ANTICANCER RESEARCH *34*: 487-492 (2014)

Review

Beyond Platinums: Gold Complexes as Anticancer Agents

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Abstract. *The accidental discovery of the anticancer properties of cisplatin in the mid-1960s triggered the development of alternative platinum-based drugs. However, the platinum-based treatment of tumor diseases is massively hampered by severe side-effects and development of resistance. Sulfur-containing biomolecules play a significant role in platinum anticancer chemotherapy because of their high affinity to the platinum(II) ion. Sulfur is involved in the entire metabolic processing of platinum drugs. Strong and irreversible binding of cisplatin to intracellular thiolato ligands is considered a major step of inactivation, and reactions with sulfur donors in proteins are believed to affect enzymatic processes. Consequently, the development of novel metal-based compounds with a pharmacological profile different from that of clinically-established platinum drugs is a major goal of modern medicinal chemistry and drug design. Among the non-platinum antitumor agents, gold(III) complexes have recently gained increasing attention due to their strong tumor cell growth-inhibiting effects, generally achieved by exploiting non-cisplatin-like mechanisms of action. The real breakthrough is not simply the use of gold compounds to treat cancer, but the rational design of gold-based drugs which may be very effective, non-toxic and potentially selective towards cancer cells, their potential impact relying on the possible site-specific delivery in localized cancer, thus strongly improving cellular uptake and minimizing unwanted side-effects. Cancer cells are known to overexpress specific proteins and receptors needed for tumor growth. Among them, two integral plasma membrane proteins mediate the cellular uptake of di- and tripeptides and peptide-like drugs. They are present predominantly in epithelial cells of the small intestine, bile duct, mammary glands, lung, choroid plexus, and kidney but are also localized in other tissues and are up-regulated in some types of tumors. Accordingly, we have been designing gold(III)-peptide dithiocarbamato derivatives which combine both the antitumor properties and reduced side-effects of the previously reported gold(III) analogues with enhanced bioavailability and tumor selectivity achieved by exploiting peptide transporters. Our compounds showed interesting cytotoxic properties towards a number of cancer cell lines in vitro and in vivo on xenograft models, together with negligible organ and acute toxicity. With respect to their mechanisms of action, we identified mitochondria and proteasome as major in vitro and in vivo targets. These results allowed the filing of an international patent for the use of gold(III) peptidomimetics in cancer chemotherapy, as well as providing a solid starting point for them to enter phase I clinical trials in a few months.*

Despite the majority of chemotherapeutics currently employed in anticancer therapy being organic molecules, an important and promising class of drugs is nowadays represented by metal-based compounds. After the approval of cisplatin [$Pt^{II}Cl_2(NH_3)_2$] by the Food and Drug Administration (FDA) in 1978 and its great success in treating genitourinary cancer, a great effort was put into the development of second- and third-generation analogs to overcome the well-known limitations related to this therapy (1). Cisplatin has become one of the best-selling anticancer drugs in the world, although its administration induces the onset of severe side-effects (mainly represented by nephro-, oto- and neurotoxicity, as a consequence of the high affinity of platinum center for sulfur-containing biomolecules) and is poorly selective towards cancerous cells. Cisplatin is currently used for the treatment of testicular cancer, is one of the most effective drugs against melanoma and non-small cell lung carcinoma and, administered in combination with other therapeutics, it is considerably active against ovarian cancer (2). Moreover, the narrow spectrum of action and the induction of resistance in some cancer types (intrinsic or acquired after few cycles of therapy) encouraged researchers to design new metal complexes, either based on platinum, or on other transition metals, aimed at improving the selectivity

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Key Words: Cancer, gold, metal complexes, proteasome, apoptosis, inorganic compounds, review.

toward cancerous cells and reducing the side-effects (3). Therefore, in the past decade, a large number of metal-based compounds were synthesized and tested, each one characterized by different physicochemical properties and biological behavior, achieved by exploiting the peculiarities of various metal oxidation states and different ligands (4, 5).

