Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent antitumor activity

James Turkson,1,4 Shumin Zhang,1,4 Jay Palmer,3,7,8 Heidi Kay,8 Joseph Stanko,3,7 Linda B. Mora,1,4 Said Sebti,3,4,5 Hua Yu,2,4 and Richard Jove1,4,5,6

1Molecular Oncology, 2Immunology and 3Drug Discovery Programs, H. Lee Moffitt Cancer Center & Research Institute; and Departments of 4Interdisciplinary Oncology, 5Biochemistry and Molecular Biology, 6Pathology, 7Chemistry, and 8College of Public Health, University of South Florida, Tampa, Florida

Abstract
DNA-alkylating agents that are platinum complexes induce apoptotic responses and have wide application in cancer therapy. The potential for platinum compounds to modulate signal transduction events that contribute to their therapeutic outcome has not been extensively examined. Among the signal transducer and activator of transcription (STAT) proteins, Stat3 activity is frequently up-regulated in many human tumors. Various lines of evidence have established a causal role for aberrant Stat3 activity in malignant transformation and provided validation for its targeting in the development of small-molecule inhibitors as novel cancer therapeutics. We report here that platinum-containing compounds disrupt Stat3 signaling and suppress its biological functions. The novel platinum (IV) compounds, CPA-1, CPA-7, and platinum (IV) tetrachloride block Stat3 activity in vitro at low micromolar concentrations. In malignant cells that harbor constitutively activated Stat3, CPA-1, CPA-7, and platinum (IV) tetrachloride inhibit cell growth and induce apoptosis in a manner that reflects the attenuation of persistent Stat3 activity. By contrast, cells that do not contain persistent Stat3 activity are marginally affected or are not affected by these compounds. Moreover, CPA-7 induces the regression of mouse CT26 colon tumor, which correlates with the abrogation of persistent Stat3 activity in tumors. Thus, the modulation of oncogenic signal transduction pathways, such as Stat3, may be one of the key molecular mechanisms for the antitumor effects of platinum (IV)–containing complexes. [Mol Cancer Ther 2004;3(12):1533–42]

Introduction
Cellular responses to growth factors and cytokines are characterized by the activation of the signal transducer and activator of transcription (STAT) family of cytoplasmic transcription factors (1–10). STATs are activated by tyrosine phosphorylation, which is induced by protein tyrosine kinases of growth factor receptors and receptor-associated cytoplasmic kinases, such as the Janus-activated kinase (Jak) or Src kinase families. This in turn allows phosphotyrosine (pTyr)-SH2 interactions between two STAT monomers, and the formation of dimers, which then translocate to the nucleus. In the nucleus, active STATs bind to specific DNA response elements and regulate the expression of genes essential for cell proliferation, differentiation, development, and survival.

Normal physiologic STAT activation is tightly regulated and has a short duration, which is in keeping with the cellular requirements for mounting a response to external stimuli. However, persistent activation of specific STAT proteins, particularly Stat3 and Stat5, occurs with high frequency in a wide range of tumors (11–21). Furthermore, compelling evidence supports persistently active Stat3 as having a causal role in malignant transformation by promoting growth and survival of cells. This has been observed in solid and hematologic cancers, including breast and prostate cancers, head and neck squamous carcinoma, lymphomas and leukemias [reviewed in (refs. 22–29)]. Of clinical importance is the observation that the blockade of aberrant Stat3 signaling induces tumor cell apoptosis and tumor regression (15, 18, 21, 30), which provides the rationale for developing small-molecule Stat3 inhibitors as anticancer drugs [31, 32, for reviews see (refs. 25, 26, 29, 33)].

Platinum complexes, the prototype of which is cisplatin, are widely used as active anticancer agents (34, 35) in a variety of human tumors, including testicular, ovarian and bladder carcinomas, head and neck squamous cell carcinoma, and non–small cell lung cancers. The biological outcome of cisplatin and other platinum-containing complexes is strongly linked to their alkylating effects on DNA. The effects on DNA, as well as possible interactions with proteins, may be part of a series of molecular events induced by platinum complexes (36) that influence their overall therapeutic outcome. In this context, earlier reports show that cisplatin induces the activation of members of
the mitogen-activated protein kinase (MAPK) family (37, 38). Thus, understanding the interactions of platinum compounds with signal transduction pathways may provide new approaches to enhance the therapeutic benefits of platinum complexes.

We determined the effects of platinum-containing compounds on persistently active Stat3 signaling and biological functions in the context of malignant transformation. Evaluation of a series of novel platinum complexes identified two, CPA-1 and CPA-7, which interfere with Stat3 and disrupt its ability to bind to DNA in vitro. Treatment with CPA-1 or CPA-7 of v-Src–transformed mouse fibroblasts, human breast and prostate cancer cells, as well as mouse melanoma and colon cancer cells blocks constitutive Stat3 activity that is harbored in these cells, and induces growth inhibition and apoptosis. Along with CPA-1 and CPA-7, both of which are platinum (IV) complexes, platinum (IV) tetrachloride (Pt(IV)Cl₄) induces similar effects in the same cellular background. Moreover, the administration of CPA-7 in a mouse tumor model of colon cancer attenuates aberrant Stat3 activity in this tumor, and induces tumor regression. These platinum (IV) compounds may represent a new group of agents that inhibit Stat3 activation.

