Published in final edited form as:

J Med Chem. 2012 March 8; 55(5): 2437–2451. doi:10.1021/jm2005942.

Gold-Containing Indoles as Anti-Cancer Agents that Potentiate the Cytotoxic Effects of Ionizing Radiation

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Abstract

This report describes the design and application of several distinct gold-containing indoles as anti-cancer agents. When used individually, all gold-bearing compounds display cytostatic effects against leukemia and adherent cancer cell lines. However, two gold-bearing indoles show unique behavior by increasing the cytotoxic effects of clinically relevant levels of ionizing radiation. Quantifying the amount of DNA damage demonstrates that each gold-indole enhances apoptosis by inhibiting DNA repair. Both Au(I)-indoles were tested for inhibitory effects against various cellular targets including thioredoxin reductase, a known target of several gold compounds, and various ATP-dependent kinases. While neither compound significantly inhibits the activity of thioredoxin reductase, both showed inhibitory effects against several kinases associated with cancer initiation and progression. The inhibition of these kinases provides a possible mechanism for the ability of these Au(I)-indoles potentiate the cytotoxic effects of ionizing radiation. Clinical applications of combining Au(I)-indoles with ionizing radiation are discussed as a new strategy to achieve chemosensitization of cancer cells.

Keywords

Gold; indoles; chemotherapy; radiosensitizing agents; apoptosis

Introduction

Metals such as magnesium, iron, and cobalt play essential cellular roles in biological systems by performing catalytic roles in biochemical reactions.^{1–3} However, other metals including copper, gold, and platinum possess properties such as redox reactivity, Lewis acidity, variable coordination modes, and reactivity towards biological macromolecules that can unleash lethal effects on cells.^{4–6} The toxicity of these metals can, under certain conditions, be controlled and subsequently used to efficiently kill cells that are associated with pathogenic conditions such as cancer. One important example is the widespread use of platinum-containing compounds such as cisplatin which damage DNA and induce apoptosis in various cancer cell lines.^{7, 8} Indeed, the efficacy of cisplatin, carboplatin, and oxaliplatin

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Supplemental Information: Details regarding the characterization of each compound using ¹H-NMR, ¹³C-NMR, ³¹P-NMR, and mass spectroscopy are provided as Supplemental Figures 1–???. The crystal structure of compound **3** is provided as Supplemental Figure ???. Data for the reactivity of compound **3** with BSA or cysteine is provided as Supplemental Figure ???. This information is available free of charge via the Internet at <http://pubs.acs.org>

against testicular and ovarian cancer has sparked significant interest in developing other metal-containing complexes as potential therapeutic agents.^{9,10}

Gold(I) complexes (hereafter referred to as Au(I) complexes) are gaining attention for their favorable toxicity toward malignant cells. Over the past five years, there have been several reports of active gold complexes and organometallic derivatives that show cytostatic and/or cytotoxic effects against various cancer cell lines.^{11–16} Unfortunately, many of these compounds are non-selective due to the ability of Au(I), a soft Lewis acid, to bind cysteine, selenocysteine, and (less so) histidine residues found in biological systems. One relevant example is auranofin, a glucopyranose containing a triethylphosphine complex of Au(I).^{17–22} Auranofin is used as a treatment against rheumatoid arthritis and also produces cytostatic and cytotoxic effects against various cancer cells *in vitro*.^{23–25} Despite these therapeutic uses, however, auranofin causes immunosuppression by inhibiting T-cell proliferation.²² The mechanism accounting for auranofin's cytotoxicity appears to differ from that of cisplatin as the gold(I) compound does not directly damage DNA.^{23–25} Instead, Rigobello *et al.* demonstrated that auranofin and related Au(I) compounds induce cell death through effects on mitochondrial integrity such as swelling and decreases in mitochondrial membrane potential.²⁵ These effects are attributed to the inhibition of mitochondrial thioredoxin reductase (TrxR)* caused by the binding of Au(I) to the active site selenocysteinate.

One goal of this work was to optimize the therapeutic potential of Au(I)-bearing compounds by encapsulating Au(I) in sterically hindered phosphine ligands to reduce metal ion loss to thiols or selenols in proteins. To this end, we synthesized several indoles substituted with (phosphine)gold(I) fragments at C-5, and we have surveyed their activity as potential anti-cancer agents. We chose to attach gold covalently to various indolyl-scaffolds since indole is an important bioorganic molecule that serves as a mimic for purines associated with ribose- and deoxyribose nucleos(t)ides.²⁶ As such, indole is often used as a key component of various pharmacological agents including staurosporine and indole-3-carbinol.^{26–28} Based upon these precedents, we hypothesize that gold-bearing indoles would form ideal candidates to deactivate adenine-binding proteins such as kinases that are often deregulated in cancer.²⁹ In this respect, tethering Au(I) to indole was predict to create a surrogate for adenine that would allow delivery of the metal to adenine-binding proteins. The inclusion of a biocompatible gold fragment could expand the chemical space of the simple indole scaffold and produce important pharmacological effects such as increased potency and/or selectivity for a particular target. Alternatively, the inclusion of gold could produce other biological effects through reactions with active site amino acids. Indeed, numerous chemical and biochemical studies indicate that many Au(I) compounds function as prodrugs that undergo specific chemical transformations to generate a pharmacologically active species.³⁰ These chemical transformations usually consist of fast ligand substitution reactions with rapid aquation in which the resulting cationic species has a strong reactivity with biomolecules. This report demonstrates that (phosphine)Au(I) indole derivatives act as therapeutic anti-cancer agents by inhibiting kinases associated with cancer progression rather than inhibiting typical targets such as TrxR.^{15,16} In addition, two Au(I)-compounds

* Abbreviations: TrxR, thioredoxin reductase; DSBs, double-strand DNA breaks; IC₅₀, concentration of a compound that inhibits 50% cell growth; LD₅₀, concentration of compound required to kill 50% of members of a tested population after a specified time period; FACS, fluorescence-activated cell sorting; PI, propidium iodide; G₁, period in the cell cycle during interphase; G₂, phase of the cell cycle following the successful completion of S-phase and that precedes mitosis; M, phase of the cell cycle where mitosis occurs; S-phase, phase of the cell cycle in which genomic DNA is replicated; sub-G₁, discontinuous fragmentation of nuclear DNA during apoptosis; BSA, bovine serum albumin, DTNB, 5,5'-dithiobis(dinitrobenzoic acid); EDTA, ethylenediaminetetraacetate sodium salt; Gy, SI unit of absorbed radiation; ATM, Ataxia telangiectasia mutated; ATR, Ataxia telangiectasia and Rad3 related; DNA-PK, DNA-dependent protein kinase; cdk, cyclin-dependent kinase; γ-H2AX, a phosphorylated form of histone H2A. PBS, phosphate-buffered saline.

show unique behavior by increasing the cytotoxic effects of ionizing radiation. One compound prevents the cellular detection of double strand DNA breaks (DSBs) by inhibiting the formation of phosphorylated histone H2A(γ H2AX) foci after exposure to ionizing radiation. The other (phosphine)Au(I) indole appears to block steps associated with DSB repair. In either case, the functional outcome is identical as inhibiting DNA repair leads to an increase in apoptotic cell death. Collectively, the results from these studies provide a novel therapeutic strategy to use Au(I) compounds as radiosensitizing agents against cancer.

Results

Design and Synthesis of Gold(I)-Indoles

The design of (phosphine)Au(I) indoles unites two themes in medicinal chemistry. The first is the use of indole as a pharmacological scaffold to target specific proteins involved in cancer.²⁶ The second is the use of gold as a relatively benign metal that can be activated to react with biomolecules, particularly “soft” ligands such as sulfur, selenium, and nitrogen groups present on amino acids such as cysteine, selenocysteine, and histidine. Since several important cellular targets bind indole derivatives, we envision that binding of the Au(I)-compound could allow covalent attachment of gold to any of these amino acids that are near the binding site. This reaction could cause irreversible inhibition of important cellular proteins and initiate a biological cascade that leads to eventual cell death by apoptosis (Figure 1A). Indeed, it has been demonstrated that Au(I)-containing compounds can react with enzymes such as TrxR, and that the subsequent inhibitory effects can cause apoptotic effects against various cancer cell lines.^{15,16}

Figure 1B summarizes the syntheses of the Au(I) conjugated indole analogs used in this study. Gold is hardwired to indoles by direct C–Au σ -bonds. Au(I) complexes are predominantly two-coordinate and linear. In the analogs described here, one ligand is an indole (bound through carbon); the other is a capping phosphine. The steric bulk of phosphine ligands can be readily altered. The phosphorus ligands herein are triphenylphosphine, tricyclohexylphosphine, and a dicyclohexylbiphenylphosphine. Phosphine-gold(I) organometallics were prepared in transmetalation reactions developed by Gray and co-workers.^{31–33} Protecting groups were added to N1 of indole to prevent coordination at nitrogen. The starting reagent is an indole boronic acid or pinacol boronate ester. (Phosphine)Au(I) fragments substitute specifically at the boron-bonded carbon; the boron moiety is displaced. Transmetalation proceeds even with bulky phosphorus ligands, such as dicyclohexylbiaryl phosphines.³³ Purity was >95% as judged by high-performance liquid chromatography. In addition, purity of all biologically active compounds was >95% as judged by microcombustion analyses (C, H, N, P and Au). Details regarding the characterization of each compound using ¹H-NMR, ¹³C-NMR, ³¹P-NMR, and mass spectroscopy are provided as Supporting Information, as is the crystal structure of compound **3**.

Anti-Cancer Effects of (phosphine)Au(I) Indoles

The cellular effects of these (phosphine)Au(I) indoles were tested against several cancer cell lines including HeLa (cervical cancer), MCF-7 (breast cancer), HCT-116 (colon cancer), and CEM-C7 (leukemia). The dose-dependency of each modified indole on cell viability was assessed using a cell-titer blue assay as previously described.³⁴ In these experiments, cells were exposed to variable concentrations (0.01 – 100 μ M) of each (phosphine)gold(I) indole for up to 48 hours and then assessed for viability. Representative data provided in Figure 2A shows the dose-dependency of 5-(triphenylphosphine gold(I))-tert-butyl 1H-indole-1-carboxylate (compound **3**) on HeLa cell viability. These data, representing an average of at least three (3) independent determinations, show that cell viability decreases as

the concentration of compound **3** is increased. A fit of the data to equation 1 provides an IC₅₀ of 2.5 $\pm$ 0.1 μ M. This anti-cancer effect depends upon the presence of the (phosphine)gold(I) ligand as the non-metalated indole derivatives do not produce significant anti-cancer effects even at the highest concentration of 100 μ M used.

Identical analyses were performed to measure IC₅₀ values for the other (phosphine)Au(I) indoles, and their values are summarized in Table 1. These data indicate that each compound functions as an independent anti-cancer agent, displaying potencies ranging from high nM to low mM. Despite the presence of a common indole scaffold, however, the potency of each compound is influenced by the nature of the substituent group present at both N1 and C5 of indole. For example, indoles containing electron-donating groups at the N1 position such as compounds **6** and **7** are more potent than compound **5**, which contains an electron-withdrawing group. Likewise, differences in the potencies of structurally similar compounds such as **3** and **4** highlight the pharmacological importance of the phosphine ligand. In particular, the π -electron system within the gold phosphine ligand appears to be important, as the IC₅₀ value for compound **3** against adherent cancer cells are at least 2-fold lower than those measured with compound **4** (Table 1).

