cells in the $\leq 5 \mu\text{m}$ size range, which represents the normal *E. coli* cell size. Thus, filamentation is only associated with the light-activated form of **1**.

It has been observed that many compounds induce filamentous growth of *E. coli*. In order to determine if this morphological feature corresponds to the compounds' mechanisms of action, we compared the metal-based compounds, preliminarily classified as DNA crosslinkers, to two commonly used antibiotics that inhibit transcription or translation, and one gyrase inhibitor. Rifampicin prevents transcription by binding and inhibiting the bacterial DNA-dependent RNA polymerase (RNAP)³⁸⁻⁴⁰ and tetracycline

inhibits translation via binding to the 30S subunit of the ribosome, preventing entrance of aminoacyl-tRNAs to the A-site. These compounds were selected as agents that do not induce DNA damage.⁴¹⁻⁴³ Nalidixic acid, which inhibits gyrase and induces DNA double strand breaks, was investigated as a DNA damaging agent with a distinct mechanism of action from cisplatin.⁴⁴⁻⁴⁵

All antibiotics were able to induce *E. coli* filaments, but the populational size analysis revealed the major populations of *E. coli* varied significantly in length. Tetracycline treatment at the MIC resulted in a large fraction (88%) of the cell population of normal length, with only 22% forming short (5–10 μm) filaments. In marked contrast, nalidixic acid induced very long filamentation, and the filaments were the only population (100%; average length of 51 μm). For rifampicin, a concentration of 10x MIC was required to induce any filaments. Treatment at this concentration resulted in 30% of the population forming short filaments of 5–10 μm ; 70% of cells were normal length ($\leq 5 \mu\text{m}$; Figure S2). This initial analysis made clear that compound **1**, cisplatin, and nalidixic

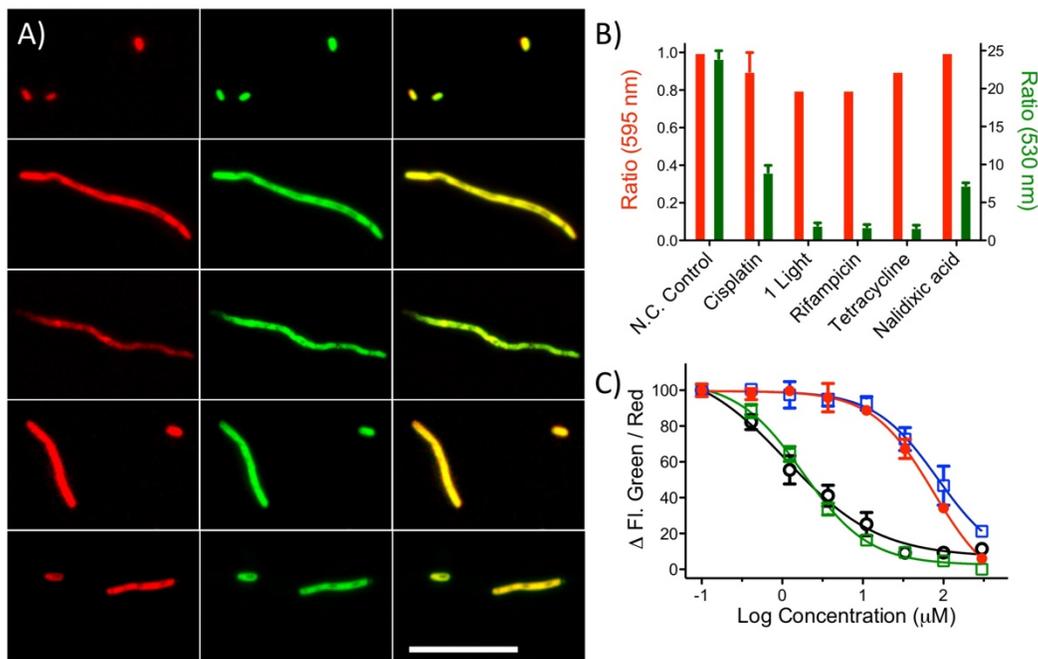


Figure 3. Dendra2 expression in *E. coli* cells. A) Dendra2 distribution in cells and filaments; Red Dendra2 (left), Green Dendra2 (middle), merge (right). From top to bottom: N. C. control; cisplatin (100 μM); compound **1**, light (100 μM); rifampicin (3 μM); tetracycline (48 μM). Cells were treated for 6 hours before imaging. The scale bar is 20 μM. B) Dendra2 production inhibition after 16 hours treatment. Compound treatment only affects the production of new, green Dendra2. C) Dose response of Dendra2 production inhibition measured at 0 and 16 hours after treatment with cisplatin (blue), compound **1** with light (red), rifampicin (green), tetracycline (black).

acid all induced longer filaments that were a larger portion of the population under all treatment conditions than antibiotics that inhibited transcription or translation.