Our research group focused particularly on the design, synthesis and characterization of several innovative metal complexes (based on Pt, Pd, Zn, Cu, Ru and Au) functionalized with different dithiocarbamato ligands. In the past, dithiocarbamates (in their free form) were clinically administered a few hours after cisplatin for, resulting in lowering of intrinsic toxicity and hampering the resistance mechanisms in cancer cells. In particular, dithiocarbamates were proved to reduce renal failure, either preventing platinum from strongly and irreversibly binding to intracellular renal sulfur-containing enzymes, or reversing platinum–sulfur bonding (6). Thus, the choice of associating a gold(III) metal ion with a chelating dithiocarbamato ligand, has allowed us to successfully combine the metal center with antitumor activity with the chemoprotective action of the sulfur-containing moiety.

In the second half of the last century, some gold (I) complexes entered clinical use (e.g., Auranofin was FDA-approved in 1985) for the treatment of rheumatoid arthritis, due to their immunosuppressive and anti-inflammatory properties (7). Moreover, gold in the oxidation state +3 shares with platinum(II) the same d^8 electronic configuration, thus reflecting similar physicochemical and geometrical properties. The connection between cancer and inflammation, along with the aforementioned chemical features made gold(III) complexes suitable candidates for testing as anticancer compounds.

Among other metal complexes, our gold(III)-dithiocarbamato derivatives stand out as very promising anticancer agents, with fine-tuned stability, good antiproliferative activity, and encouraging selectivity associated with lower toxicity towards healthy tissues (8, 9). First-generation complexes of the type $[Au^{III}X_2(L)]$ (X=Br, Cl; L=various dithiocarbamates) were synthesized and tested for anti-proliferative activity *in vitro* on different cancer cell lines and *in vivo* on xenografts, always showing anti-proliferative activity and tumor growth inhibition greater than that of cisplatin and no cross-resistance to the reference drug, clearly highlighting a different mechanism of action (6). In particular, unlike cisplatin, the main intracellular targets were detected in proteasome, the thioredoxin system and mitochondria (10, 11). Remarkably, the higher activity was coupled with lower toxicity towards normal tissues, as confirmed both by lethal dose 50 (LD_{50}) values (three-times higher than that recorded for the reference drug cisplatin) and by histopathological analysis on mice organs excised from animals treated with gold(III)-dithiocarbamate complexes. No metal accumulation or tissue lesions were detected in any of the examined organs, and the urine biomarkers for kidney damage (monitored after injection of different single doses of complex) highlighted no alteration of normal renal function (12). This was consistent with the rapid excretion of gold from the animals, which occurs mainly through feces (>89%) within 48 h and only about 10% *via* urinary system.

Present and Future Perspectives

Based on the encouraging results obtained for the first-generation compounds, we focused our attention on improving their bioavailability and their cellular uptake, exploiting the possibility of functionalizing the dithiocarbamato moiety with small oligopeptides to be recognized by specific transporters. Since cancerous cells are known to overexpress specific biomarkers and receptors needed for carcinogenesis and tumor growth, targeted chemotherapies actually aim to block cancer cells proliferation exploiting such specific up-regulated biomolecules. This led to the development of efficient and innovative delivery systems in which conjugated drugs incorporating a tumor-targeting group can selectively reach the tissue of interest and deliver the cytotoxic agent directly into the tumor cells (13). In particular, specific peptide transporters (PEPTs) were selected as potential targets for our compounds. They are integral membrane proteins expressed in mammals in two different isoforms, PEPT1 and PEPT2, which are known to promote cellular uptake of potentially all physiologically-occurring di- and tripeptides with different sequence, charge and hydrophobicity (14). They are present predominantly in epithelial cells of small intestine, bile duct, mammary glands, lung, choroid plexus and kidney, but are also localized in other tissues (such as pancreas, liver, gastrointestinal tract) and are up-regulated in different types of cancer (15). Owing to their specificity in recognition and transport, they represent an excellent target for the delivery of some pharmacologically active peptidomimetics [*e.g.* β-lactam antibiotics and angiotensin-converting-enzyme (ACE) inhibitors], and might also have a central role in the uptake of our gold(III) compounds (16). Indeed, we recently designed and reported on the synthesis, characterization and biological evaluation of new gold(III)-dithiocarbamato complexes functionalized with short oligopeptides, of the type $[Au^{III}X_2$(dct-Sar-AA)] (X=Br, Cl; Sar=sarcosine, *N*-methylglycine; AA=different amino acids). Among the library of tested compounds, three complexes (Figure 1) with greater *in vitro* antiproliferative activity were selected, namely AuD6 $[Au^{III}Br_2$(dct-Sar-Gly-OtBu)], AuD8 $[Au^{III}Br_2$(dct-Sar-Aib-OtBu)] and AuD9 $[Au^{III}Cl_2$(dct-Sar-Gly-OtBu)]. These complexes were tested on a large panel of cancer cell lines representatives of different cancer types, recording IC_{50} in the low micromolar range, with values up to about four-times