Another important feature is the unique pharmacological properties of compounds **3** and **4** compared to other the other (phosphine)Au(I) indoles. Specifically, compounds **5**, **6**, and **7** display IC₅₀ values that are essentially invariant across the four cancer cell lines tested here. The identity in IC₅₀ values suggests that **5–7** cause cell death by a non-specific mechanism, i.e., reacting with one or more macromolecules common to all cell types and that are essential for cellular proliferation or survival. In contrast, the potencies for compounds **3** and **4** vary more significantly across these cell lines. This result suggests that these compounds differentially influence various biological targets present in these diverse types of cancer. In this respect, compound **3** is unique as it is categorically more potent against all adherent cell lines compared to the leukemia cell line, CEM-C7. The selectivity for compound **3** contrasts that of the structurally related compound **4** which is more efficacious against CEM-C7 cells compared to any of three adherent cell lines tested.[#] The differences in potency and selectivity for compounds **3** and **4** were deciding factors in further characterizing their anti-cancer effects alone and in combination with other therapeutic modalities such as ionizing radiation (vide infra).

Cell Death Occurs via Distinct Mechanisms

To further investigate the underlying mechanisms for the effects of compounds **3** and **4**, fluorescence microscopy was used employing propidium iodide (PI) uptake and annexin V staining as two well established biomarkers of cell death.³⁵ Figure 2B provides representative microscopy images of HeLa cells treated with DMSO (vehicle) or equimolar concentrations of compound **3** or compound **4**, (50 μ M). After 12 hours, cells treated with either (phosphine)Au(I) indole show significantly higher levels of annexin V staining compared to cells treated with DMSO and indicates that both compounds Au(I)indoles cause early stage apoptosis. Furthermore, the lack of significant propidium iodide uptake after 12 hours indicates that neither compound induces necrotic cell death at this early time point. However, treatment with compound **4** for 24 hours leads to more intense PI staining with a concomitant decrease in annexin V staining. These results suggest that cells transit from early- to late-stage apoptosis within 12-hours. In contrast, significant PI uptake is not observed in cell treated with compound **3**, even after 24 hours. Instead, a steady increase in the amount of annexin V staining is observed over the 24 hour time period.

[#]Selectivity is used to describe the observation that compound **3** shows higher potency against adherent cancer cells compared to the leukemia cell line, CEM-C7. This difference argues that this Au(I)-indole could avoid potential side effects such as thrombocytopenia and leukopenia that are caused by inadvertently killing thrombocytes and leukocytes, respectively.

Dual parameter FACS analyses with PI and annexin V staining was next performed to quantify these differences and shed more insight into the mechanism of cell death. Representative data averaged over three (3) independent determinations are provided in Figure 2C. These data show that treatment with compound **3** for 24 hours produces significantly higher amounts of early- and late-stage apoptosis compared to cells treated with DMSO. As summarized in Table 2, compound **3** causes a 6-fold increase in early stage apoptosis and a ~10-fold increase in late stage apoptosis compared to treatment with DMSO. In addition, the lack of PI uptake again supports the conclusion that compound **3** does not cause necrosis.

Several important differences are noted in HeLa cells treated with compound **4**. One distinction is that lower amounts of early stage apoptosis are detected with compound **4** compared to compound **3** (5.3% versus 8.8%, respectively). In addition, compound **4** generates a 2-fold higher amount of late-stage apoptotic cells compared to compound **3** (8.1% versus 4.3%, respectively). However, the most striking difference is that compound **4** causes necrotic cell death as evidenced by extensive PI uptake in the absence of appreciable annexin V staining. Quantitative analyses reveals that treatment with compound **4** causes a ~9-fold higher amount of necrosis compared to treatment compound **3**. Collectively, the differences in the mechanism and timing of cell death upon treatment with compounds **3** versus **4** highlight how subtle differences in the structure of the Au(I)-ligand can produce significant pharmacological effects.

We next analyzed the effects of compounds **3** and **4** on cell-cycle progression after 24 hours post-treatment using PI staining to measure cellular DNA content. Figure 2D provides a histogram of HeLa cells treated with DMSO. This represents a standard cell-cycle distribution for asynchronous cells as the vast majority of cells exist at G1 (45.6 $\pm$ 0.1%) and S-phase (43.6 $\pm$ 0.1%) while a significantly smaller population exists at G2/M (10.8 $\pm$ 0.1%). Treatment with compound **3** does not cause any significant alterations in cell-cycle progression over a 24 hour period (Table 3). Thus compound **3** induces apoptosis without overtly perturbing cell cycle progression. A different phenomenon is observed after treating the cells with compound **4** due to the significant accumulation at G1 (60.3 $\pm$ 3.2%) with a concomitant decrease at S-phase (28.3 $\pm$ 1.3%) (Table 3). These combined effects indicate that compound **4** blocks cellular entry into S-phase. However, this blockade evokes more of a necrotic form of cell death as opposed to a classic apoptotic response. Regardless, these data again highlight the ability of structurally related Au(I) compounds to produce different effects on cell-cycle progression.

These effects prompted us to evaluate the cytotoxicity of the unprotected Au(I)-phosphine ligand, BrAuPPh₃. When tested against the leukemia cell line, CEM-C7, this unprotected Au(I)-complex displayed potent cytostatic and cytotoxic effects (Supplemental Information, Figure S26). The dose-dependency of BrAuPPh₃ in generating a cytotoxic effect yielded an LD₅₀ of 0.22 $\pm$ 0.05 μ M, a value which is ~40–80-fold lower than that measured for compound **3** and **4**. Despite this higher potency, treatment of CEM-C7 cells with BrAuPPh₃ causes necrosis as evidenced by significant uptake in propidium iodide (A. Berdis, unpublished results). This distinction is important as it indicates that encapsulating Au(I) with sterically hindered phosphine ligands reduces its ability to non-selectively react with biological targets to cause necrosis. Defining this mechanism of cell death is important as necrosis can cause various side-effects including septic shock and kidney failure that can obviously compromise patient health. Surprisingly, the unprotected Au(I)-phosphine ligand, BrAuPPh₃, does not produce significant cytostatic or cytotoxic effects against the adherent cell line, HeLa, up to a concentration of 100 μ M (Supplemental Information, Figure S27). As such, the protected Au(I)-indoles have significantly higher potencies against adherent cells. The dichotomy in the potency of BrAuPPh₃ against the leukemia cell line versus

adherent cells is not clear at this time. However, likely possibilities include non-selective reactions between cellular proteins and the unprotected BrAuPPh₃ and/or inhibition of thiol- and selenocysteine-containing enzymes such as TrxR that is involved in maintaining nucleoside homeostasis (vide infra).

Reactivity of Au(I)-compounds to Biological Thiols

The most abundant plasma protein and principal extracellular source of sulfhydryl groups is serum albumin. This protein plays important roles by transporting numerous compounds including metals, amino acids, hormones, fatty acids, and medicinal drugs. Although serum albumin contains 35 cysteines, all but one exist as disulfide bonds. Cys34 is the only residue in serum albumin that can exist as a reduced thiol or as a mixed disulfide of cysteine or glutathione. The pK_a of Cys34 is approximately 5.0 and thus more acidic than cysteine or glutathione, which have pK_as of 8.5 and 8.9, respectively. Collectively, the biological abundance and lower pK_a value of Cys34 predicts that it is highly reactive toward Au(I) and would favor exchange reactions with Au(I)-containing complexes.

We tested the ability of various Au(I)-complexes to non-selectively react with BSA using modifications to protocols established by Shaw *et al.*³⁷ and Roberts *et al.*³⁸ In a typical experiment, variable concentrations of BSA (0–120 μM) were treated with a fixed concentration of compound **3** (120 μM) or compound **4** (120 μM) yielding Au(I)/BSA ratios of 4:1, 2:1, 1:1, and 0:1. After incubating for one hour, the reaction mixtures were applied to Penefsky spun columns using P2 gel filtration resin and centrifugal force to rapidly and efficiently separate unreacted Au(I)-containing complexes from BSA. P2 resin was effective in retaining Au(I)-containing compounds including compound **3** and compound **4**. However, BSA was not retained in the resin as >95% of the BSA loaded into the Penefsky column is recovered in the eluant. After recovery from the column, the -SH titer of BSA was determined using DTNB as previously described for reactions performed at varying concentrations of Au(I)-indole. Values obtained from reactions containing BSA incubated with the various Au(I) complexes were compared to identical reactions containing BSA alone and eluted through Penefsky columns. Data provided as Supporting Information (Figure S28) show that the relative thiol content of BSA remains unchanged in the presence of compound **3**, even at the highest Au(I)/BSA ratio of 4:1. Identical experiments performed with compound **4** yield similar results (Supplemental Information, Figure S29). Positive control experiments measured the reactivity of BrAuPPh₃ with BSA and demonstrated facile interactions of the unprotected Au(I)-ligand with the reactive cysteine residue present on BSA (Supplemental Information, Figure S30).

To further interrogate the stability of these Au(I)-complexes, we next measured the reactivity of compounds **3** and **4** toward L-cysteine. In this case, the -SH content of variable concentrations of cysteine was determined using DTNB as described above. Varying the concentration of L-cysteine generates a linear standard curve (Supplemental Information, Figure S31). Compound **3** alone gives an absorbance reading equivalent to background, indicating that the Au(I)-indole does not react with DTNB. Incubation of 400 μM L-cysteine with an equivalent concentration of compound **3** does not cause a change in the amount of free -SH present on L-cysteine. If L-cysteine had reacted with Au(I) present on compound **3**, then a decrease in the amount of free or unliganded -SH on L-cysteine would have been observed. The identity in A₄₁₂ for L-cysteine in the absence and presence of compound **3** provides additional evidence for the lack of a displacement reaction by biological thiol groups.

Inhibitory Effects Against Thioredoxin Reductase

The inhibitory effects of compound **3**, compound **4**, and the unprotected Au(I)-phosphine ligands (BrAuPPh₃ and BrAuPCy₃) were measured against rat liver TrxR. The activity of TrxR was measured using a standard DTNB assay as previously described.³⁹ In this assay, TrxR uses DTNB as a substrate to generate two molecules of 5'-thionitrobenzoic acid (TNB) with the concomitant generation of NADP⁺ from NADPH and H⁺. Time courses in product formation are generated as increases in the absorbance at 412 nm due to the generation of 2 equivalents of TNB from the reduction of DTNB. Figure 3 provides representative time courses in TrxR activity in the absence and presence of compound **3**, compound **4**, and the unprotected Au(I)-phosphine complex, BrAuPPh₃. Each time course represents an average of three (3) independent determinations. The unprotected Au(I)-phosphine complex, BrAuPPh₃, inhibits 85% of TrxR activity at a low concentration of 1 μ M. This result indicates that BrAuPPh₃ causes nearly 100% inhibition at stoichiometric levels of TrxR. The ability of BrAuPPh₃ to inhibit TrxR activity highlights the ability of unprotected gold compounds to undergo facile reaction with selenoenzymes. In contrast, both compounds **3** and **4** are poor inhibitors of TrxR activity. Specifically, compound **3** inhibits 17% TrxR activity at a fixed concentration of 40 μ M while compound **4** inhibits 39% of TrxR activity at an equivalent concentration. Although the Au(I)-indoles can affect TrxR activity, it should be noted that this low level of inhibition occurs at a concentration of 40 μ M which is significantly higher than the IC₅₀ values for either compound measured using the viability assay (vide supra). These results, coupled with the results of experiments validating the stability of both compounds **3** and **4**, collectively indicate that these gold-containing indoles do not cause cellular effects by reacting with thiol- or selenol-containing proteins like TrxR.

