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LETTER

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# Inhibition of DNA Synthesis by a Platinum−Acridine Hybrid Agent Leads to Potent Cell Kill in Nonsmall Cell Lung Cancer

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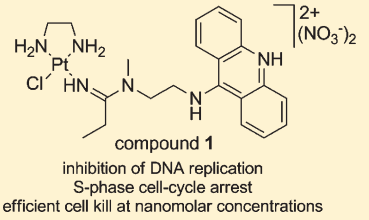
Supporting Information

**ABSTRACT:** The platinum−acridine anticancer agent $[PtCl(en)(LH)](NO_3)_2$ (**1**) [en = ethane-1,2-diamine, LH = *N*-(2-(acridin-9-ylamino)ethyl)-*N*-methylpropionimidamide, acridinium cation] and the clinical drug cisplatin were studied in chemoresistant nonsmall cell lung cancer (NSCLC) cell lines for their cytotoxic potency and cell kill mechanisms. In the three cell lines tested (NCI-H460, NCI-H522, and NCI-H1435), compound **1** shows a pronounced cytotoxic enhancement of 40−200-fold as compared to cisplatin at inhibitory concentrations reaching the low nanomolar range. On the basis of changes in cell adhesion and cell morphology, monitored in real time by impedance measurements, compound **1** kills NCI-H460 cells significantly more efficiently than cisplatin at equitoxic concentrations. Flow cytometry analysis of NCI-H460 cells reveals a robust S phase arrest of cells treated with compound **1**, whereas cells treated with cisplatin progress to G2/M of the cell cycle. A pronounced inhibition of DNA replication in 75% of viable cells is observed in NCI-H460 cells treated with compound **1** at an $IC_{90}$ molar concentration for 48 h, based on the reduced incorporation of the fluorophore-clickable nucleoside analogue 5-ethynyl-2′-deoxyuridine (EdU) into newly synthesized DNA. The distinct cell cycle perturbations and cell kill potential of compound **1** are discussed in the light of the DNA interactions of this agent and its potential to overcome cisplatin resistance in NSCLC.




**KEYWORDS:** Platinum−acridine, DNA replication, cytotoxicity, cell cycle arrest, chemoresistant lung cancer

Lung carcinomas, of which more than 80% are histologically classified as nonsmall cell lung cancers (NSCLC), continue to be the leading cause of cancer mortality worldwide.[1] NSCLC, a notoriously chemoresistant form of the disease, was identified by the WHO as an incurable metastatic (category 3) cancer in which conventional chemotherapies will shrink tumors and prolong survival in a small population of patients and for a short duration.[2] More than a decade after this sobering assessment and despite the development of novel molecularly targeted therapies,[1] the clinical outcome of the disease remains poor. Regimens based on cisplatin (Chart 1) continue to be the cornerstone of NSCLC treatment.[3,4] Recent biological and clinical studies indicate that the overexpression of proteins of the nucleotide excision repair (NER) machinery is responsible for tumor resistance and the failing of platinum-based chemotherapy.[5−7] NER recognizes and removes DNA adducts that severely distort and thermodynamically destabilize double-stranded DNA, such as the cross-links formed by the drug cisplatin [*cis*-diamminedichloroplatinum(II)].[8] To overcome this problem and to generate an agent that produces its cytotoxic effect at the DNA level by a mechanism different from that of current clinical platinum drugs, we designed a noncross-linking platinum−acridine hybrid pharmacophore.[9,10] Here, we have used NSCLC cell lines of varying degrees of cisplatin resistance in conjunction with cell proliferation assays and cell cycle analysis to demonstrate that the prototypical agent, $[PtCl(en)(LH)](NO_3)_2$ [compound **1**, Chart 1; en = ethane-1,2-diamine, LH = *N*-(2-(acridine-9-ylamino)ethyl)-*N*-methylpropionimidamide, acridinium cation],[10] is a potent inducer of cancer cell kill significantly superior to cisplatin.