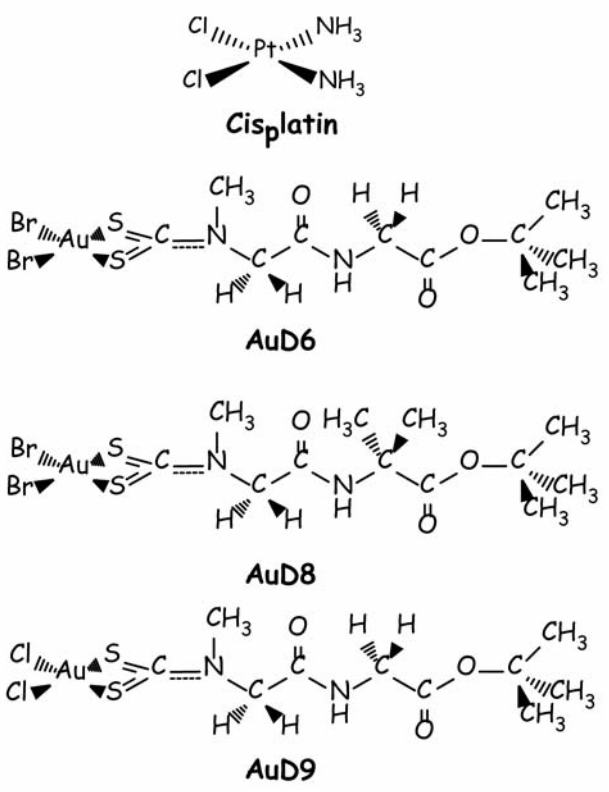

Figure 1. *Chemical structure of cis-diaminodichloroplatinum(II) (cisplatin) and three of our gold(III) peptidomimetics (32): dibromido[1-(1,1-dimethylethyl)N-dithiocarboxy-κS,κS'-N-methylglycylglycinato]gold-(III) (AuD6), dibromido[1-(1,1-dimethylethyl)N-dithiocarboxy-κS,κS')-N-methylglycyl-2-methylalaninato]gold(III) (AuD8) and dichlorido[1-(1,1-dimethylethyl)N-dithiocarboxy-κS,κS')-N-methylglycylglycinato]gold-(III) (AuD9).*

lower than the reference drug cisplatin, showing no cross-resistance with cisplatin (17, 18).

After this preliminary screening was carried out on several human tumor cell lines, we decided to perform a deeper biochemical evaluation in order to better-understand their biological activity. Thus, we have taken into account two specific types of adenocarcinoma, namely human triple-negative breast cancer (TNBC) and two androgen-resistant types of cancer, breast and prostate cancer these being among the most frequent causes of tumor death in women and men, respectively (19).

In particular, for further in-depth studies, we exploited the breast cancer MDA-MB-231 cell line and PC3 and DU145 prostate cancer cell lines. The choice of these tumor subtypes was not accidental. In fact, the MDA-MB-231 cells belong to the so-called TNBC subtype as they do not express the genes for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu), thus preventing the use of traditional hormonal or monoclonal antibody therapies (20, 21). Furthermore, these cells are highly metastatic, invasive and resistant to cisplatin, and lead patients to have frequent and early gastrointestinal metastases, with adverse prognosis.

Regarding the PC3 and DU145 prostate cancer cell lines, it is worth noting that neither is androgen-responsive, thus ruling out the benefits of androgen ablation in the early stages of the disease. Therefore, these types of androgen-independent tumors require alternative therapeutic strategies (22).

For all the cell lines, we performed a number of biological tests aimed at elucidating the mechanism of action and identifying the major targets of our gold(III) compounds (23).