Screening for Kinase Inhibition

Since neither compound **3** or **4** displays appreciable inhibitory effects against known cellular targets of gold such as TrxR, we next tested for inhibitor effects against adenine-binding proteins including kinases. It is well established that dysfunctional and unregulated kinase activity plays significant roles in cancer initiation and progression.²⁹ Both compounds **3** and **4** were profiled against a panel of 64 kinases using a commercially available screening assay to evaluate if the measurable differences in cell-cycle progression and cell death arise from inhibitory effects on any of these cellular targets.⁴⁰ Experiments were performed using protocols described by Luceome Biotechnologies (Tucson, AZ) maintaining the concentration of compounds **3** and **4** fixed at 10 μ M. Table 4 provides a report of the percentage inhibition exhibited by compound **3** and **4** as a function of these representative human kinases. Inspection of the data provides several interesting observations. First, it is clear that neither compound **3** or **4** exhibits high potency toward any of the kinases present in this library. Despite the low potencies, however, a small number of kinases are inhibited by ~40% when the concentration of either compound **3** or **4** are maintained at 10 μ M. These inhibitory effects at this concentration are consistent with their measured IC₅₀ values that are also in the μ M range (Table 1). In addition, several kinases display overlapping inhibitory responses to both Au(I) compounds. Many of these are of particular interest due to their involvement in cancer initiation and/or progression. For example, the Aurora kinase family members, Aurora A and Aurora B, as well as the MARK2 and MARK3 kinases function during mitosis to regulate cell division.^{41,42} These kinases are important therapeutic targets as their activities are often deregulated in many types of cancers.⁴³ Other kinases such as RPS6KA3 and MLK3 are involved in the MAP kinase and JNK pathways which are also important in cancer progression.^{44–46} Thus, despite the low potency of Au(I)-compounds, the ability to weakly inhibit multiple therapeutic targets provides a plausible mechanism to explain for their anti-cancer effects. Indeed, several recent reports have established a new treatment paradigm for using compounds that display low potencies toward inhibiting multiple targets associated with a cancer phenotype.^{47–49} It is argued that

using a single, less-specific drug to inhibit various cellular targets may have therapeutic benefits compared to multi-drug regimens that produce similar outcomes.

While compounds **3** and **4** display some overlap in inhibitory effects, compound **4** is a more promiscuous kinase inhibitor compared to compound **3**. In this respect, only six kinases show greater than 20% inhibition with 10 μ M of compound **3** while an equivalent concentration of compound **4** inhibits 18 kinases to the same extent. The ability of compound **4** to inhibit certain kinases such as SNF1LK, YES1, and SNARK is noteworthy as they are involved in various pathological conditions. For example, YES1 is proto-oncogene that plays a role in cancer metastasis by functioning as a tyrosine protein kinase.⁵⁰ SNARK is another potential anti-cancer target as this kinase, normally involved in regulating glucose metabolism, may fuel carcinogenesis.⁵¹ In general, the ability of compound **4** to inhibit these kinases provides a new strategy to generate selective modulators of these therapeutic targets.

Enhancing the Cytotoxicity of Ionizing Radiation via (phosphine)gold(I) Indoles

Since compounds **3** and **4** inhibit kinases associated with cancer progression, we next tested their ability to enhance the anti-cancer effects of existing therapeutic modalities. In this case, we measured the effects of combining these (phosphine)Au(I) indoles with ionizing radiation (IR), a widely used therapy used to treat solid tumors. Experiments were performed pre-treating HeLa cells with compounds **3** or **4**, using sub-lethal concentrations, i.e., concentrations that produce <10% cell death over a 24 hour period. After this time frame, media containing the (phosphine)Au(I) indole was removed and replaced with fresh media. The cells were then irradiated in a dose-dependent fashion from 0 to 6 Gy. Cell viability was assessed using a clonogenic assay that measures colony formation and thus accurately defines the cytotoxic effects of IR exposure.⁵² Figure 4A shows the relationship between radiation dose with the fraction of cells that survive exposure to these doses of IR. HeLa cells treated with IR alone show a typical linear-quadratic survival curve characterized by an initial linear cell killing phase that is proportional to the dose of radiation followed a second cell killing phase that is proportional to the square of the dose. The initial linear phase is particularly important as this reflects the ability of the cell to repair damaged DNA inflicted by clinically-relevant doses of IR (~2 Gy). This initial phase is eliminated when cells are pre-treated with compound **3** (4 μ M) or compound **4** (7 μ M) (Figure 4A). The ability of either compound **3** or **4** to increase the slope of this initial phase provides clear evidence that both Au(I)-containing compounds enhance the cell killing activity of IR. In fact, the radiosensitizing effect exhibited by these compounds is reminiscent of survival curves generated in cells defective in kinases involved in repairing DNA damage such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad-3 related (ATR).^{53, 54} Quantitative analyses reveal that cells treated with either compound **3** or **4** are ~4-fold more sensitive to the effects of IR exposure than cells treated with IR alone. Compounds **6** and **7** do not enhance the cell killing effects of IR. This dichotomy implies that compounds **3** and **4** exert their effect by binding selective cellular proteins including kinases while the non-specific analogs **6** and **7** do not.

The underlying mechanism for these radiosensitizing effects was further interrogated by quantifying the number of DSBs formed after IR exposure in the absence and presence of compounds **3** and **4** using γ H2AX as a biochemical marker (Figure 4B).⁵⁵ Histograms provided in Figure 3C – H show plots of the percentage of cells containing γ H2AX foci after exposure to 2 Gy IR after 30 minutes or 4 hours, respectively. Cells treated with DMSO show an increased number of γ H2AX foci 30 minutes post-IR exposure (Figure 4C). This rapid response is indicative of DSB formation via radical damage.⁵⁶ The vast majority of these DSBs are repaired within 4 hours as judged by fewer γ H2AX foci (Figure 4D). HeLa cells treated with compound **4** also show an increase in the number of γ H2AX foci 30

minutes post IR exposure (Figure 4E). However, a significant number of γ H2AX foci persist 4 hours after IR exposure in cells pre-treated with compound **4** (Figure 4F). The attenuation in γ H2AX foci disappearance indicates that compound **4** inhibits DSB repair, and this inhibition likely accounts for the enhancement in IR cytotoxicity caused by this gold-containing indole.

A different effect on γ H2AX foci formation is observed combining compound **3** with 2 Gy of IR. As illustrated in Figure 4G, cells pre-treated with compound **3** have fewer γ H2AX foci 30 minutes after IR exposure compared to cells treated with either DMSO or compound **4**. This reduction in γ H2AX foci formation suggests that compound **3** inhibits H2AX phosphorylation without influencing the overall number of DSBs formed after IR exposure. The ability of compound **3** to prevent phosphorylation is reasonable as we have shown that this Au(I)-indole inhibits the activity of several kinases (Table 4). Indeed, phosphorylation of H2A is catalyzed by several PI3K-like kinases including ATM, ATR, and DNA-dependent protein kinase (DNA-PK).⁵⁷ After DSB formation, each kinase is rapidly activated, and their ability to phosphorylate key proteins such as H2AX is essential for the timely repair of these lesions.⁴⁴ While we do not know if any of these specific kinases are influenced by compound **3**, it is tempting to speculate that they are either directly or indirectly inhibited by this gold compound. Current efforts are exploring this possibility. Regardless, the net effect for inhibiting H2AX phosphorylation is a reduction in DSB repair that causes a concomitant increase in the cytotoxic effects of IR observed in our clonogenic assays.

Discussion

This report describes the development and application of unique (phosphine)gold(I) indoles that function as anti-cancer agents when used individually and in combination with therapeutic doses of IR. The anti-cancer activity of these compounds is not unprecedented as several Au(I)- and Au(III)-complexes have been reported to generate favorable anti-cancer effects.^{11–16, 25} One of the most relevant cellular targets for many gold compounds is TrxR, the only known enzyme that catalyzes the reduction of thioredoxin.⁵⁸ Thioredoxin plays important roles ranging from maintaining nucleotide pools associated with DNA replication and repair to defending against oxidative stress directly and indirectly via redox signaling.⁵⁸ As such, inhibiting TrxR activity can produce robust anti-cancer effects. For example, Rubbiani *et al.* reported that Au(I)-complexes with benzimidazole derived N-heterocyclic carbene ligands displayed more potent inhibition against TrxR compared to the closely related redox enzyme, glutathione reductase.¹¹ In this case, the selective inhibition of TrxR activity coincided with significant cytostatic effects against various cancer cell lines. In addition, Yan *et al.* reported that Au(I)-containing N,N'-disubstituted cyclic thiourea complexes inhibit TrxR with nanomolar potencies.¹² As expected, several of these compounds also exerted significant cytotoxic effects against cancer cells presumably as a result of this inhibition. Another recent example comes from the work of Vergara *et al.* demonstrating that Au(I) complexes containing phosphine ligands such as 1,3,5-triaza-7-phosphaadamantane and 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1] attached to thionate ligands show cytostatic effects against cisplatin sensitive (A2780S) and resistant (A2780R) ovarian cancer cells.¹³ Similar to results published by Rubbiani *et al.*, these Au(I)-phosphine complexes inhibited both cytosolic and mitochondrial TrxRs while being relatively inert against the related glutathione reductase. Finally, Maiore *et al.* recently published on the synthesis and biological characterization of Au(I) and Au(III) complexes based on the saccharinate ligand.¹⁴ In general, Au(III) complexes displayed moderate cytotoxicities against the A2780S ovarian cancer cell line while only modest activities were observed with disaccharinato gold(I) complexes. The higher reactivity of Au(III)

saccharinate derivatives compared to the Au(I) counterparts correlate with their increased cytotoxic effects.

While TrxR is an excellent target for many Au(I)-containing complexes, other cellular enzymes can be efficiently inhibited by related gold-complexes. For example, Zhang *et al.* demonstrated that Au(I)-dithiocarbamate species show inhibitory effects against another therapeutic target, i.e., the proteolytic activity of 20S proteasome and 26S proteasome in human cancers.¹⁵ Inhibiting the proteasome caused the accumulation of ubiquitinated proteins and proteasome target proteins and ultimately caused cell death. Likewise, Trani *et al.* showed that aurothiomalate, a gold compound similar to auranofin that is used to treat rheumatoid arthritis, shows unique pro-apoptotic effects in cancer cells via the disruption of the PKC α -Par6 complex.¹⁶ Disruption of this complex leads to the activation of the ERK pathway, causing caspase-3 activation and subsequent apoptosis. In addition, aurothiomalate activates the p38 and JNK MAP kinases. Finally, Bagowski *et al.* demonstrated that Au(I) phosphine complexes containing a naphthalimide ligand display anti-angiogenic effects in two different zebrafish angiogenesis models, including a tumor-cell induced neovascularization assay.⁵⁹ While the exact cellular target responsible for this anti-cancer effect has yet to be conclusively identified, the beneficial therapeutic effect is unlikely to be related to TrxR inhibition. Collectively, these studies highlight the ability of gold-complexes to exert cytotoxic effects by inhibiting cellular targets other than TrxR.