To assess the relative chemosensitivities of NSCLC cancer cells to cisplatin and compound **1**, colorimetric (MTS) cell proliferation assays were performed in three cell lines, NCI-H460, NCI-H522, and NCI-H1435. The cisplatin resistance of these cell lines previously has been shown to correlate with transcript levels of DNA repair and multidrug resistance genes, with NCI-H1435 being the most and NCI-H460 the least resistant cell line.[11] The inhibitory concentrations in the micromolar range extracted from drug−response curves for 72 h incubations of cisplatin with the three cell lines confirm this trend (Table 1). By contrast, compound **1** shows a dramatic increase in cytotoxicity in all three cell lines. In NCI-H460, an $IC_{50}$ of 8 ± 2 nM was determined, while in NCI-H522, the $IC_{50}$ was 18 ± 2 nM, corresponding to a 150- and 200-fold cytotoxic enhancement as compared with cisplatin, respectively (Table 1). In the most resistant cell line, NCI-H1435, compound **1** maintains micromolar activity at an

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**Chart 1. Structures of Compounds Studied**

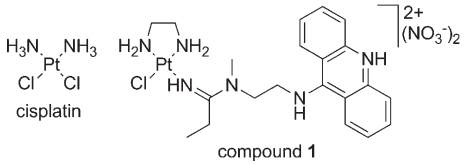


**Table 1. Summary of Cytotoxicity Data ($IC_{50}$ Values ± SD, μM) in NSCLC Cell Lines**

| | NCI-H460[a] | NCI-H522[b] | NCI-H1435[c] |
|---|---|---|---|
| cisplatin | 1.2 ± 0.2 | 3.7 ± 0.8 | 85 ± 20 |
| compound **1** | 0.008 ± 0.002 | 0.018 ± 0.002 | 2.1 ± 0.3 |

[a] $n = 5$. [b] $n = 3$. [c] $n = 4$; $n$ is the number of independent experiments performed.

inhibitory concentration approximately 40-fold lower than that observed for cisplatin (Table 1).

To gain insight into the dynamics of cancer cell kill produced by compound **1** and cisplatin, we studied the interaction of the two complexes with NCI-H460 cells using impedance measurements. In this assay, a fixed number of cells are treated with drug in 96-well microtiter plates in which each well contains a microelectrode responsive to changes in cell index due to cell adhesion/detachment and changes in cell morphology.[12,13] Thus, the assay provides a means of monitoring in real-time the effects of the drugs on cell growth, spreading, and proliferation. Cells growing in log phase were treated with varying concentrations of platinum drug, and impedance changes were recorded for a period of 100 h. The kinetic traces recorded for compound **1** (Figure 1A) show a decrease in cell index relative to untreated cells, consistent with a concentration-dependent cytotoxic effect caused by the hybrid agent. At the highest lethal concentration ($IC_{90}$) and over the 100 h treatment period, compound **1** efficiently kills the entire population of cancer cells. When cisplatin was incubated with NCI-H460 cells under the same conditions, but at specific concentrations relevant to the cell growth inhibition effected by this drug, an entirely different outcome was observed (Figure 1B). While cisplatin causes an overall reduction of cell proliferation relative to untreated cells, the effect is significantly delayed and less pronounced than in the case of compound **1**, and a concentration-dependent effect is observed only during the first 48 h of treatment. Most strikingly, while cisplatin initially causes the most pronounced retardation of cell growth at the highest concentration ($IC_{90}$) studied, the drug fails to reduce the terminal cell index. This contrasts the situation for compound **1**, which causes complete kill of adherent cells at the highest concentration.