2) Membrane Integrity. The membrane stain FM4-64 was used to confirm that the observed filaments were single cells and to visualize membrane integrity. As shown in Figure 2A and B, filaments were formed by single cells upon compound treatment. No disruption of the cell membrane was observed, indicating that the phenotypic changes were not associated with cell lysis. This is consistent with results that were obtained utilizing Trypan blue staining of HL60 cells, which indicated that neither of the two metal compounds act as membrane damaging agents. Thus, the abnormal features observed occur in live cells and are not an artifact resulting from physical disruptions of cellular integrity. In addition, the mechanism of action does not entail membrane damage.

3) Nucleoid Morphology and Number. As *E. coli* contain a single chromosome, DNA staining and analysis allows for detection of DNA fragmentation or other morphological changes due to compound treatment. Over 30 cells per treatment condition were analyzed, and distinct effects were observed for the impact of the different compounds on *E. coli* nucleoids (Figure 2A and B).

Both rifampicin and tetracycline treatment produced filaments with a regular distribution of DNA. Rifampicin treatment (at 10x MIC) produced the fewest nucleoids, with the majority of filaments containing a single nucleoid that spread along the length of the cell. Tetracycline, in contrast, produced a number of nucleoids in each of the filaments, and the nucleoids were compact and regularly distributed throughout the cell.

In marked contrast, nalidixic acid, cisplatin, and light irradiated **1** caused expansion, fragmentation, and irregular distribution of nucleoids. Both the size and distribution of the

nucleoids within the cells were quite varied. In order to quantify this observation, the % STD (the ratio of the standard deviation to the average nucleoid size, used as a measurement of variability; Figure S12) was calculated. The % STD was 38% for the no treatment control, and was 36–49% for the transcription and translation inhibitors. In the metal complex and nalidixic acid treated systems, however, the % STD was 121–160%. Values greater than 100% indicate the standard deviation of nucleoid size exceeded the average size of the nucleoids. This large range of nucleoid size implicates issues of DNA fragmentation and failure of DNA segregation after DNA replication.⁴⁶ The morphological changes in the bacterial nucleoids treated with the metal compounds and nalidixic acid demonstrate a multifaceted process as a consequence of DNA damage, in contrast with compounds that act to inhibit transcription or translation, which did not result in DNA fragmentation.

It is well established that the processes of transcription and translation are closely coordinated in *E. coli*, and the “transertion model” posits, in part, that coupled transcription-translation and membrane association of the growing protein impacts nucleoid morphology. Thus, any process that interferes with mRNA production and protein synthesis could be reflected in the nucleoids. A recent report demonstrated that transcription and translation inhibitors affected *E. coli* nucleoid shape and spatial distribution, with expansion observed with treatment of rifampicin and compaction with tetracycline.⁴⁶ This is qualitatively similar to our results. In addition, treatment with nalidixic acid resulted in the observation of fragmented nucleoids,⁴⁶ similar to our imaging results with this compound and the metal complexes. This supports our hypothesis that nucleoid morphology can be used as a phenotypic indicator of DNA damage.

1 A colony forming assay was performed to provide further
2 support for the assignment of a DNA damaging mechanism of
3 action (Figure 2C and D). Cells were treated at the MIC for each
4 compound, and then media removed and the cells were spread
5 on an agarose plate. Only cells treated with the transcription
6 and translation inhibitors were able to form colonies; the metal
7 complexes and nalidixic acid were clearly cytotoxic at their
8 MIC. This supports a conclusion that these three compounds
9 induce irreversible damage to the *E. coli*, likely through DNA.