In this report, we describe two Au(I)-indoles that show unique properties by killing cancer cells without adversely affecting TrxR activity. This ability contrasts that of the unprotected Au(I)-ligand, BrAuPPh₃, which produces potent cytotoxic effects in leukemia cells by inhibiting TrxR activity. As such, the low reactivity displayed by compounds **3** and **4** against BSA or inhibiting TrxR activity is consistent with our original hypothesis that encapsulating Au(I) with sterically hindered phosphine ligands reduces the reactivity of this metal with biological thiols or selenols. This result is also consistent with structure-activity relationships reported by Shaw *et al.* showing that the ease of displacing trialkylphosphines from their albumin-gold complexes to be Me₃P > Et₃P > *i*-Pr₃P.³⁷ As such, using bulky ligands such as triphenylphosphine or tricyclohexylphosphine reduces reactivity toward thiol groups. In addition, the potencies of compounds **3** and **4** vary amongst the cancer cell lines tested here, suggesting that they inhibit selective molecular targets within the cell. Screening of both Au(I) compounds against a panel of human kinases provides evidence that for their inhibitory effects against several potential therapeutic targets. The data provided in Table 4 indicates that the structurally related compounds inhibit some common kinases with different potencies. In particular, compound **4** shows a higher degree of promiscuity by interacting with three-times as many kinases compared to compound **3**. The combination of variable potencies and promiscuity for inhibiting different targets provides a reasonable explanation for the nuances observed in their physiological effects. We emphasize that these screening efforts cover ~10% of the reported 520 different protein and lipid kinases that constitute the human kinome. As such, it is likely that these Au(I)-indoles also inhibit other therapeutically-relevant kinases not present in this initial screen. In fact, the ability of compound **4** to inhibit G1 to S-phase progression suggests that this gold compound inhibits other kinases including cyclin-dependent kinases (cdks 2, 4, and 6) that are involved in cell-cycle progression.⁶⁰ Current efforts are exploring these possibilities.

Another important feature of this work is that these Au(I)-indoles potentiate the cytotoxic effects of IR exposure at concentrations that are fractions of their respective LD₅₀ values. IR is an important therapeutic modality used in approximately one-half of all cancer patients and is particularly effective against cancers of the brain, cervix, breast, and colon that are inaccessible to surgery and/or refractory to chemotherapy.^{61–64} Although the primary target of therapeutic IR is water in tissue, the radicals derived from water eventually damage DNA.

While IR produces several forms of DNA damage, the most lethal are double-stranded DNA breaks (DSBs).⁶⁵ In general, the inability of a cancer cell to effectively repair these DSBs causes both cytostatic and cytotoxic effects to reduce tumor growth.

IR is relatively effective in amply oxygenated tissue, and this reflects the ability of long-lived oxygen-centered radicals to be more effectively propagated to DNA.⁶⁶ Unfortunately, the inner volumes of many solid tumors are hypoxic and cause radiation-resistant zones.⁶⁷ As such, a major complication in IR therapies is effectively killing radiation-resistant cells. Approaches to improve the efficacy of IR have focused on using DNA damaging agents such as cisplatin to enhance cell death by overwhelming DNA repair pathways.⁶⁸ Alternatively, anti-metabolites such as 5-fluorouracil can be used to reduce intracellular dNTP pools needed to re-synthesize DNA during repair.^{69, 70} While these approaches work, these specific chemotherapeutic agents are themselves highly cytotoxic and thus kill both cancerous and healthy cells.⁷¹ Indeed, the non-selective killing of healthy, normal cells produces severe side effects including anemia, leukopenia, and thrombocytopenia that are significant complications in the effectively treating patients. The results described here with our Au(I)-containing compounds provide an alternative approach to potentiate the cytotoxic effects of IR. Our data show that compounds **3** and **4** function as radiosensitizers to inhibit DSB repair through two mutually exclusive mechanisms. As illustrated in Figure 5A, exposure to IR produces DSBs that cause the phosphorylation of H2AX. This acts as a key signaling event that initiates DSB repair which allows cells to survive the insult to genomic DNA. Compound **3** inhibits H2AX phosphorylation, leading to a decrease in γ H2AX foci formation (Figure 5B). By blocking this key step, compound **3** causes a significant number of DSBs to be left unrepaired to enhance the extent of apoptosis. The structurally related analog, compound **4**, also inhibits DSB repair and increases the cytotoxicity of IR. However, compound **4** does this via a different mechanism that involves the inhibition of steps occurring after γ H2AX foci formation (Figure 5C). It is tempting to speculate that the potentiating effect caused by compound **4** reflects inhibition of cell cycle progression. However, more data are needed to prove this hypothesis.

Regardless, we envision that the potentiating effect by either Au(I)-compound is caused by reversible inhibition of key cellular proteins such as kinases involved in DNA repair and/or cell-cycle progression. This argument is strengthened by the observation that other (phosphine)gold(I) indoles including compounds **6** and **7** do not enhance the cell killing effects of IR (data not shown). This dichotomy implies that compounds **3** and **4** exert their effect by binding selective cellular proteins while the non-specific analogs, **6** and **7**, do not. Another possible mechanism is that IR exposure leads to radical induced cleavage of the Au(I)-containing indole which then inflicts irreversible damage on these cellular components. This prediction is based upon the results of density-functional theory calculations for these gold-bearing compounds. As illustrated in Figure 6, the dissociation energy of the gold-carbon bond into radicals is approximately 58 kcal mol⁻¹ while the dissociation energy of HO-H is 119 kcal mol⁻¹.⁷² Since metal-carbon bonds are weaker than the O-H bond of water, it is possible that radicals initiated by IR are transduced from water radicals to these Au(I) compounds to produce gold- and carbon-centered radicals. If so, then Au(I) organometallics containing homolyzable carbon-gold bonds have therapeutic prospects by potentiating damage caused by radicals caused by IR or other radical generating systems.

Finally, while both (phosphine)gold(I) indoles increase the cytotoxic effects of IR, compound **3** may prove to be more efficacious than compound **4**. This is based on the fact that compound **3** shows higher potency against adherent cancer cells compared to the hematological cancer cell line, CEM-C7. The lower potency against systemic cancer cells implies that this novel gold-indole analog could avoid potential side effects such as

thrombocytopenia and leukopenia that are caused by inadvertently killing thrombocytes and leukocytes, respectively. This selectivity combined with the measured dose-modifying factor of 4 indicates that this (phosphine)Au(I) indole could be used to increase the effectiveness of ionizing radiation, especially for clinical protocols that require fractionation of large IR doses. By increasing the efficacy of IR, these innovative gold-bearing indoles can be used to reduce total exposure to ionizing radiation. This will provide additional therapeutic benefits by lowering the risk of developing complications associated with excessive exposure to ionizing radiation that include side effects such as inflammation, gastrointestinal ailments, and immunosuppression. Current efforts are further exploring the therapeutic utility of these and other gold-bearing indole derivatives.

Methods and Materials

Reagents and General Methods

Acetonitrile (Acros Organics) was distilled from CaH_2 . Tetrahydrofuran (Acros Organics) was distilled from Na and benzophenone. Anhydrous isopropanol was purchased from Acros Organics. Thioredoxin reductase, EDTA, 5,5'-dithio-bis-(2-nitrobenzoic acid), bovine serum albumin, and NADPH were obtained from Sigma-Aldrich. Bio-Gel P2 resin and Bradford reagent dye were obtained from BioRad, Incorporated. Apoptosis Kit #2 was purchased from Invitrogen. Cell-titer blue reagent was purchased from Promega. All other commercial reagents, including 5-indole boronic acid pinacol ester and 1-methylindole-5-boronic acid pinacole ester used for synthetic procedures were purchased from Sigma-Aldrich or Strem Chemicals and were used without further purification. All ^1H and ^{13}C NMR spectra were recorded on a Varian AS-600 spectrometer, at 400 and 150 MHz, respectively, using tetramethylsilane as the internal standard. Mass spectral analyses were performed using the Ohio State University Analytical Facility. ^{31}P NMR spectra were recorded on a Varian AS-400 and 600 spectrometers. Purity of all biologically active compounds was >95% as judged by microcombustion analyses (C, H, N, P and Au) performed by Robertson Microлит Laboratories (Ledgewood, NJ). In addition, purity was >95% as judged by high-performance liquid chromatography. Reverse phase-HPLC used a linear gradient of 25% acetonitrile in water to 100% acetonitrile over a 25 minutes with a flow rate of 1 mL/min monitored at 220 nm and 280 nm using a Vadac C18 column; 4.6 mm $\times$ 250 mm. RP-HPLC was performed using a JASCO analytical HPLC system.

Tert-butyl 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indole-1-carboxylate (1)—5-indole boronic acid pinacol ester (1.5g, 6.1mmol) was treated with tert-butoxycarbonyl [(Boc) $_2$ O](2.02g, 9.3mmol) in the presence of dimethylaminopyridine (148 mg, 1.2 mmol) in 20 ml of anhydrous CH_3CN . The reaction was stirred until completion, which was monitored by thin layer chromatography. After completion the crude product **1** was concentrated *in vacuo* then purified through flash chromatography (silica gel:hexanes/EtOAc 9:1) to produce a white solid in 90% yield.

^1H -NMR (400MHz, CDCl_3) δ (ppm): 8.15-8.13(m, 1H), 8.05(m, 1H), 7.74-7.76(m, 1H), 6.56-6.57(d, $J=4.0$ Hz, 1H), 1.67(s, 9H), 1.37(s, 12H).

^{13}C -NMR(150 MHz, CDCl_3 , ppm) δ : 149.91, 130.74, 130.36, 128.44, 126.09, 107.74, 83.95, 28.40, 25.12.

(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indol-1-yl)methyl pivalate (2)—To a solution of 5-indole boronic acid pinacol ester (350 mg, 1.45 mmol) in 10 ml of anhydrous THF, sodium hydride (63 mg, 2.17 mmol) was added. After solution was allowed to stir for 30min, the reaction was chilled to 0°C then pivaloyloxymethylchloride (433 mg, 2.17 mmol) was added dropwise. The reaction was allowed to stir for 4 hr. After completion

the reaction was quenched with ice-water and extracted with EtOAc, washed with brine and dried over anhydrous magnesium sulfate. The solution was then gravity filtered and concentrated under reduced pressure. The residue was then purified by silica gel flash chromatography eluting with hexanes/EtOAc (9:1) to yield a white solid at a 54% yield.

^1H -NMR (400MHz, CDCl_3) δ (ppm): 8.13(m, 1H), 7.72-7.69(m, 1H), 7.50-7.48(m, 1H), 7.32-7.24(m, 1H), 6.54-6.52(m, 1H), 6.09(s, 2H), 1.37(s, 12H), 1.12(s, 9H).

^{13}C -NMR (150 MHz, CDCl_3) δ (ppm): 178.42, 138.41, 129.00, 128.79, 109.21, 104.18, 83.74, 68.82, 39.13, 29.93, 27.12, 25.11.