In previous work, we demonstrated that cancer cells treated with our platinum−acridines show the morphological and molecular hallmarks of apoptotic cell death.[14,15] Because compound **1** kills NSCLC cells significantly more efficiently than cisplatin, we reasoned that differences might exist between the preapoptotic mechanisms of the two compounds. To answer this question, the effects of the two agents on cell cycle progression were studied using flow cytometry (Figure 2). NCI-H460 cells incubated with drug at 90% inhibitory concentrations for 24 and 48 h were studied along with untreated cells. Cisplatin caused a

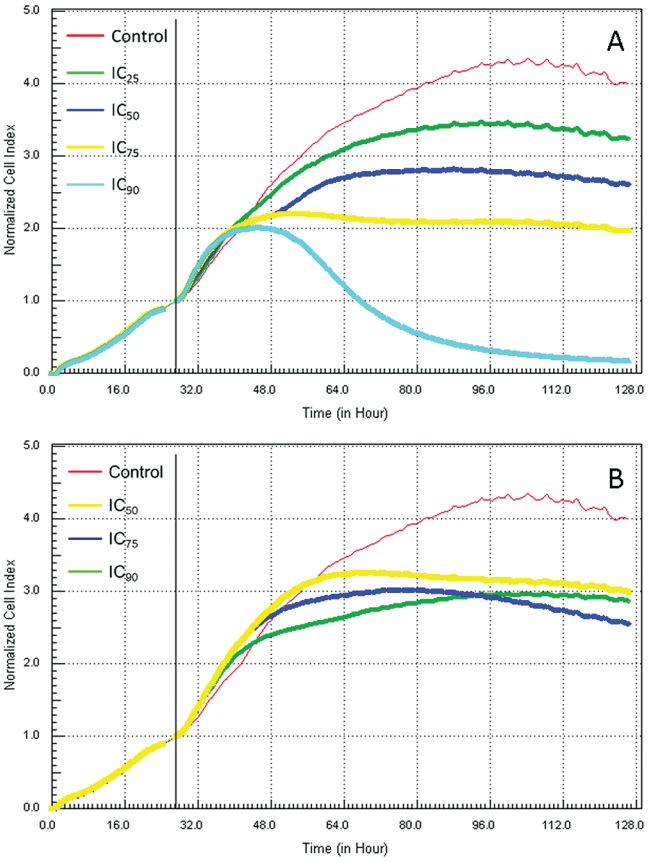


**Figure 1.** Interaction of compound **1** (A) and cisplatin (B) with NCI-H460 cells monitored by real-time cell electronic sensing (RT-CES). For compound **1**, $IC_{25}$ = 4.4 nM, $IC_{50}$ = 10 nM, $IC_{75}$ = 35 nM, and $IC_{90}$ = 94 nM; for cisplatin, $IC_{50}$ = 1.6 μM, $IC_{75}$ = 4.7 μM, and $IC_{90}$ = 11 μM. The vertical lines indicate the start of treatment after allowing the cells to grow and adhere to the microelectrodes for 27.5 h. Cell indices were normalized to account for differences in cell counts that exist across the wells prior to treatment. Incubations were performed in triplicate with 20 000 cells/well using inhibitory drug concentrations determined for 72 h incubations in a colorimetric cell viability assay. Qualitatively similar results were obtained for experiments performed with 40 000 cells/well (Supporting Information).

significant slowing of progression of viable cells through the S phase and an accumulation in the G2/M phase based on the calculated percentages of cells in each phase of the cell cycle after 24 and 48 h of drug exposure (Figure 2C,D). A transient slowing of cell growth in the S phase in response to the inhibitory effects of cisplatin−DNA adducts on DNA replication and arrest of cells in the G2/M phase are common cell cycle perturbations observed in solid tumor cells treated with platinum-based drugs.[16−18] By comparison, the histograms generated for compound **1** show a build-up of cells at the G1/S border and in the S phase but not in the G2/M phase (Figure 2E,F). In addition, nuclear debris from apoptotic or necrotic cells is observed as a sub-G1 population for cells treated with compound **1**, which is virtually absent for cisplatin-treated cells (Figure 2C−F). The cell cycle perturbations caused by compound **1** proved to be dependent on incubation concentrations. At the 24 h time point, for instance, in incubations at an $IC_{70}$, compound **1** causes a small increase in G0/G1 and G2/M populations of 8 and 2%, respectively, and a decrease in S population by 10%. The opposite, and a

more pronounced, effect is observed at the $IC_{90}$, at which an increase in cell population in the S phase of 28% is observed relative to untreated cells, reminiscent of a robust S phase arrest at the highest lethal concentration studied (Supporting Information).