10 **4) Protein Production.** Cisplatin and other platinum-based
11 agents are known to interfere with protein production. Some
12 question remains, however, if this is an important feature that
13 induces cell death, or simply a side effect of the DNA damage.
14 Several experiments have quantified the impact on protein
15 production after transfection of already metalated plasmids into
16 living systems.⁴⁷⁻⁴⁸ To study the process and impact of DNA
17 metalation, we treated *E. coli* with the metal complexes and
18 subsequently monitored protein production. This experiment
19 couples the quantitation of protein levels in the detection of the
20 fluorescent protein to the preceding natural sequence of events
21 that impact transcription/translation, and allows for
22 observation of important features that may play a role, such as
23 compound uptake, localization within the cell, or sequence-
24 dependant interactions with the nucleic acid.

25 Cells undergoing death will slow or cease protein production,
26 which produces a similar phenotype to cells that are under the
27 influence of a transcription or translation inhibitor. To
28 discriminate between inhibition of protein production and
29 induction of cell death, we used a photo-convertible protein,
30 Dendra2, as a reporter, since it is able to provide information on
31 both aspects of cell viability and new protein production
32 simultaneously.⁴⁹ Dendra2 undergoes a photochemical reaction,
33 transforming from a green fluorescent protein to a red
34 fluorescent protein when exposed to 405 nm light. The
35 photoconverted "Red" Dendra2 emission provided a stable
36 internal reference for cell health and cell number for all samples,
37 while new protein production (after light exposure) is reflected
38 in the "Green" Dendra2 emission. Both forms are stable and
39 persist in living cells with half-lives ($t_{1/2}$) on the order of 50 to
40 70 hrs.⁵⁰⁻⁵² The two forms of the protein thus provide spatial
41 and temporal tracking of Dendra2 formed before and after light
42 exposure.

43 Dendra2 production was induced in *E. coli* with IPTG and
44 allowed to proceed for 3 hours before photoconversion,
45 followed by compound treatment. A clear negative correlation
46 was seen between protein production and cell size, where
47 filamentous cells with longer filament lengths exhibited a lower
48 fluorescence intensity, reflecting a reduction in the amount of
49 new Dendra2 protein being produced. As shown in Figure 1,
50 after 24 hours of treatment with compound **1**, the average
51 fluorescence intensity of the cell population with the largest
52 length ($> 40 \mu\text{m}$) dropped by over 70% compared to the control
53 population. Other populations with increased cell lengths
54 exhibited a 30 – 70% decrease in fluorescence intensity. The
55 same trend was seen in cisplatin treated cells, where the
56 fluorescence intensity decreased by 15 – 80%, depending on the
57 length of the filament. Both compound dose and the time of
58 treatment was found to have an effect on filament formation and
59 protein production (see Figure S3 - S5).

60 The production of Dendra2 was quantified by dose response,
providing IC_{50} values for inhibition of protein production.
Protein production was quantified using the ratio of the average
fluorescent intensity of the two forms of Dendra2, as shown in

Figure 3. The transcription inhibitor rifampicin and translation
inhibitor tetracycline exhibited IC_{50} values for inhibition of
Dendra2 production that matched well with growth inhibition
(within 3 – 10-fold; see Table 1 and Figure 3). In contrast, both
compound **1** and cisplatin displayed a greater disparity between
inhibition of protein production and cell growth inhibition. The
30 – 40-fold decrease in potency reflects that the mechanism of
action of cisplatin and compound **1** is not solely (or primarily)
through transcription or translation inhibition. In contrast,
nalidixic acid, which induces DNA double strand breaks, was
far more effective at inhibiting protein production.

While cisplatin has been described as a transcription inhibitor,
it was the least effective of the five compounds tested for
inhibition of protein production. The impact of the DNA
damage induced by platinum compounds on protein production
has been comprehensively and conclusively proven, along with
the restoration of protein production when the appropriate DNA
repair mechanisms are activated to remove the lesions. However,
our studies suggest that the inhibition of protein production by
cisplatin is of secondary importance for the health of *E. coli*, as
the concentrations required to observe a significant impact on
this process far exceeded the toxic dose for the compound.