General Procedure of the Indole Gold(I) Phosphine Ligands Scaffolds—To a round bottom flask, compound **1** or **2** [350 mg, 1 equiv.], cesium carbonate (653 mg, 2 equiv.) and gold phosphine ligand (AuPR_3) [320 mg, 0.5 equiv.] were added, followed by 10 ml of anhydrous isopropyl alcohol. The reaction was heated at 40°C for 16 hr. After completion of reaction, the mixture was concentrated *in vacuo*. To the crude solid, toluene was then added and the residue was filtered through Celite. The solution was concentrated under reduced pressure. The crude residue was precipitated from n-pentane.

[5-{Triphenylphosphine gold(I)}-tert-butyl 1H-indole-1-carboxylate](3)—white solid, 51% yield. ^1H -NMR (400MHz, C_6D_6) δ (ppm): 8.37-8.35(d, $J=5.2\text{ Hz}$, 1H), 8.24-8.21(dd, $J=5.2, 7.6\text{ Hz}$, 1H), 7.46-7.42(m, 6H), 6.98-6.97(m, 1H), 6.96-6.90(m, 9H), 6.52-6.51(m, 1H), 1.35(s, 9H), $^{31}\text{P}\{^1\text{H}\}$ -NMR (243 MHz, C_6D_6) δ (ppm): 44.66

$^{13}\text{C}\{^1\text{H}\}$ -NMR (158MHz, CDCl_3) δ (ppm): 165.88-165.10, 135.61, 134.68-134.59, 131.58, 131.29, 129.26-129.19, 128.44, 125.51, 124.50, 114.31, 107.94, 28.49.

(TOF MS-ES+): calculated $m/z = 698.1499$; found 698.1503 [$\text{M}+\text{Na}$]; $m/z = [\text{Au}(\text{PPH}_3)_2] = 721.1484$.

[5-{Tricyclohexylphosphine gold(I)}-tert-butyl 1H-indole-1-carboxylate](4)—white solid, 50% yield. ^1H -NMR (400MHz, CDCl_3) δ (ppm): 7.99(m, 1H), 7.70-7.69(m, 1H), 7.46(m, 2H), 6.47-6.46(m, 1H), 2.06-2.04(m, 10H), 1.87-1.86(m, 7H), 1.74-1.73(m, 3H), 1.65(s, 9H), 1.37-1.25(m, 12H), 0.895-0.870(m, 1H).

$^{31}\text{P}\{^1\text{H}\}$ -NMR (243 MHz, C_6D_6) δ (ppm): 58.09

$^{13}\text{C}\{^1\text{H}\}$ -NMR (150 MHz, CDCl_3) δ (ppm): 170.23-169.50, 135.16, 131.16, 124.82, 114.23, 107.97, 33.55-33.39, 30.93, 28.48, 27.5-27.43, 26.30.

(TOF MS-ES+): calculated $m/z = 716.2908$; found 716.2897 [$\text{M}+\text{Na}$]

[5-{[1,1'-biphenyl]-2-ylidicyclohexylphosphine aurate(I)}-tert-butyl H-indole-1-carboxylate] (5)—white solid, 52% yield. ^1H -NMR (400MHz, C_6D_6) δ (ppm): 8.12-8.10(d, $J=8.0\text{ Hz}$, 1H), 7.94-7.91(dd, $J=4.8, 8.0\text{ Hz}$, 1H), 7.61-7.57(m, 1H), 7.28-7.27(m, 5H), 7.21-7.19(m, 1H), 7.10-7.08(m, 4H), 6.57-6.56(m, 1H), 1.99-1.94(m, 4H), 1.70-1.60(m, 3H), 1.51(m, 4H), 1.46-1.43(m, 3H), 1.38(s, 9H), 1.05-0.88(m, 8H), $^{31}\text{P}\{^1\text{H}\}$ -NMR (243 MHz, C_6D_6) δ (ppm): 52.31

$^{13}\text{C}\{^1\text{H}\}$ -NMR (150 MHz, CDCl_3) δ (ppm): 167.51-166.76, 149.36-149.29, 142.18, 135.56, 135.35-135.30, 132.37-132.33, 131.19, 130.23, 129.73, 128.50, 128.30, 128.02, 127.39, 124.14, 113.80, 107.97, 36.82-36.65, 31.14-31.10, 29.77, 28.51, 27.03-26.95, 26.08.

(TOF MS-ES+): calculated $m/z = 786.2751$; found 786.2722 [$\text{M}+\text{Na}$]

[5-{{1, 1'-biphenyl}-2-ylidicyclohexylphosphine aurate(I)}-(1H-indol-1-yl)methyl pivalate](6)—white solid, 50% yield. $^1\text{H-NMR}$ (400MHz, CDCl_3) δ (ppm): 8.21-7.86(m, 1H), 7.58-7.57(m, 1H), 7.44-7.42(m, 5H), 7.48-7.32(m, 1H), 7.29-7.26(m, 4H), 7.08-7.07(m, 1H), 6.40-6.39(m, 1H), 6.03(s, 2H), 2.35-2.16(m, 2H), 2.11-1.95(m, 2H), 1.64-1.54(m, 5H), 1.52-1.51(m, 6H), 1.31-1.41(m, 7H), 1.10(s, 9H).

$^{31}\text{P}\{^1\text{H}\}$ -NMR (243MHz, CDCl_3) δ (ppm): 53.80

$^{13}\text{C}\{^1\text{H}\}$ -NMR (150MHz, CDCl_3) δ (ppm): 178.62, 164.69-163.99, 149.19-149.12, 142.17, 135.76-135.69, 135.34, 133.67, 132.34-132.29, 131.13, 130.21, 129.73, 129.52-129.33, 128.61-128.41, 128.47, 128.17, 128.01, 127.36, 126.83, 108.37, 103.46, 69.21, 39.14, 36.84, 31.15, 29.90-29.81, 27.18-26.82, 26.06, 25.99-25.07.

(TOF MS-ES+): calculated m/z = 800.2908; found 800.2902 [M+Na]

[5-{{1,1'-biphenyl}-2-ylidicyclohexylphosphine aurate(I)}-1-methyl-1H-indole](7)—white solid, 62% yield. $^1\text{H-NMR}$ (400MHz, C_6D_6) δ (ppm): 8.27-8.26(d, J = 8 Hz, 1H), 7.90-7.87(dd, J = 4.4, 7.6 Hz, 1H), 7.74-7.69(m, 1H), 7.30-7.27(m, 5H), 7.10-7.05(m, 4H), 6.68-6.69(m, 1H), 6.64-6.63(m, 1H), 3.06(s, 3H) 1.98-1.92(m, 4H), 1.72-1.70(m, 2H), 1.57-1.43(m, 10H), 0.97(m, 6H), $^{31}\text{P}\{^1\text{H}\}$ -NMR (243 MHz, C_6D_6) δ (ppm): 53.45.

$^{13}\text{C}\{^1\text{H}\}$ -NMR (150 MHz, CDCl_3) δ (ppm): 163.19-162.43, 149.17-147.10, 142.17, 135.96-135.84, 132.82, 132.32-132.27, 131.00, 129.73, 128.93-128.89, 128.56-128.33, 128.48, 128.02, 127.34-127.28, 127.01, 108.00, 100.49, 36.85-36.68, 32.77, 31.20-31.16, 29.83, 27.05-26.96, 26.08.

(TOF MS-ES+): calculated m/z = 700.2383; found 700.2382 [M+Na]

General Cell Culture Procedures

HeLa, MCF-7, HCT-116, and CEM-C7 were obtained from the American Type Culture Collection (Manassas, VA, USA). All adherent cell lines were maintained in Dulbecco's modified Eagle's medium (Mediatech) with 100 U/ml penicillin (Invitrogen), 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen), 0.25 $\mu\text{g}/\text{ml}$ amphotericin B (Invitrogen), and 10% fetal bovine serum (USA Scientific) and incubated at 37° C with 5% CO_2 . CEM cells were maintained in RPMI-1640 media supplemented with 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, and 10% fetal bovine serum and incubated at 37° C with 5% CO_2 .

Cell Proliferation Assays

Cells were plated at a density of 7,000–13,000/well in 200 μl of media overnight in a 96-well plate. Each (phosphine)gold(I)-indole was added to wells in a dose-dependent manner (0.01–100 μM). Cells were treated with compounds for variable time periods (8–48 hours). With adherent cell lines, medium was removed from the wells and then 100 μl of fresh medium was added into each well followed by the addition of 20 μl of cell titer-blue reagent (Promega). Cells were incubated with reagent for 1–4 hrs and the optical density of samples was read at 560 nm using a microplate reader. The background absorbance of dye with media was subtracted from each sample. Cell viability was then normalized against cells treated with DMSO. IC_{50} values were obtained using a fit of the data to equation 1

$$Y = 100\% / [1 + (\text{IC}_{50} / \text{Inhibitor})] \quad (1)$$

where y is the fraction of viable cells, IC_{50} is the concentration of inhibitor that inhibits 50% cell growth, and Inhibitor is the concentration of compound tested. Each experiment

represents an average of at least three (3) independent determinations performed on different days.

Measurements of Apoptosis

Cells were plated at 200,000/ml, Au(I)-Indole analogs were added in a dose-dependent fashion for 12–24 hr. Cells were trypsinized and then washed with cold PBS. After discarding the supernatant, a 100 μ L solution containing 1X annexin-binding buffer, 5 μ L of Alexa Fluor 488 annexin V and 1 μ g/ml of PI solution was added to each sample. The cells were incubated at room temperature for 15 min. After this incubation period, an additional 400 μ L of 1X annexin-binding buffer was added. Cells were analyzed using a band pass filters with wavelengths of 525/40 nm and 620/30 nm with a Beckman Coulter XL flow cytometer.

Cell Cycle Analyses

Cells were plated at a density of 200,000/ml. Au(I)-Indole analogs were then added in a dose-dependent manner for time periods varying from 1 to 3 days. Cells were treated with 0.25% trypsin and harvested by centrifugation. The supernatant was removed and then washed with PBS. After aspiration of PBS, 500 μ L of 70% ethanol was added and cells were incubated on ice for 15 minutes followed by centrifugation and the removal of ethanol. One ml of PI staining solution [(10 ml of 0.1 Triton X-100/PBS, 0.4 ml of 500 Mg/ml of PI, and 2 mg/ml of DNase-free RNase)] was added to the cell suspension, placed on ice for 30 minutes, and then analyzed using a Beckman Coulter XL flow cytometer with a red filter. Each experiment represents an average of three (3) independent determinations performed on different days.

Microscopy

Cells were plated at 75,000–125,000/ml for 24 hours. (Phosphine)gold(I) indoles then were added at concentrations equal to their LD₅₀ values for 12 and 24 hours. Alexa Fluor 488 annexin V and propidium iodide were added to each well and images were taken on Leica CTR 6500 microscope using green (480/40 nm) and red (560/40 nm) excitation band pass filters.

Clonogenic Survival Assay

HeLa cells were plated at a density of 250,000–300,000 cells/mL. After 24 hr cells, the cells were irradiated in a dose dependent manner (0–4 Gy) using a ¹³⁷Cs gamma source. After treatment, the cells were trypsinized and plated at a density between 300–4000 cells in 60mm dishes. Cells were allowed to grow colonies (1 colony $\geq$ 50 cells) for 10–14 days, stained with 0.25% crystal violet, and manually counted to measure the number of colonies. Survival fractions were normalized against positive controls (colony formation with no radiation) and plotted as the log percent survival versus dose of radiation. Each experiment was performed an average of four (4) times with cells propagated on several different days.