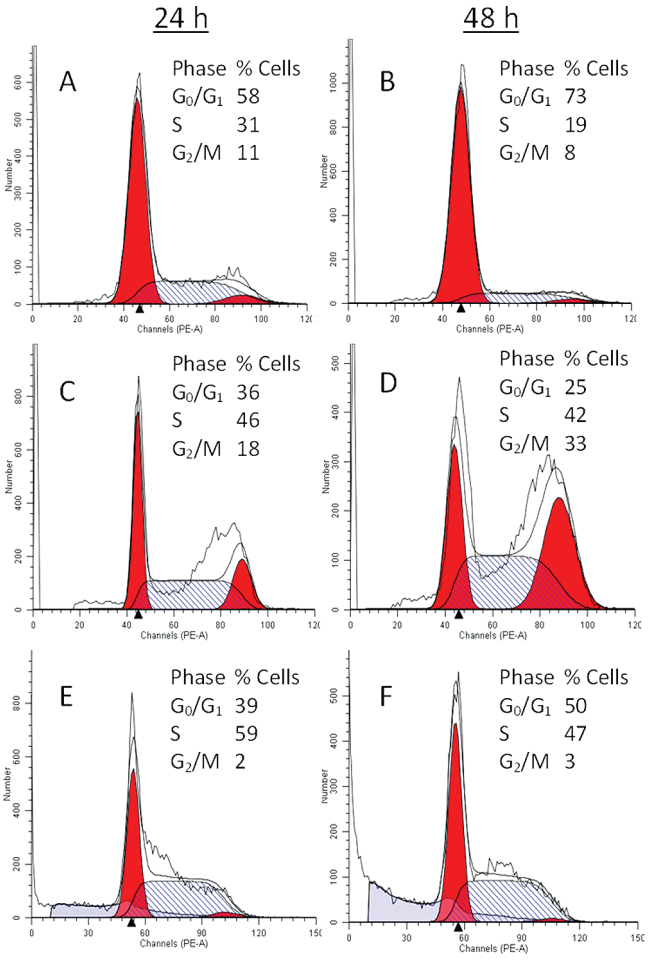


**Figure 2.** Cell cycle analysis of untreated NCI-H460 cells (A and B) and cells treated with cisplatin (C and D) or compound **1** (E and F) at $IC_{90}$ doses for 24 and 48 h. Typical histograms are shown with modeled distributions of cells in G0/G1 and G2/M phases (red), S phase (blue pattern), and sub-G1 (solid blue). The percentages given are average values of three incubations.