In an analogous study, Lippard and coworkers tested cisplatin
in mammalian cells containing a genetically encoded
fluorescent reporter system.⁵³ Very good agreement was
observed between the concentrations required to inhibit protein
synthesis and to induce cytotoxicity evaluated via a colony
counting assay in that report. The reason for the disparity in the
ability of cisplatin to inhibit protein production in *E. coli*
compared to the HeLa cells used by Lippard is unclear. It is
particularly surprising, given the very similar values we found
for DNA and RNA metalation in *E. coli* and HL60 cells, as
described above. However, due to the sensitivity of DNA
damage detection mechanisms in mammalian cells, it seems
unlikely that translation would be affected before initiation of
the DNA damage response.

5) Protein distribution. The use of a photoconvertible protein
allows for a spatiotemporal analysis of protein content. This
provided the opportunity to address intriguing questions such as
the impact of interruption of cell division and filamentous
growth on the activity of ribosomes for new protein production,
and the re-distribution of existing protein within a filamentous
cell. Fluorescent imaging was performed to probe the effects of
the different compounds on protein distribution in single cells.
Compounds were dosed after Dendra2 photoconversion, and
imaging was performed 6 hours later. As shown in Figure 3 A,
both the "Red" Dendra2 (the internal control of pre-treatment
protein level) and the "Green" Dendra2 (reflecting protein
synthesis after treatment) were distributed throughout the cell
as the healthy cells underwent multiple cell divisions. Both Red
and Green forms of Dendra2 were also found within the
filamentous cells where cell division was blocked by either
DNA damaging agents or transcription/translation inhibitors. It
has been reported that disruption of DNA replication and double
strand breaks resulting from nalidixic acid treatment could lead
to uneven distribution of ribosomes in filamentous cells.⁴⁶
However, we did not observe any particular spatial
sequestration of active ribosomes; alternatively, protein
diffusion is sufficiently rapid to prevent observation of any
localization during the time scale of the experiment.

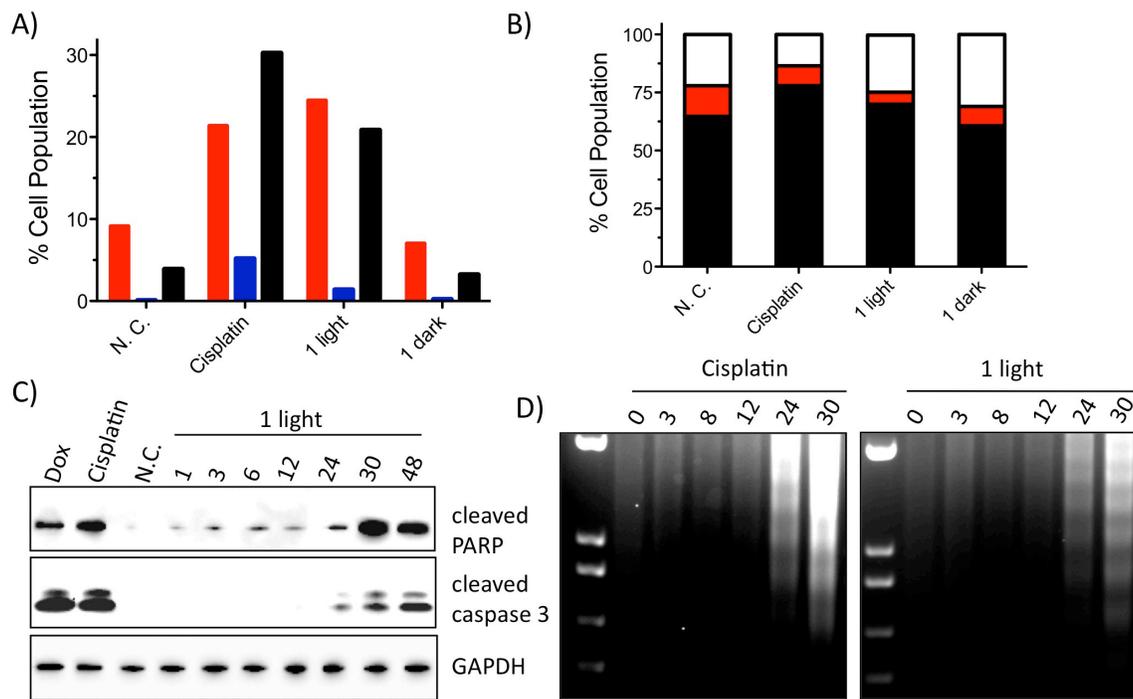


Figure 4. Compound **1** induces apoptosis in HL60 cells without cell cycle arrest. A) Flow cytometry by PI/Annexin V in HL60 cells; Red = apoptotic cells, Blue = necrotic cells, Black = dead cells. B) Flow cytometry by PI in HL60 cells; Black = G1; Red = G2; White = S phase. C) Immunoblotting of cleaved PARP and cleaved caspase 3 in HL60 cells. GAPDH was blotted as loading control. D) Agarose gel electrophoresis of DNA laddering. HL60 cells were treated for 24 hours for flow cytometry. All panels: compound **1**, 20 μ M; cisplatin, 20 μ M; doxorubicin, 1 μ M.