Detection of γ H2AX formation

HeLa cells were plated at a density of 40,000 cells/ml in 24-well glass bottom plate for 24 hr. After this time, cells were treated with Au(I)indole compounds at desired concentration for an additional 24 hr. The cells were irradiated at 2 Gys. Cells were fixed with 4% paraformaldehyde for 15 min at 37°C at time points corresponding to 0.5, 1, 2, and 4 hrs. Cells were washed with 1X PBS and permeabilized in 0.2% Triton X-100 for 15 min at 37°C. Cells were washed with 1X PBS and subsequently blocked with 1% BSA, 0.1% Tween in 1X PBS for one hour at room temperature. Mouse monoclonal anti-phospho-histone H2AX antibody (Millipore) was applied (1:500 dilution) to each well for one hour. After washing

several times with blocking buffer, a 1:500 dilution of goat anti-mouse secondary antibody conjugated with Alexa 647 (Invitrogen) was added for 1 hr. The wells were then washed several times, and the number of γ H2AX foci per nuclei was measured using an iCyte laser scanning cytometer with a red long pass filter (650 nm). To collect accurate data, a threshold was set to minimize noise produced by unrelated events using iNovator software (version 3.4.2.52). Each well was scanned using a 40X magnification with 0.25 μ m X step and a field size of 250 $\times$ 186 μ m (1000 $\times$ 768 pixels). Data were plotted as the percentage of cells as a function of foci number. The resulting histogram was fit to the equation for a Gaussian distribution (equation 2).

$$Y = 1 / (2\pi\sigma^2)^{1/2} e^{-[(x-\mu)^2 / 2\sigma^2]} \quad (2)$$

where Y is the percentage of cells, μ is the mean, and σ is the variance used to define the width of the mean.

Reactivity of Au(I)-Compounds with Biological Thiols

BSA concentrations were determined measuring A_{280} ($\epsilon=36,600 \text{ M}^{-1}\text{cm}^{-1}$) or using the Bradford assay measuring samples at A_{595} as described.⁷³ The -SH titer of BSA was determined using DTNB ($\epsilon_{414} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$) as previously described.⁷⁴ BSA (0–120 μ M) was treated with variable concentrations of compound **3** (120 μ M) and compound **4** (120 μ M) yielding Au(I)/BSA ratios of 4:1, 2:1, 1:1, and 0:1. Phosphate-buffered saline (PBS) was used as the buffer in these experiments. After incubating for one hour at 37°C, the reaction mixtures were applied to Penefsky spun columns using Bio-Gel P2 gel filtration resin and centrifugal force to rapidly and efficiently separate unreacted Au(I)-containing complexes from BSA. Penefsky spun columns were prepared using the following procedure: P2 resin was pre-swelled in 10 mM Tris, pH 7.5 and 1 mM EDTA. The resin was then loaded into 1 mL tuberculin syringes (Becton-Dickinson) and spun in a fixed-angle rotor at 2,000 rpm for 2 minutes. Reaction mixtures described above were then loaded into the column and spun at 2,000 rpm for 2 minutes. The eluants were removed and analyzed for Au(I)-containing compounds and BSA. Compound **3** and compound **4** were analyzed measuring absorbances at 212 nm and 280 nm before and after spinning through the Penefsky column. Note that these experiments were performed in the absence of BSA; thus, there is no spectral overlap between the Au(I)-complexes and protein. In all cases, free Au(I)-containing complexes were retained in the P2 resin as their presence was not detected in the eluant. The concentration of BSA was quantified before and after spinning through the Penefsky column using Bradford assay dye. In these cases, the concentration of BSA was reduced less than 5% after elution through the Penefsky column. This result indicates that BSA was not retained in the P2 resin under these conditions. In some experiments, a wavelength of 319 nm was used to quantify the presence of Au(I)-indole compounds when the Au(I)-indole (compound **3**) was incubated with BSA. This higher wavelength avoids spectral overlap with protein. Finally, the -SH titer of BSA before and after elution through the Penefsky column was determined using DTNB ($\epsilon_{414} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$) as described.⁷⁴

Interactions of compound **3** with cysteine were measured via the quantitation of reactive sulfhydryls using DTNB ($\epsilon_{414} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$) as previously described.⁷⁴ A linear standard curve for L-cysteine was measured by reacting variable concentrations of L-cysteine (0–800 μ M) with 2 mM DTNB at 37°C for 60 minutes. PBS was used as the reaction buffer. The reactivity of DTNB was measured using an absorbance of 412 nm ($\epsilon_{414} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$). Incubation of 400 μ M L-cysteine with an equivalent concentration of compound **3** does not change the titer of free -SH present on L-cysteine.

Inhibitory Effects of Au(I)-Compounds against Thioredoxin Reductase

The inhibitory effects of compound **3**, and compound **4**, and the unprotected Au(I)-phosphine ligands (BrAuPPh₃ and BrAuPCy₃) were measured against rat liver TrxR. All assays were performed at 25° C. Experiments were performed adding 600 nM TrxR to a pre-incubated solution containing 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mg/mL BSA, and 240 μM NADPH in the absence and presence of Au(I)-containing compounds. The amount of TNB formed as a function of time was measured by examining changes in absorbance at 412 nm. Under all conditions tested, the change in absorbance was linear under the time frame tested (5 minutes). Time courses in product formation were fit using equation 3

$$y=mt \quad (3)$$

where y is the change in absorbance at 412 nm, m is the rate of the reaction, and t is time.

Computations

Spin-unrestricted density-functional theory computations were performed within the Gaussian 03 program suite.⁷⁵ Calculations employed the exchange functional of Becke⁷⁶ and the correlation functional of Lee, Yang, and Parr.⁷⁷ Nonmetal atoms were described with the TZVP basis set of Godbelt, Andzelm, and co-workers.⁷⁸ Gold orbitals were described with the Stuttgart effective core potential and the associated basis set.⁷⁹ Gas-phase equilibrium geometries were optimized in redundant internal coordinates without imposed symmetry. Harmonic frequency calculations confirm the structures so generated to be energy minima. All other calculated properties reported here include implicit water solvation, which was incorporated in single-point calculations of the gas-phase geometries with Tomasi's polarizable continuum model.⁸⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

T.G.G. thanks the National Science Foundation (grant CHE-1057659) and the Alfred P. Sloan Foundation for support. A.J.B. is supported by funding from the National Institutes of Health (CA118408) and the Department of Defense, US Army Medical Research (CA091380). We thank Nihal Deligonul for assistance with the X-ray diffraction crystallography.

References

1. Cowan JA. Structural and catalytic chemistry of magnesium-dependent enzymes. *Biometals*. 2002; 15:225–235. [PubMed: 12206389]
2. Qi W, Cowan JA. Structural, Mechanistic and Coordination Chemistry of Relevance to the Biosynthesis of Iron-Sulfur and Related Iron Cofactors. *Coord Chem Rev*. 2011; 255:688–699. [PubMed: 21499539]
3. Brown KL. The enzymatic activation of coenzyme B12. *Dalton Trans*. 2006:1123–1133. [PubMed: 16482346]
4. Brewer GJ. Risks of copper and iron toxicity during aging in humans. *Chem Res Toxicol*. 2010; 23:319–326. [PubMed: 19968254]
5. Moulis JM. Cellular mechanisms of cadmium toxicity related to the homeostasis of essential metals. *Biometals*. 2010; 23:877–896. [PubMed: 20524046]
6. Butterworth RF. Metal toxicity, liver disease and neurodegeneration. *Neurotox Res*. 2010; 18:100–105. [PubMed: 20369313]

7. Brabec V, Kasparkova J. Modifications of DNA by platinum complexes. Relation to resistance of tumors to platinum antitumor drugs. *Drug Resist Update*. 2005; 8:131–146.
8. Wang X. Fresh platinum complexes with promising antitumor activity. *Anticancer Agents Med Chem*. 2010; 10:396–411. [PubMed: 20545618]
9. Fléchon A, Rivoire M, Droz JP. Management of advanced germ-cell tumors of the testis. *Nat Clin Pract Urol*. 2008; 5:262–276. [PubMed: 18398406]
10. Adams G, Zekri J, Wong H, Walking J, Green JA. Platinum-based adjuvant chemotherapy for early-stage epithelial ovarian cancer: single or combination chemotherapy? *BJOG*. 2010; 117:1459–1467. [PubMed: 20560942]
11. Rubbiani R, Kitanovic I, Alborzinia H, Can S, Kitanovic A, Onambele LA, Stefanopoulou M, Geldmacher Y, Sheldrick WS, Wolber G, Prokop A, Wölfl S, Ott I. Benzimidazol-2-ylidene gold(I) complexes are thioredoxin reductase inhibitors with multiple antitumor properties. *J Med Chem*. 2010; 53:8608–8618. [PubMed: 21082862]
12. Yan K, Lok CN, Bierla K, Che CM. Gold(I) complex of N,N'-disubstituted cyclic thiourea with in vitro and in vivo anticancer properties-potent tight-binding inhibition of thioredoxin reductase. *Chem Commun (Camb)*. 2010; 46:7691–7693. [PubMed: 20623063]
13. Vergara E, Casini A, Sorrentino F, Zava O, Cerrada E, Rigobello MP, Bindoli A, Laguna M, Dyson PJ. Anticancer therapeutics that target selenoenzymes: synthesis, characterization, in vitro cytotoxicity, and thioredoxin reductase inhibition of a series of gold(I) complexes containing hydrophilic phosphine ligands. *Chem Med Chem*. 2010; 5:96–102. [PubMed: 19937669]
14. Maiore L, Cinellu MA, Michelucci E, Moneti G, Nobili S, Landini I, Mini E, Guerri A, Gabbiani C, Messori L. Structural and solution chemistry, protein binding and antiproliferative profiles of gold(I)/(III) complexes bearing the saccharinato ligand. *J Inorg Biochem*. 2011; 105:348–355. [PubMed: 21421122]
15. Zhang X, Frezza M, Milacic V, Ronconi L, Fan Y, Bi C, Fregona D, Dou QP. Inhibition of tumor proteasome activity by gold-dithiocarbamate complexes via redox-dependent and -independent processes. *J Cell Biochem*. 2010; 109:162–172. [PubMed: 19911377]
16. Trani M, Sorrentino A, Busch C, Landström M. Pro-apoptotic effect of aurothiomalate in prostate cancer cells. *Cell Cycle*. 2009; 8:306–313. [PubMed: 19164922]
17. Shaw CF III. Gold-based therapeutic agents. *Chem Rev*. 1999; 99:2589–2600. [PubMed: 11749494]
18. Ho, SY.; Tiekink, ERT. ⁷⁹Au Gold-Based Metallotherapeutics: Use and Potential. In: Gielen, M.; Tiekink, ERT., editors. *Metallotherapeutic Drugs & Metal-based Diagnostic Agents: The Use of Metals in Medicine*. Wiley; New York: 2005. p. 507-528.
19. Crooke ST, Mirabelli CK. Molecular mechanisms of action of auranofin and other gold complexes as related to their biologic activities. *Am J Med*. 1983; 75:109–113. [PubMed: 6419591]
20. Mirabelli CK, Johnson RK, Hill DT, Faucette LF, Girard GR, Kuo GY, Sung CM, Crooke ST. Correlation of the in vitro cytotoxic and in vivo antitumor activities of gold(I) coordination complexes. *J Med Chem*. 1986; 29:218–223. [PubMed: 3081721]
21. Messori L, Marcon G. Gold complexes in the treatment of rheumatoid arthritis. *Met Ions Biol Syst*. 2004; 249:279–304. [PubMed: 15206120]
22. Ward JR. Role of disease-modifying antirheumatic drugs versus cytotoxic agents in the therapy of rheumatoid arthritis. *Am J Med*. 1988; 85:39–44. [PubMed: 3052055]
23. Simon TM, Kunishima DH, Vibert GJ, Lorber A. Cellular antiproliferative action exerted by auranofin. *J Rheumatol Suppl*. 1979; 5:91–97. [PubMed: 226702]
24. Bhabak KP, Bhuyan BJ, Mugesh G. Bioinorganic and medicinal chemistry: aspects of gold(I)-protein complexes. *Dalton Trans*. 2011; 40:2099–2111. [PubMed: 21321730]
25. Rigobello MP, Scutari G, Folda A, Bindoli A. Mitochondrial thioredoxin reductase inhibition by gold(I) compounds and concurrent stimulation of permeability transition and release of cytochrome c. *Biochem Pharmacol*. 2004; 67:689–696. [PubMed: 14757168]
26. de Sa Alves FR, Barreiro EJ, Fraga CAM. From nature to drug discovery: The indole scaffold as a 'privileged structure'. *Mini Rev Med Chem*. 2009; 9:782–793. [PubMed: 19519503]