As far as the epithelial breast adenocarcinoma is concerned, the complexes AuD6 and AuD8 (Figure 1) were tested. First, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to assess their *in vitro* cytotoxicity after 24 or 72 h treatment. Both complexes inhibited tumor cell growth in a dose-dependent manner, AuD8 being about three times more potent than AuD6,with IC_{50} values in the low micromolar range. It should be noted that MDA-MB-231 cells were resistant to cisplatin under the same experimental conditions. In fact, after 24 h treatment, the IC_{50} value was not reached using concentrations ranging from 25 to 100 μM, in agreement with literature data. After 72-h treatment with the reference drug at 100 μM, about 23% cell viability was recorded, in agreement with the awareness that its antitumor activity occurs slowly (23).

In the past 10 years, after FDA approval of bortezomib (N-acyl-dipeptidyl boronic acid; Velcade®) in 2003 for the treatment of relapsed multiple myeloma (MM) and mantle cell lymphoma, several families of organic and inorganic compounds were tested as proteasome inhibitors (5, 24). The 26S proteasome is an ATP-dependent multicatalytic protease, playing a role of paramount importance in cytoplasmic and nuclear homeostasis *via* hydrolysis of a large collection of proteins previously labeled with poly-ubiquitin chains. The ubiquitin-proteasome system (UPS) is indeed involved in several intracellular processes including the removal of abnormal, misfolded or improperly assembled proteins, cell cycle progression, apoptosis, drug resistance, cell differentiation, angiogenesis and stress response (25). From the structural point of view, 26S proteasome is made up of a central barrel-shaped complex termed the 20S proteasome (consisting of 28 subunits), capped at each end by a regulatory component referred to as 19S complex. 19S units recognize ubiquitinated client proteins and regulate their unfolding and translocation into the 20S proteasome proteolytic chamber. Eukaryotic 20S core contains only three proteolytically active subunits (double present and placed within the complex according to a C2 symmetry), namely β1, β2 and β5, which recognize distinguishing side chains of different peptides, thus resulting in specific activity for each catalytic site (peptidyl glutamyl peptide hydrolyzing-like, trypsin-like and chymotrypsin-like, respectively).

Interestingly, our compounds were shown to trigger cell death *via* a different mechanism of action if compared to clinically-established platinum drugs as they exhibited proteasome-inhibitory activity both *in vitro* and *in vivo* (23). Tumor cell proteasome targeting is critical as the homeostasis of the UPS is unbalanced and some regulatory proteins either circulate longer due to decreased degradation (*e.g.* anti-apoptotic proteins) or lost due to accelerated degradation (*e.g.* pro-apoptotic proteins).

In order to elucidate to what extent cellular proteasome is inhibited by our compounds, and to provide insights into the mechanism of action and cell death type, we carried out dose-dependent and time-dependent studies. MDA-MB-231 cells were treated with either i) AuD6 or AuD8 for 24 h at different concentrations (5, 10, 15 and 20 μM), or ii) each complex at 20 μM for 4, 8, 16 or 24 h. Overall, upon western blot analysis of the cell extracts, we observed accumulation of ubiquitinated proteins, the proteasomal target proteins IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and p27 (pro-apoptotic), thus highlighting proteasome inhibition *in vitro*. Unlike AuD6, treatment with AuD8 also led to caspase-3 activation, poly (ADP-ribose) polymerase (PARP) cleavage and increased levels of the pro-apoptotic form of Bcl-2-associated X protein (BAX) (p36) with consequent decrease or complete disappearance of p21/BAX and p18/BAX fragments. AuD6 was less potent than AuD8, in agreement with its higher IC_{50} value. Indeed, we observed bands as intense as the control for some proteins, while weaker effects on the apoptotic proteins PARP and caspase-3 were detected after treatment for longer times, or at higher concentrations (23).

Based on these results obtained *in vitro*, we addressed our attention on cell-free systems, investigating the effects of both compounds on the proteasomal chymotrypsin (CT)-like, trypsin (T)-like and peptidyl-glutamyl-peptide-hydrolizing (PGPH)-like activities of MDA-MB-231 cell extracts. Both complexes were able to inhibit all the three enzymatic activities in a concentration-dependent manner (23). Thus, we incubated purified human 20S proteasome with each compound at different concentrations in order to provide direct evidence for this inhibition activity. AuD8 seemed to be slightly more selective toward the CT-like activity of the purified proteasome core. In this context, it has been reported that chymothrypsin-like pocket targeting is associated with apoptosis induction in cancerous cells (26). In terms of proteasome inhibition, both complexes proved to be about an order of magnitude more potent than the first generation compounds (10, 27).