Comparison of *In Vitro* and *In Cell* Protein Production.

Previously, we reported an *in vitro* transcription and translation assay (IVTT) with compound **1** and cisplatin using Green Fluorescent Protein (GFP) as a fluorescent reporter.³⁷ In this assay, either a plasmid containing the GFP gene or the mRNA transcript for GFP were allowed to react with varying concentration of cisplatin or compound **1**, with irradiation, before addition of the nucleic acids to a cancer cell lysate containing transcription and translation machinery. Both metal compounds inhibited GFP production with a clear dose response. Interestingly, the IC_{50} value for inhibition of protein production was ~ 3 μ M for both compounds. The ratio of DNA nucleotides or RNA nucleotides to each metal center was calculated at the IC_{50} for protein synthesis inhibition. The values for compound **1** were 1140:1 and 820:1, while the values for cisplatin were 600:1 for DNA and 610:1 for RNA respectively.

In the current uptake studies, *E. coli* cells were dosed at 20 μ M, which is approximately 10x higher than the IC_{50} value for growth inhibition, but well below the IC_{50} value for inhibition of protein synthesis, as determined by Dendra2 production (see Table 1). In order to compare the *in vitro* experiment to the cell data, extrapolation of the ratio for DNA and RNA nucleotides per metal center at the IC_{50} value for *in vivo* protein synthesis inhibition was performed as detailed in the *Supporting Information*. The calculated values were remarkably close to the values from the *in vitro* transcription and translation assay, with DNA nucleotides to metal center ratios of 520:1 for compound **1** and 700:1 for cisplatin. The RNA nucleotide to metal center ratio was 1000:1 for compound **1** and 1090:1 for cisplatin.

This analysis of the ratio of DNA or RNA bases to metal centers suggests the functional inhibition of protein synthesis by covalent adducts to DNA and mRNA by compound **1** and cisplatin is similar in *E. coli* and the *in vitro* assay. It is notable that in a living cell, where the reaction conditions are much more complex than the buffered system of IVTT assay, the IC_{50} values to inhibit protein synthesis were diminished by over 60-fold relative to the IVTT assay. However, the ratio between DNA or RNA bases and the metal center for inhibition of protein production remain quite consistent. The increase in the IC_{50} values in cells suggests two conclusions: 1) inhibition of protein synthesis is not the factor that induces cell death, and 2) both compounds suffer from off-target binding to biological molecules. The later is known to be a major issue for many currently administered drugs, especially cisplatin.

The role of off-target binding was also supported by the AAS analysis of metal content with the different nucleic acids in *E. coli* and mammalian cells, as only a minor component of the metal compounds entered the cells, and of this, only 1.30% and 1.26% of cellular ruthenium from compound **1** and 0.98% and 1.12% of the cellular platinum from cisplatin were found with genomic DNA in the two systems. If one includes the $< 2\%$ of metal present in the RNA as on-target damage, this means over 96% of the cellular metal is reacting with potentially non-relevant targets. Extending this argument, if off-target binding could be eliminated, cytotoxicity IC_{50} values would be reduced to nanomolar concentrations if the same levels of cellular uptake could be maintained. This may lead to another method to improve the potency of currently used drugs: instead of focusing on the generation of analogues that are more potent

against nucleic acids, analogues with reduced off-target binding could be more effective.

It has been shown that the vast majority of cisplatin and other Pt species bind to plasma proteins and do not reach their target *in vivo*.⁵⁴⁻⁵⁶ It was anticipated that the ruthenium compound would fare better than cisplatin in avoiding off-target binding, due to its lower affinity for hydrophobic proteins such as human serum albumin (HSA)³⁷ and thiols such as glutathione (GSH), but this has not been found to be the case in cells.^{31, 57} As the preferred binding partners are not the same for the platinum and ruthenium complexes, it will be important to identify the primary off-target biomolecules responsible for sequestering the ruthenium in order to rationally design derivatives that avoid these species to increase the potency of these inorganic compounds.