27. Fuse E, Kuwabara T, Sparreboom A, Sausville EA, Figg WD. Review of UCN-01 development: a lesson in the importance of clinical pharmacology. *J Clin Pharmacol*. 2005; 45:394–403. [PubMed: 15778420]
28. Weng JR, Omar HA, Kulp SK, Chen CS. Pharmacological exploitation of indole-3-carbinol to develop potent antitumor agents. *Mini Rev Med Chem*. 2010; 10:398–404. [PubMed: 20370707]
29. Giroux V, Dagorn JC, Iovanna JL. A review of kinases implicated in pancreatic cancer. *Pancreatol*. 2009; 9:738–754. [PubMed: 20090395]
30. Shaw CF III. The protein chemistry of antiarthritic gold(I) thiolates and related complexes. *Comments Inorg Chem*. 1989; 8:233–267.
31. Partyka DV, Zeller M, Hunter AD, Gray TG. Relativistic functional groups: aryl carbon-gold bond formation by selective transmetalation of boronic acids. *Angew Chem, Int Ed*. 2006; 45:8188–8191.
32. Gao L, Peay MA, Partyka DV, Updegraff JB III, Teets TS, Esswein AJ, Zeller M, Hunter AD, Gray TG. Mono- and di-gold(I) naphthalenes and pyrenes: synthesis, crystal structures, and photophysics. *Organometallics*. 2009; 28:5669–5681.
33. Partyka DV, Updegraff JB III, Zeller M, Hunter AD, Gray TG. Probing the steric limits of carbon-gold bond formation: (Dialkylbiarylphosphine)gold(I) aryls. *Organometallics*. 2009; 28:1666–1674.
34. Harvey AL, Cree IA. High-throughput screening of natural products for cancer therapy. *Planta Med*. 2010; 76:1080–1086. [PubMed: 20635309]
35. Willingham MC. Cytochemical methods for the detection of apoptosis. *J Histochem Cytochem*. 1999; 47:1101–1109. [PubMed: 10449530]
36. Shaw CF 3rd, Isab AA, Coffey MT, Mirabelli CK. Gold(I) efflux from auranofin-treated red blood cells. Evidence for a glutathione-gold-albumin metabolite. *Biochem Pharmacol*. 1990; 40:1227–1234. [PubMed: 2403377]
37. Shaw CF 3rd, Isab AA, Hoeschele JD, Starich M, Locke J, Schulteis P, Xiao J. Oxidation of the phosphine from the auranofin analog, triisopropylphosphine(2,3,4,6-tetra-O-acetyl-1-thio-beta-D-glucopyranosato-S)gold(I), via a protein-bound phosphonium intermediate. *Journal of the American Chemical Society*. 1994; 116:2254–2260.
38. Roberts JR, Xiao J, Schliesman B, Parsons DJ, Shaw CF 3rd. Kinetics and mechanism of the reaction between serum albumin and auranofin (and its isopropyl analogue) in vitro. *Inorg Chem*. 1996; 35:424–433. [PubMed: 11666224]
39. Holmgren A. Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. *Structure*. 1995; 3:239–243. [PubMed: 7788289]
40. Jester BW, Cox KJ, Gaj A, Shomin CD, Porter JR, Ghosh I. A coiled-coil enabled split-luciferase three-hybrid system: applied toward profiling inhibitors of protein kinases. *J Am Chem Soc*. 2010; 132:11727–11735. [PubMed: 20669947]
41. Carpinelli P, Moll J. Aurora kinases and their inhibitors: more than one target and one drug. *Adv Exp Med Biol*. 2008; 610:54–73. [PubMed: 18593015]
42. Miglarese MR, Carlson RO. Development of new cancer therapeutic agents targeting mitosis. *Expert Opin Investig Drugs*. 2006; 15:1411–1425.
43. Katayama H, Sen S. Aurora kinase inhibitors as anticancer molecules. *Biochim Biophys Acta*. 2010; 1799:829–839. [PubMed: 20863917]
44. Carrière A, Cargnello M, Julien LA, Gao H, Bonnell E, Thibault P, Roux PP. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. *Curr Biol*. 2008; 18:1269–1277. [PubMed: 18722121]
45. Kang S, Elf S, Lythgoe K, Hitosugi T, Taunton J, Zhou W, Xiong L, Wang D, Muller S, Fan S, Sun SY, Marcus AI, Gu TL, Polakiewicz RD, Chen ZG, Khuri FR, Shin DM, Chen J. p90 ribosomal S6 kinase 2 promotes invasion and metastasis of human head and neck squamous cell carcinoma cells. *J Clin Invest*. 2010; 120:1165–1177. [PubMed: 20234090]
46. Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, Lassam NJ. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO J*. 1996; 15:7026–7035. [PubMed: 9003778]

47. Patyna S, Laird AD, Mendel DB, O'Farrell AM, Liang C, Guan H, Vojkovsky T, Vasile S, Wang X, Chen J, Grazzini M, Yang CY, Haznedar JO, Sukbuntherng J, Zhong WZ, Cherrington JM, Hu-Lowe D. SU14813: a novel multiple receptor tyrosine kinase inhibitor with potent antiangiogenic and antitumor activity. *Mol Cancer Ther.* 2006; 5:1774–1782. [PubMed: 16891463]
48. Omuro AM. Exploring multi-targeting strategies for the treatment of gliomas. *Curr Opin Investig Drugs.* 2008; 9:1287–1295.
49. Rodon J, Perez J, Kurzrock R. Combining targeted therapies: practical issues to consider at the bench and bedside. *Oncologist.* 2010; 15:37–50. [PubMed: 20080862]
50. Lee JH, Pyon JK, Kim DW, Lee SH, Nam HS, Kim CH, Kang SG, Lee YJ, Park MY, Jeong DJ, Cho MK. Elevated c-Src and c-Yes expression in malignant skin cancers. *J Exp Clin Cancer Res.* 2010; 29:116. [PubMed: 20796316]
51. Koh HJ, Toyoda T, Fujii N, Jung MM, Rathod A, Middelbeek RJ, Lessard SJ, Treebak JT, Tsuchihara K, Esumi H, Richter EA, Wojtaszewski JF, Hirshman MF, Goodyear LJ. Sucrose nonfermenting AMPK-related kinase (SNARK) mediates contraction-stimulated glucose transport in mouse skeletal muscle. *Proc Natl Acad Sci USA.* 2010; 107:15541–15546. [PubMed: 20713714]
52. Zips D, Thames HD, Baumann M. New anticancer agents: *in vitro* and *in vivo* evaluation. *In Vivo.* 2005; 19:1–7. [PubMed: 15796152]
53. Derheimer FA, Kastan MB. Multiple roles of ATM in monitoring and maintaining DNA integrity. *FEBS Lett.* 2010; 584:3675–3681. [PubMed: 20580718]
54. Smith J, Tho LM, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res.* 2010; 108:73–112. [PubMed: 21034966]
55. Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J Cell Biol.* 1999; 146:905–915. [PubMed: 10477747]
56. Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, O'Driscoll M, Jeggo PA. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J.* 2006; 25:5775–82. [PubMed: 17124492]
57. Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Löbrich M, Jeggo PA. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res.* 2007; 64:2390–2396. [PubMed: 15059890]
58. Lillig CH, Holmgren A. Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal.* 2007; 9:25–47. [PubMed: 17115886]
59. Bagowski CP, You Y, Scheffler H, Vlecken DH, Schmitz DJ, Ott I. Naphthalimide gold(I) phosphine complexes as anticancer metallodrugs. *Dalton Trans.* 2009; 48:10799–10805. [PubMed: 20023909]
60. Suryadinata R, Sadowski M, Sarcevic B. Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. *Biosci Rep.* 2010; 30:243–255. [PubMed: 20337599]
61. Finch SC. Radiation-induced leukemia: lessons from history. *Best Pract Res Clin Haematol.* 2007; 20:109–118. [PubMed: 17336261]
62. Chandana SR, Movva S, Arora M, Singh T. Primary brain tumors in adults. *Am Fam Physician.* 2008; 77:1423–1430. [PubMed: 18533376]
63. Hricak H. Cancer of the uterus: the value of MRI pre- and post-irradiation. *Int J Radiat Oncol Biol Phys.* 1991; 21:1089–1094. [PubMed: 1917607]
64. MacMahon B. Epidemiology and the causes of breast cancer. *Int J Cancer.* 2006; 118:2373–2378. [PubMed: 16358260]
65. Ward JF. The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. *Int J Radiat Biol.* 1990; 57:1141–1150. [PubMed: 1971840]
66. Harrison L, Blackwell K. Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? *Oncologist.* 2004; 9:31–40. [PubMed: 15591420]
67. Vaupel P, Kelleher DK, Höckel M. Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. *Semin Oncol.* 2001; 28:29–35. [PubMed: 11395850]
68. Berry SE, Kinsella TJ. Targeting DNA mismatch repair for radiosensitization. *Semin Radiat Oncol.* 2001; 11:300–315. [PubMed: 11677655]