Previous studies have monitored incorporation of 5-bromo-2′-deoxyuridine (BrdU) to demonstrate that the S phase delay caused in cancer cells treated with cisplatin correlates with inhibition of DNA synthesis.[19,20] To confirm that the cell cycle effects caused by compound **1** are also related to efficient blockage of DNA replication, the ability to synthesize DNA of NCI-H460 cells treated with this agent was studied using bivariate flowcytometric analysis. This method detects cell cycle distribution based on total DNA content as well as the cells actively synthesizing DNA. Here, the latter subpopulation of cells was identified using the modified DNA precursor 5-ethynyl-2′-deoxyuridine (EdU), which can be detected with azide-modified fluorophores using copper-catalyzed conjugation chemistry ("click chemistry").[21] To assess the impact of compound **1** on DNA synthesis activity, NCI-H460 cells were incubated with drug for 24 and 48 h and subsequently analyzed for cell distribution and DNA content. The results of the EdU incorporation into DNA in platinum-treated cells along with control experiments are shown in Figure 3. In each of the bivariate analyses, global DNA content in treated and untreated cells is measured by propidium iodide (PI) fluorescence and plotted as abscissa, and fluorescence levels resulting from EdU incorporation are plotted as ordinate. Panels A and D in Figure 3 show the cell cycle distribution of untreated cells in the absence of EdU. In the absence of compound **1**, but with EdU present in the growth media, the fraction of normally proliferating cells entering S phase ($S_E$), cells in the S phase, and cells entering G2 ($G2_E$) are identified by elevated fluorescence levels due to incorporation of fluorophore-clickable DNA precursor (Figure 3B,E). For cells incubated with lethal concentrations ($IC_{90}$) of compound **1** for 24 and 48 h, the bivariate cytometric analysis shows both a characteristic perturbation of cell cycle distribution of cells and a pronounced suppression of DNA replication, based on the percentage of viable cells able to incorporate EdU precursor into newly synthesized DNA (Figure 3C,F). After 24 and 48 h of incubation with drug, the populations of proliferating cells are 25 and 6% of all viable cells, respectively. This corresponds to a reduction in DNA synthesizing cells relative to the no-platinum controls (Figure 3B,E) of 45 and 75%, respectively. Even more importantly, the remaining cells able to incorporate EdU (Figure 3C,F; fluorescent intensities in red box) do so with an

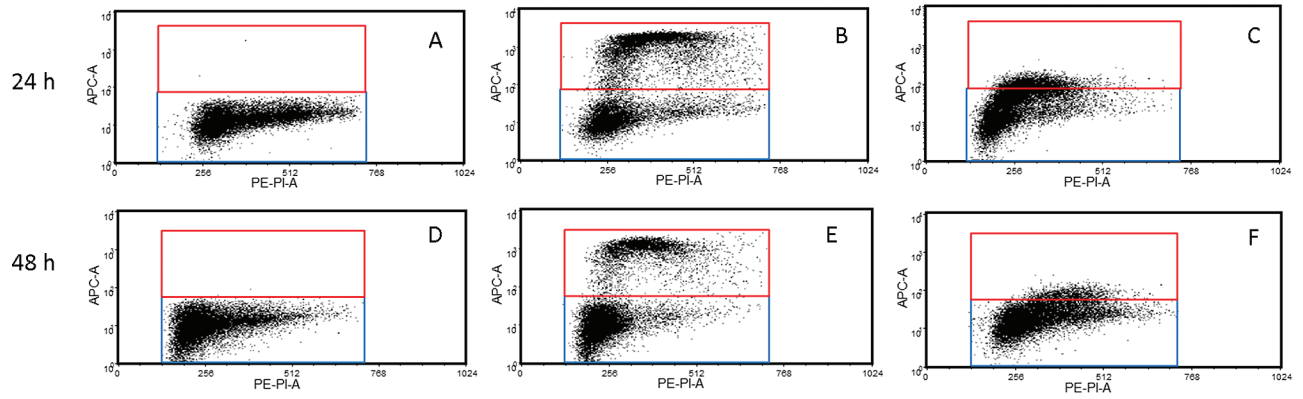


**Figure 3.** Bivariate cell cycle analysis of NCI-H460 cells incubated for 24 and 48 h. (A and D) Untreated control cultures. (B and E) Cells cultured in the presence of EdU. (C and F) Cells incubated with compound **1** ($IC_{90}$) and treated with EdU. The axis labeled PE-PI-A shows cellular DNA content based on propidium iodide (PI) staining, while the axis labeled APC-A gives the fluorescence resulting from incorporated and fluorescently detected EdU. The assay was performed with the Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA) using the red-fluorescent azide-modified fluorophore Alexa Fluor 647.


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order of magnitude reduced efficiency (note that Edu-based fluorescence is plotted on a logarithmic scale). These findings are consistent with a robust S phase arrest in NCI-H460 cells caused by lethal concentrations of compound **1**.