Ru and Pt Compounds Induce Distinct Cellular Responses in Mammalian Cells.

The cellular effect of compound **1** was also studied in mammalian cells, with a focus on proteins involved in cell signaling and cell death. As shown in Figure 4, effects on cell cycle and apoptosis were studied using flow cytometry, immunoblotting for apoptotic markers, and DNA fragmentation. No cell-cycle specific arrest point was observed with compound **1** treatment, while a sub G1 population of 20% of cells was observed after 24 hours. Flow cytometry analysis of apoptosis vs. necrosis using FITC-AnnexinV and propidium iodide indicated that compound **1** induced cell death through apoptosis as the dominant mechanism. While cisplatin induced necrosis in a small fraction (5%), less than 2% of cells treated with compound **1** were characterized as necrotic. Immunoblotting of caspase 3 and PARP showed a time dependent induction of apoptosis; in addition, isolation of genomic DNA showed fragmentation, which is consistent with apoptotic cell death. All apoptotic reporters were clearly observed at 24 hours.

The tumor suppressor protein p53 regulates cell growth and cell cycle checkpoints to eliminate proliferation. It is one of the most commonly mutated genes in cancer, resulting in loss of its regulatory function.⁵⁸⁻⁵⁹ Both p53/p21 and chk1 are involved in G1/S and G2/M cell cycle checkpoints in response to DNA damage.⁶⁰⁻⁶¹ In order to probe the role of p53 in response to compound **1**, immunoblotting was performed in A549 cells. This non-small-cell-lung cancer cell contains functional p53, in contrast with the p53 deficient HL60 cell line. While both

cisplatin and doxorubicin were able to induce apoptosis in the absence of functional p53 in HL60 cells (Figure 4), the A549 cell line demonstrated clear induction of p53 for these two compounds. In contrast, compound **1** did not induce elevated expression of p53, and didn't significantly alter its phosphorylation or expression of p21 (Figure 5A).

Another surprising difference observed between the platinum and ruthenium compounds is that p-chk1, which is involved in G2/M cell cycle arrest in response to DNA damage, was not induced by treatment of compound **1** in either cell line, while both cisplatin and doxorubicin induced phosphorylation of chk1. This finding is consistent with the fact that no cell cycle arrest point was seen with compound **1** in HL60 cells, in contrast to cisplatin and doxorubicin. However, phosphorylation of γ -H2AX, an early sensor of DNA damage, was observed after 6 hours of treatment with **1**, indicating DNA damage even in the absence of chk1 activation.

Pro-survival and pro-apoptotic pathways, including MAPK pathways were examined, and consistent signaling behaviors after compound **1** treatment were observed in both cell lines. The ERK pathway has been reported to facilitate cell survival and prevent apoptosis.⁶²⁻⁶³ As shown in Figure 5, this pathway was inactivated by compound **1** in both A549 and HL60 cell lines; in contrast, both cisplatin and doxorubicin didn't down-regulate this pro-survival pathway. The JNK pathway has been reported to act as a pro-apoptotic pathway in response to cellular stress induced by DNA damage, and is mainly activated by mismatch repair signals.^{62, 64} Both cisplatin and doxorubicin were able to induce phosphorylation of JNK in A549 and HL60 cells at 24 hours, though different phosphorylation levels in HL60 cells were observed, which might indicate possible phosphorylation time course differences. Phospho-JNK was seen as early as 6 hours after doxorubicin treatment in HL60 cells while cisplatin induced phospho-JNK was seen to increase to maximum level at 24 hours.⁶⁵⁻⁶⁶ Surprisingly, compound **1** didn't activate the JNK pathway to the same extent as cisplatin or doxorubicin. The phosphorylation level was slightly increased within 6 to 12 hours of treatment with compound **1** in both cell lines, but then decreased over time. This, along with the previously discussed markers, indicate a different DNA damage response for compound **1** either from altered cell signaling pathways or by a different class of DNA damage.