The previously described western blot analysis highlighted that our gold agents seem to induce cell death *via* apoptosis. To confirm this type of cell death commitment, we carried out an Annexin V/Propidium Iodide assay. It should be noted that the amount of cells undergoing non-apoptotic cell death was comparable to the solvent control. After 16h treatment with AuD8 at 20 μM, cell death occurred mainly by apoptosis (62.9%, late stage) and this population of cells further increased after 24 h (74.1%). Both compounds caused similar percentages of cells in early-stage apoptosis (about 7-9%), with AuD6 being less potent than AuD8, triggering apoptosis at the late stage in only 49% of cells after 24 h treatment (23).

Subsequently, we investigated the antiblastic activity of both complexes *in vivo* exploiting female athymic nude mice bearing human breast cancer MDA-MB-231 cells. Unexpectedly, our gold complexes showed similar antiproliferative activity on this xenograft breast cancer model. In fact, contrary to the results collected *in vitro*, AuD6 turned out to be as active as AuD8 both in the short- (13 days) and in the long-term (27 days) treatment at 1.0 mg kg^{-1} d^{-1}, thus highlighting the importance of animal models when considering the various factors involved in the tumor tissue physiology. In this study, we observed 53% inhibition of xenograft growth compared to control after 27 days, while after a short-term treatment some mice displayed 85% inhibition and, in some cases, tumor shrinkage. Remarkably, mice looked healthy and very active during the treatment showing neither signs of fatigue nor weight loss, thus ruling-out the onset of systemic toxicity (23).

Tumors were collected and weighed, followed by western blot analysis after two weeks and after 27 days of treatment. Our compounds showed similar levels of p27 expression and PARP cleavage in both the short- and the long-term treatments. Conversely, AuD8 was slightly more potent than AuD6 toward accumulation of IκBα and Bax fragmentation. To validate the apoptosis-inducing and proteasome-inhibitory activities *in vivo*, we performed two immunohistochemical stainings after 27 days of treatment, showing enhanced p27 levels in both AuD6- and AuD8-treated xenografts, thus indicating proteasome inhibition, and increased TUNEL positivity, representing apoptotic cell death *in vivo* (23).

Regarding the PC3 and DU145 androgen-resistant prostate adenocarcinomas (28), the cytotoxic effect of two among the most active gold(III) peptidodithiocarbamato derivatives, AuD8 and AuD9 (Figure 1), was evaluated *in vitro* after 72 h treatment considering cisplatin as the reference drug. Additionally, cell growth recovery experiments were performed to check whether tumor cells are able to proliferate again upon gold compound removal. In particular, following 24-h incubation with increasing concentrations of both derivatives, the medium was replaced with drug-free one and cells were incubated for further 72 h, recording IC_{50} values comparable to those observed after 72 h treatment (in the low-micromolar range). These results pointed out that the cytotoxic activity is not reversed, thus ruling-out a cytostatic effect of our compounds. Notably, both complexes showed higher anticancer activity than cisplatin, with IC_{50} values about 2- to 4-fold lower than the reference drug against these androgen receptor-negative prostate cancer cells.

Afterwards, the biological investigation was focused on PC3 cells as they are more invasive and more resistant to chemotherapy than DU145 cells (28).

Based on an annexin-V/PI (propidium iodide) staining, our complexes caused cell death by inducing apoptosis after 24 h incubation at 10 μM (for both compounds about 60% early apoptosis and about 20% late apoptosis were detected), with cisplatin inducing no death effect under the same experimental conditions. Since BCL-XL, BCL-2 and BAX proteins have a critical role in regulating the intrinsic apoptotic pathway, we analyzed whether our complexes are able to affect their expression. Thus, we treated PC3 cells for 24 h with either AuD8/AuD9 or cisplatin (10 μM). Both gold complexes significantly reduced the expression of the pro-survival BCL-XL protein while increasing the levels of the pro-apoptotic BAX protein. However, only AuD8 treatment led to down-regulation of the expression of the anti-apoptotic BCL-2 protein. Under these experimental conditions, cisplatin reduced BCL-XL expression but had no effect on BAX and BCL-2 counterparts (28).