The present study demonstrates that a noncross-linking agent based on platinum is able to produce a pronounced cytotoxic enhancement relative to the clinical drug cisplatin in NSCLC cell lines of varying degrees of chemoresistance, which, for the most part, is mediated by increased DNA repair activity. The potent activity observed for compound **1** may be, in part, due to the fact that the DNA adducts formed by this agent evade NER. DNA damage recognition (DDR) proteins belonging to the class of high-mobility group (HMG) proteins, which recognize cisplatin−DNA cross-links,[22] did not recognize the monofunctional adduct formed by compound **1** (unpublished results). Similarly, these adducts may not be efficiently detected by DDR proteins belonging to the NER machinery, a possibility currently explored in our laboratory. It is noteworthy to mention that compound **1** maintains superior activity as compared to cisplatin in the nanomolar range irrespective of *KRAS* oncogene status and status of the tumor suppressor gene *p53* in NCI-H460 (mutated *KRAS*/wild-type *p53*) and NCI-H522 (wild-type *KRAS*/mutated *p53*) cells. This is an important finding as these mutations have been correlated with tumor resistance and aggressiveness.[23,24]

On the basis of the time-dependent impedance measurements, which detect the cumulative effects of changes in cell count, cell adhesion, and cell morphology due to drug toxicity, compound **1** kills NCI-H460 cells significantly more efficiently than cisplatin at equitoxic concentrations determined in the MTS assay. Compound **1**, but not cisplatin, produces a concentration-dependent decrease in impedance, suggesting that the reduced cell viability determined in the colorimetric assay translates into cell death. By contrast, an increase in cisplatin dose does not kill cells to an extent that would be expected from the MTS assay-based $IC_{50}$ values. These findings suggest that critical differences exist in the rate and mechanisms of cell kill caused by the two agents. Previous studies have shown that platinum drugs, while generally believed to induce apoptotic cell death, may require concentrations significantly higher than $IC_{50}$ values to produce the morphological features of apoptosis.[25] On the other hand, the preapoptotic signaling in various cancer cell lines, including NCI-H460, has been demonstrated to be defective causing inefficient cell kill by cisplatin.[26,27] Thus, it appears that compound **1** is a more potent inducer of apoptosis than cisplatin.

The distinct differences in cell kill observed for the two agents tested in this study may be ultimately caused at the DNA level. Cisplatin−DNA adducts typically inhibit DNA replication to an extent that slows cell cycle progression through the S phase but allows cells to accumulate in the G2/M phase.[16] Efficient repair of the cross-links and replicative bypass of the adducts by damage-tolerant translesion DNA polymerases contribute to the survival of cisplatin-treated cells.[28] Conversely, the fact that no significant build-up of cells in the G2/M phase and a more pronounced cell kill are observed in NCI-H460 cells treated with compound **1** suggests that inhibition of DNA synthesis by adducts of this agent is more lethal to the cells than the effects of cisplatin type cross-links.

In conclusion, the current study reveals critical mechanistic differences between the cell kill effected by the classical anticancer drug cisplatin and the new platinum−acridine hybrid agent **1**. These differences render compound **1** a potent antiproliferative agent far superior to current clinical platinum-based therapies. In previous work,[29] we demonstrated that compound **1** produces permanent DNA adducts much more rapidly than cisplatin (half-lives of 20 min and 2 h, respectively), which, in addition to inefficient repair by NER, may contribute to the hybrid agent's ability to inhibit DNA replication more efficiently than the clinical drug. Structurally and mechanistically unique DNA-targeted agents like compound **1** and its derivatives, one of which has already demonstrated activity in a very aggressive NCI-H460 xenograft model,[10] may lead to treatments for chemoresistant cancers and should be pursued rigorously for further preclinical development.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Experimental procedures and supporting data for cell cycle analysis and impedance measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

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