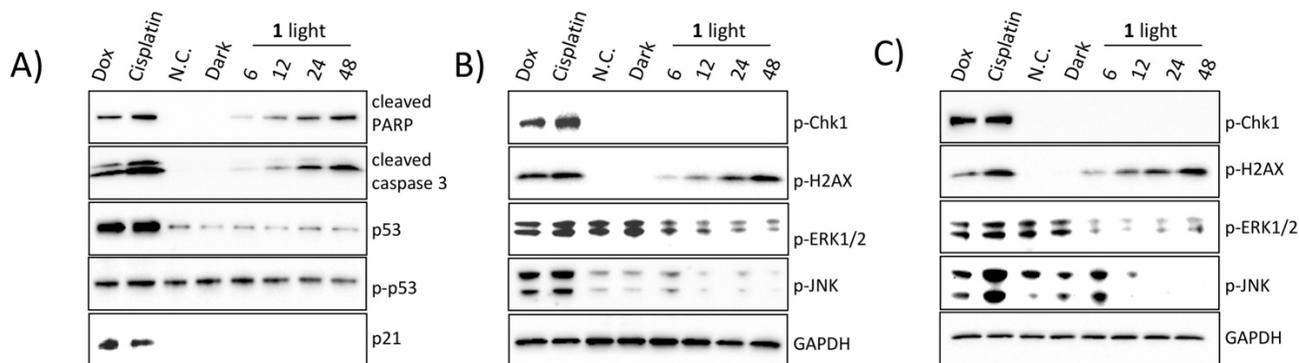


Figure 5. Immunoblotting of apoptotic markers and cell signaling proteins in A) and B) A549 cells; C) HL60 cells. Cells were treated with 20 μ M of compound **1** for specified time periods, cisplatin (20 μ M) and doxorubicin (2 μ M) at 24 hours of treatment were used as controls. GAPDH was used as loading control.

Recent reports have raised questions as to the source of the biological effects of compound **1** and analogous Ru(II) systems, with data suggesting that it is the liberated bipyridyl ligand, rather than the Ru(II) metal center **2**, that is responsible for activity.⁶⁷⁻⁶⁸ This study demonstrates such striking similarities between the behavior of the light activated Ru(II) complex and cisplatin, both in *E. coli* and mammalian cancer cells, that we find it improbable that the ligand, rather than the metal center, is responsible for the phenotypic effects. The cytological profile, nucleic acid metallation, and DNA damage response is consistent with metal-mediated DNA damage. However, it is possible that the ligand is inducing other effects that are not observed with these assays. A more detailed investigation is underway.

DISCUSSION

This work demonstrates that a combination of phenotypic screening based on *E. coli* imaging and protein production using Dendra2 as a fluorescent reporter allows for rapid investigations of mechanisms of action for cytotoxic agents which may have similar activities in mammalian cells. We found that a combination of these two experimental parameters facilitates discriminating DNA damaging agents from agents that work solely as transcription or translation inhibitors. While filaments are formed by all classes of compound, filament size and population distribution was radically different depending on the mechanism of action. Furthermore, the observation of irregular bacterial nucleoids, easily visualized using Hoechst staining, was associated with DNA damage, while regular nucleoid size, shape, and distribution was found with compounds that do not directly affect DNA. Such experiments could not be performed in eukaryotic cells, given the number of chromosomes.

E. coli have been used in the past as model systems to probe binding characteristics of drugs, such as cisplatin, with proteins using NMR.⁶⁹⁻⁷⁰ Phenotypic analysis in bacteria by microscopy is now gaining more attention, primarily to identify the cellular pathways impacted by antibiotics,⁷¹⁻⁷² but a recent report identified anticancer activity for a molecule characterized by cytological profiling in bacteria.⁷³ In that case, identification of the mechanism of action of the molecule under investigation motivated later studies in cancer cells, and the authors characterized bacterial cytological profiling (BCP) as a tool for drug repurposing.

We are taking the reverse approach by using bacteria to elucidate mechanistic features of compounds already identified as promising anticancer agents. We believe that these studies in bacterial and mammalian cells highlight the capacity of performing rapid studies of anticancer agents in a simple biological model system. Phenotypic studies and quantitative analysis reveal similarities in biological activities between complex **1** and cisplatin. Some limitations are apparent, though, as the cellular response to the compounds in cancer cells suggest diverse pathway regulation following the DNA damage. Altering the metal center thus offers a possibility to maintain efficacy without experiencing the same resistance.