Moreover, cell-cycle progression and DNA fragmentation were evaluated by PI staining and flow cytometric analysis. After 12 h of incubation, both complexes caused an increase of the S phase population, with comparable losses (with respect to the control) from both the G_2/M and G_0/G_1 phases. Interestingly, an S phase population comparable to that of the control (upon percentage reduction with respect to 12 h incubation) and a block in G_0/G_1 step, due to a loss of the G_2/M phase population with respect to the medium, were detected after 24 h. Such a situation (*viz*. G_0/G_1 block) remained steady, with comparable percentages in the different phases, after 48 and 72 h incubation. DNA fragmentation was significant (about 20%) only after 48 or 72 h treatment. Conversely, after 72 h of incubation, an equivalent concentration of cisplatin caused a blockade in the G_2/M phase of the cell cycle, with no significant DNA fragmentation, thus suggesting a different mechanism of action for our compounds compared to Pt-based drugs (28).

It has been reported that the epidermal growth factor receptor (EGFR) is overexpressed in androgen-refractory and metastatic prostate cancer. Furthermore, EGFR phosphorylation affects the migration and proliferation of prostate cancerous cells (29). Contrary to cisplatin, treatment of PC3 cells with our compounds (at 10 μM for 24 h) resulted in a remarkable decrease of the surface expression of EGFR and its phosphorylated form (pEGFR) as evaluated by flow cytometry. The effect of both complexes on PC3 cells migration was then studied by means of a 12- and 24-h cell scratch assay (30). Treatment with each gold complex at 4 μM for 24 h reduced PC3 cell migration rate measured as the area of the scratch covered by the migrating cells after 12 and 24 h by imaging software, resulting in failure to form a confluent monolayer (28).

Remarkably, similarly to the previously described breast cancer cells and xenografts (10, 23, 27), our gold(III) derivatives also significantly inhibited proteasome activity in PC3 cells (analysis of cytosolic extracts after treating cells at 10 μM for 12 h at 37˚C). Under the same experimental conditions, cisplatin was much less active than both compounds (28).

Similarly to breast cancer described above, we investigated the anti-neoplastic activity of AuD8 against prostate tumor xenografts in nude mice. Following *s.c*. injection of 2 mg/kg AuD8 every other day for 19 days of treatment, we observed a reduction of 70% in tumor volume with neither decreased activity nor weight loss, contrary to mice treated with cisplatin. Furthermore, no organ damage was histologically-detected in the spleen, liver and kidney of animals except for the observation of focal lung lymphocyte infiltration (28).

Recently, we have been investigating acute toxicity in 80 mice by two administration routes (endovenous and oral) taking into account different doses, much higher than those used in chemotherapy (xenografts treatment at only 1-2 mg/kg). So far, we have recorded outstanding results (unpublished data). To sum up, no deaths, nor signs of toxicity have been observed during the study in any of the treated animals. There were no clear clinical findings in any of the survivors. It is worth highlighting that body weight for any mouse was constant or increased during the 14-day observation period. On necropsy, no alterations or gross anatomical findings in the main organs and tissues were observed.

In conclusion, the selected gold(III)-dithiocarbamato complexes were shown to be able to inhibit the proliferation of each studied cancer cell line, both *in vitro* and *in vivo*. We have herein shed some light on the mechanisms of action and the biological targets of our gold(III) compounds. In spite of initial skepticism of the scientific community, proteasome inhibition has been emerging as a winning strategy to treat malignancies and we have here reported that our compounds proved to be proteasome inhibitors, both *in vitro* and *in vivo*, in two distinct human tumor cell lines.

Overall, the anticancer activity and toxicological data described highlight that the design of our potential drugs is very successful both in stabilizing the transition metal center and in avoiding the non-specific reactivity of each complex towards healthy tissues (4, 6, 31).

At present, we are completing a transcriptomic study on MDA-MB-231 and MCF7 breast cancer cells upon treatment with two compounds in order to determine differentially expressed genes and pathways.

All the obtained results are very encouraging and account for the interest in ongoing advanced pre-clinical studies on these compounds, evaluating the possibility for them to approach phase I clinical trials (32).

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Received September 20, 2013
Revised December 3, 2013
Accepted December 4, 2013