69. Grem JL. 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Invest New Drugs*. 2000; 18:299–313. [PubMed: 11081567]
70. Lawrence TS. Radiation sensitizers and targeted therapies. *Oncology (Williston Park)*. 2003; 17:23–28. [PubMed: 14723003]
71. Lyman GH. Impact of chemotherapy dose intensity on cancer patient outcomes. *J Natl Compr Canc Netw*. 2009; 7:99–108. [PubMed: 19176210]
72. Blanksby SJ, Ellison GB. Bond dissociation energies of organic molecules. *Acc Chem Res*. 2003; 36:255–263. [PubMed: 12693923]
73. Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. *Biotechnol Appl Biochem*. 1999; 29:99–108. [PubMed: 10075906]
74. Ellman GL. A colorimetric method for determining low concentrations of mercaptans. *Arch Biochem Biophys*. 1958; 74:443–450. [PubMed: 13534673]
75. Frisch, MJ.; Trucks, GW.; Schlegel, HB.; Scuseria, GE.; Robb, MA.; Cheeseman, JR.; Montgomery, JA., Jr; Vreven, T.; Kudin, KN.; Burant, JC.; Millam, JM.; Iyengar, SS.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, GA.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, JE.; Hratchian, HP.; Cross, JB.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, RE.; Yazyev, O.; Austin, AJ.; Cammi, R.; Pomelli, C.; Ochterski, JW.; Ayala, PY.; Morokuma, K.; Voth, GA.; Salvador, P.; Dannenberg, JJ.; Zakrzewski, VG.; Dapprich, S.; Daniels, AD.; Strain, MC.; Farkas, O.; Malick, DK.; Rabuck, AD.; Raghavachari, K.; Foresman, JB.; Ortiz, JV.; Cui, Q.; Baboul, AG.; Clifford, S.; Cioslowski, J.; Stefanov, BB.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, RL.; Fox, DJ.; Keith, T.; Al-Laham, MA.; Peng, CY.; Nanayakkara, A.; Challacombe, M.; Gill, PMW.; Johnson, B.; Chen, W.; Wong, MW.; Gonzalez, C.; Pople, JA. Gaussian 03, Revision D.01. Gaussian, Inc; Wallingford CT: 2004.
76. Becke AD. Density-functional exchange-energy approximation with correct asymptotic behavior. *Phys Rev A*. 1988; 38:3098–3100. [PubMed: 9900728]
77. Lee C, Yang W, Parr R. Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys Rev B*. 1988; 37:785–789.
78. Godbout N, Salahub DR, Andzelm J, Wimmer E. Optimization of gaussian-type basis sets for local spin density functional calculations. Part I Boron through neon, optimization technique and validation. *Can J Chem*. 1992; 70:560–571.
79. Dolg M, Wedig U, Stoll H, Preuss H. Energy-adjusted ab initio pseudopotentials for the first row transition elements. *J Chem Phys*. 1987; 86:866–872.
80. Miertus S, Scrocco E, Tomasi J. Electrostatic interaction of a solute with a continuum. A direct utilization of ab initio molecular potentials for the prevision of solvent effects. *Chem Phys*. 1981; 55:117–129.

Figure 1.

(A) Model describing the potential anti-cancer effects of (phosphine)gold(I) indoles. The indole scaffold is a mimic of purine that can interact weakly with ATP-binding proteins, most notably kinases. The inclusion of the phosphine gold(I) ligand can influence the chemical space of this minimal scaffold to increase potency and/or generate selectivity for various targets (*i*). In addition, the gold molecule can irreversibly react with selected amino acids (cysteine, selenocysteine, and/or histidine) to inactivate potential therapeutic targets (*ii* and/or *iii*). In all cases, inhibition of these potential targets can create a variety of downstream effects leading to beneficial cytostatic and/or cytotoxic effects. (B) Synthesis of (phosphine)gold(I) indoles. See text for complete details.

Figure 2.

(A) Dose-dependent effects of compound **3** against the adherent cancer cell line, HeLa, demonstrate its anti-cancer activity. Data were fit to the equation: $y = 100\% / [1 + (IC_{50}/[(3))]$ to yield an IC_{50} of $2.5 \pm 0.1 \mu M$ (B) Microscopy images using propidium iodide (PI) uptake and annexin V staining in HeLa cells treated with DMSO, 50 μM compound **3**, and 50 μM compound **4** for 12 and 24 hours, respectively. (C) Quantitative flow cytometry analyses using propidium iodide (PI) uptake and annexin V staining in HeLa cells treated with DMSO, 50 μM compound **3**, and 50 μM compound **4** after 24 hours. (D) Cell cycle analyses of HeLa cells treated with DMSO, 50 μM compound **3**, and 50 μM compound **4** after 24 hours.

Figure 3.

Inhibition of thioredoxin reductase activity by Au(I)-indoles. Experiments were performed by adding 600 nM TrxR to a preincubated solution containing 100 mM potassium phosphate, pH 7.0, 10 mM EDTA, 5 mM DTNB, 0.2 mg/mL BSA, 240 μ M NADPH in the absence (●) or presence of 40 μ M compound **3** (◇), 40 μ M compound **4** (◆), or 1 μ M BrAuPPh3 (□). The background rate in DTNB reduction (○) was determined by performing identical reactions in the absence of TrxR and Au(I)-containing compound.

Figure 4.

(A) Plot of the survival fraction versus dose of IR exposure for HeLa cells in the absence (■) or presence of 4 μ M compound 3 (●) or 7 μ M compound 4 (○). (B) γ H2AX foci formation for HeLa cells treated with DMSO and 2 Gy IR after 30 minutes (left panel) or 4 hours (right panel). Histograms for HeLa cells treated with 2 Gy of IR under the following conditions: (C) DMSO, 30 minutes post-IR exposure; (D) DMSO, 4 hours post-IR exposure; (E) compound 4 (7 μ M), 30 minutes post-IR exposure; (F) compound 4 (7 μ M), 4 hours post-IR exposure; (G) compound 3 (4 μ M), 30 minutes post-IR exposure; and (H) compound 3 (4 μ M), 4 hours post-IR exposure.

Figure 5.

Proposed models for the enhancement of IR-induced cytotoxicity by compounds **3** and **4**.

(A) Under normal conditions, exposure to IR induces DNA damage that can be repaired through activation of ATM, ATR, and DNS-PK. (B) Compound **3** inhibits DNA repair by directly or indirectly blocking the phosphorylation of H2AX. Reductions in γ H2AX foci formation leads to a reduction in DNA repair. (C) Compound **4** inhibits the repair of DSBs through mechanisms independent of γ H2AX foci formation. See text for further details.

$$\Delta G^\circ = 57.6 \text{ kcal mol}^{-1}$$

UBLYP/TZVP calculations

Figure 6. Homolytic bond dissociation energy of a model Au(I) indole. See text for details.

Table 1

Summary of IC₅₀ values for (phosphine)gold(I) indoles against adherent and systemic cancer cell lines^a.

Compound	HeLa (μM)	MCF-7 (μM)	HCT116 (μM)	CEM (μM)	Selectivity Factor ^b
3	2.5 +/- 0.1	16.2 +/- 0.1	11.5 +/- 0.1	19.9 +/- 0.1	1.2-8.0
4	16.2 +/- 0.1	36.4 +/- 0.1	22.8 +/- 0.1	9.7 +/- 0.1	0.3-0.6
5	2.4 +/- 0.1	2.3 +/- 0.1	3.4 +/- 0.1	2.1 +/- 0.1	0.6-0.9
6	0.66 +/- 0.05	0.96 +/- 0.02	1.8 +/- 0.1	0.42 +/- 0.10	0.2-0.6
7	0.46 +/- 0.01	3.25 +/- 0.05	0.38 +/- 0.03	1.43 +/- 0.05	0.4-3.8

^a Assay were performed as described in Methods and Materials. IC₅₀ values were obtained using a non-linear regression curve fit of the data to $y = 100\%/[1 + (IC_{50}/Inhibitor)]$ where y is the fraction of viable cells, IC₅₀ is the concentration of inhibitor that inhibits 50% cell growth, and Inhibitor is the concentration of compound tested. All values represent an average of at least three (3) independent determinations performed on different days.

^b Selectivity factor is defined as the ratio of IC₅₀ values measured against leukemia cells (CEM-C7) versus adherent cells (SF = IC₅₀ Leukemia/IC₅₀ Adherent Cells). Values greater than 1 indicate that the anti-cancer effects are more selective for adherent cells compared to the leukemia cell line.

Table 2

Summary of the effects of treating HeLa cells with (phosphine)gold(I) indoles.

Compound	Live (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic (%)
DMSO	94.8 +/- 0.6	1.5 +/- 0.1	0.10 +/- 0.02	3.6 +/- 0.4
3^a	84.4 +/- 3.0	8.8 +/- 0.7	4.3 +/- 1.8	2.4 +/- 0.8
4^b	68.4 +/- 0.8	5.3 +/- 1.1	8.1 +/- 1.8	18.8 +/- 2.5

^a A concentration of 50 μ M was used which is 20-fold higher than the IC₅₀ value reported in Table 1. All values represent an average of at least three (3) independent determinations performed on different days.

^b A concentration of 50 μ M was used which is 3-fold higher than the IC₅₀ value reported in Table 1. All values represent an average of at least three (3) independent determinations performed on different days.

Table 3Summary of the effects on cell-cycle progression by (phosphine)gold(I) indoles.^a

Compound	G1 (%)	S-Phase (%)	G2/M (%)
DMSO	45.6 +/- 0.1	43.6 +/- 0.1	10.8 +/- 0.1
3 ^b	45.4 +/- 2.1	41.6 +/- 0.1	13.0 +/- 2.1
4 ^c	60.3 +/- 0.8	28.3 +/- 1.3	11.4 +/- 4.9

^aHeLa cells were used in these experiments. Cells were analyzed 24 hours post-treatment. All values represent an average of at least three (3) independent determinations performed on different days.

^bThe concentration of 50 μ M used in this experiment is 20-fold higher than its IC₅₀ value.

^cThe concentration of 50 μ M is 3-fold higher than its IC₅₀ value.

Table 4

Summary of the inhibitory effects of (phosphine)gold(I) indoles on a panel of various human kinases.^a

Kinase	Compound 3 (% Inhibition)	Compound 4 (% Inhibition)	Kinase	Compound 3 (% Inhibition)	Compound 4 (% Inhibition)
AKT1	0	0	MARK3	6.5	34
AKT2	25.8	33.6	MET	15.1	23.3
AKT3	0	0	MLK1	32.5	42.2
AMPK- α 1	0	0	MLK3	31.2	43
AMPK- α 2	0	0	MST2	0	0
AURKA	26.7	41.7	P38- γ	0	0
AURKB	11.1	24.5	PAK1	0	0
AURKC	23.9	35.0	PDGFRB	8.1	15.2
BLK	0	0	PDK1	17.4	24.3
CAMK1	0	0	PIM1	0	10.6
CAMK1D	0	0	PIM2	0	8.7
CAMK1G	0	0	PKAC- α	0	12.4
CAMK2B	0	0	PKC- ϵ	0	5.9
CAMK2D	0	0	PKC- δ ;	0	3.8
CAMKK1	0	0	PKC- η	3.2	0
CAMKK2	0	7.9	PRKD2	0	3.8
CHEK1	0	0	PKG1	5.4	3.2
CLK1	0	0	RPS6KA1	0	0
CLK2	0	0	RPS6KA3	16.7	34.9
DDR2	0	0	RPS6KA4	1.0	21.8
FGFR2	0	0	RPS6KA5	0	5.0
FLT1	0	9.4	SNF1LK	0	40.6
FLT2	0	0	SNF1LK2	0	32.9
FLT3	0	0	SLK	0	19.6
FYN	0	1.2	SNARK	0	39.5
GSK3 α	0	5.8	SRC	14.3	5.2
IGF1R	0	4.7	SYK	21.6	40.7

Kinase	Compound 3 (% Inhibition)	Compound 4 (% Inhibition)	Kinase	Compound 3 (% Inhibition)	Compound 4 (% Inhibition)
ITK	0	3.1	TNK2	0	2.2
LYN	0	0	VEGFR2	0	5.8
MARK1	5.1	11.9	YES1	0	31.7
MARK2	18.9	26.2	YSK1	0	20.9

^aThe concentration of compounds 3 and 4 were maintained fixed at 10 μ M. Assays were performed as described.⁴⁰