While these studies bring us closer to understanding the mechanism of action of a particular cytotoxic ruthenium complex, it also raised several provocative questions. These include the following: 1) Why is the cytotoxic potency of cisplatin and other inorganic putative DNA damaging agents the same in *E. coli* and mammalian cells when DNA damage

recognition and repair pathways that are unique to eukaryotic systems are implicated as playing a key role in their mechanism of action? 2) While more of the metal complexes are taken up in *E. coli* than the HL60 cells, the nt / mc ratio remains quite consistent for both DNA and RNA. What biological entities are responsible for the enhanced sequestration of the metals in the *E. coli*, and is it possible that similar molecules play a role in cisplatin-resistant cancer cells? 3) Why does DNA packing not play a greater role in determining the degree of metallation? DNA is packaged in different ways and to different degrees of compaction in the two cells types, and if the more highly exposed, transcriptionally active sequences were the primary target, we would anticipate greater potency for inhibition of Dendra2 production. We believe that addressing these basic questions may be very important to the rational development of improved DNA targeting agents, and see *E. coli* as an excellent system to seek the answers.

ASSOCIATED CONTENT

Supporting Information. Supporting figures and tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

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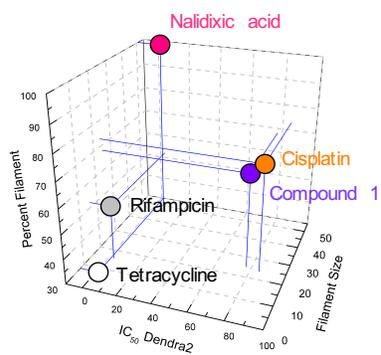
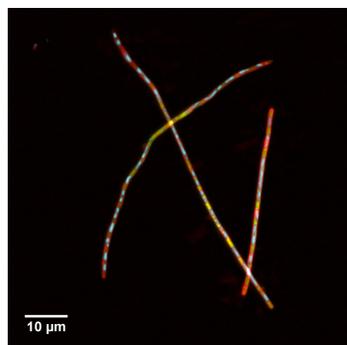
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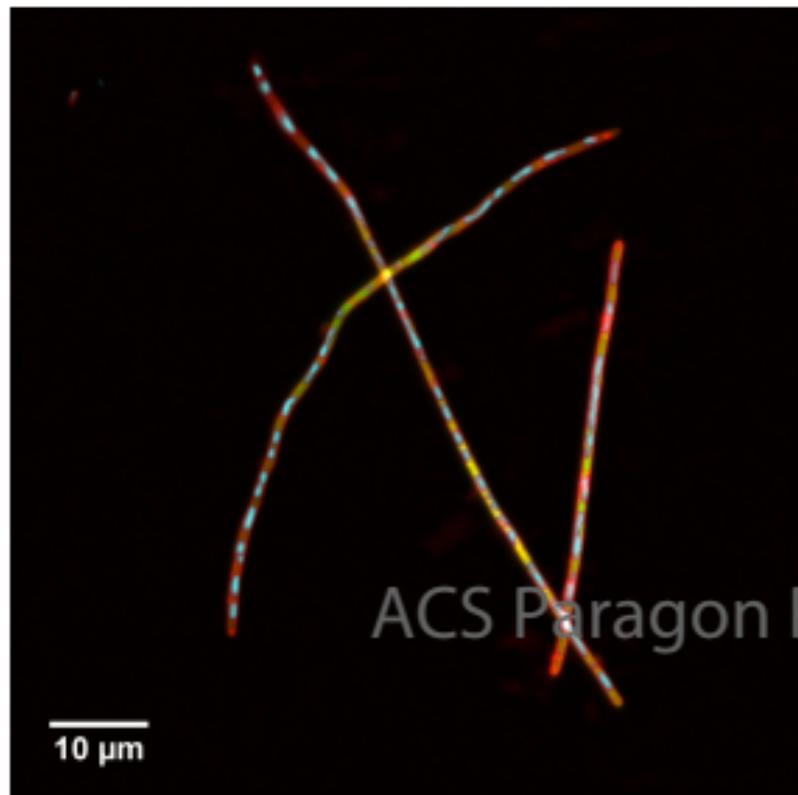
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