Materials and Methods

Cells, Plasmids, and Other Reagents

v-Src–transformed (NIH3T3/v-Src) mouse fibroblasts and their counterparts stably expressing Stat3 reporter, pLucTKS3 (NIH3T3/v-Src/pLucTKS3) or Stat3-independent plasmid, pRLSRE (NIH3T3/v-Src/pRLSRE), and Ras-transformed (NIH3T3/v-Ras) mouse fibroblasts, human breast carcinoma MDA-MB-231, MDA-MB-435, MDA-MB-453, MCF-7, and MDA-MB-468 cells, mouse melanoma B16 and M2 cells, human prostate cancer DU145 cells, human lung carcinoma A459 cells, as well as colon cancer cells CT26 (mouse) have been previously described (21, 31, 39–41). HCT116p53−/− cell line was generously provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). Plasmids pLucTKS3 (driving expression of the firefly luciferase gene) and pRLSRE (driving renilla luciferase gene) and pLucTKS3 (driving expression of the firefly luciferase gene) and pRLSRE (driving renilla luciferase gene expression) have been previously described (31, 42). Recombinant human epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA), recombinant murine interferon gamma (IFN-γ) from Research Diagnostics, Inc. (Flanders, NJ), and the Src tyrosine kinase inhibitor, SU6656 from Sugen (South San Francisco, CA). The EGF and IFN-γ were used at 9 ng/μL and 10 units/mL, respectively. Cells were grown in DMEM containing 5% iron-supplemented bovine calf serum, with or without G418. Cisplatin and (Pt(IV)Cl₄) were purchased from Sigma-Aldrich (Milwaukee, WI). Novel platinum (II) and (IV) complexes, CPA-3, or CPA-1, and CPA-7 were synthesized according to previously published procedures (43, 44).

Cytosolic Extract Preparation and Luciferase Assays

Cytosolic lysate preparation from fibroblasts for luciferase assays or from baculovirus-infected Sf-9 insect cells have been previously described (12, 31, 42, 45). Luciferase assays were done as outlined in the supplier’s (Promega, Madison, WI) manual and measured with a luminometer.

Nuclear Extract Preparation and Gel Shift Assays

Nuclear extracts were prepared from cell lines and used for electrophoretic mobility shift assay (EMSA) as previously described (12, 40, 46). In some cases, cells were pretreated with platinum complexes for the indicated times prior to harvesting. Where cells were stimulated with EGF (9 ng/μL) or IFN-γ (10 units/mL), duration of treatment was 15 to 30 minutes. Nuclear extracts (normalized for equal amounts of total protein) were preincubated with compounds for 30 minutes at room temperature prior to incubation with radiolabeled probe. The 32P-labeled oligonucleotide probes used were high-affinity sis-inducible element (hSIE, m67 variant, 5'-AGCTTACATTCCCCG-TAAATCCCTA), which binds both Stat1 and Stat3 (46, 47), mammary gland factor element (MGFe) from the bovine β-casein gene promoter, 5'-AGATTCTTGGATTAATTCCAA, which binds Stat1 and Stat5 (48, 49), the NF-κB-binding oligo (5'-TCGAGAGGGACTTCTCGAGAGGC-3'), and the 90-bp oligonucleotide sequence from the dihydrofolate reductase promoter that binds E2F1 (50).

Western Blot Analyses

Whole-cell lysates were prepared in boiling SDS sample-loading buffer to extract total proteins from the cytoplasm and nucleus as well as preserve the in vitro phosphorylation states. Equivalent amounts of total cellular protein were electrophoresed on a SDS-10% polyacrylamide gel and transferred to nitrocellulose membranes. Probing of nitrocellulose membranes with primary antibodies and detection of horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (Amersham, Piscataway, NJ) were done as previously described (12, 42, 45). The probes used were anti-Stat3 (Santa Cruz, Santa Cruz, CA), anti-pTyr705Stat3 (Cell Signaling, Beverly, MA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA), anti-pTyr705Stat3 (Cell Signaling, Beverly, MA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA), anti-pTyr705Stat3 (Cell Signaling, Beverly, MA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA), antiphosphotyrosine, clone 4G10 (Santa Cruz, Santa Cruz, CA).

Cell Proliferation and TUNEL Staining

Proliferating cells were counted by phase-contrast microscopy for viable cells (using trypan blue exclusion). Thymidine incorporation assays were done in triplicate with 5 × 10³ cells per well exposed to the indicated concentrations of compounds for 30 hours. Cells were pulsed with [3H]-thymidine (0.5 μCi/well) for 6 hours of culture, then transferred onto glass fiber filters to determine the [3H]-thymidine incorporated using liquid scintillation counter. Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) staining was carried out according to supplier’s (Roche, Indianapolis, IN) instructions to detect apoptotic cells. Cells were first treated with or without compounds for 24 to 48 hours prior to staining.
Mice and \textit{In vivo} Tumor Studies

Six-week-old female Balb/C mice were purchased from the National Cancer Institute (Frederick, MD) and maintained in the institutional animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. Balb/C mice were shaved in the left flank area and injected s.c. with $2 \times 10^5$ colon carcinoma CT26 cells in 100 $\mu$L of PBS. After 5 to 10 days, tumors with a diameter of 3 to 6 mm were established. Animals were stratified so that the mean tumor sizes in all treatment groups were nearly identical. Tumor volume was calculated according to the formula $V = \frac{0.52 \times a^2 \times b}{2}$, where $a$, smallest superficial diameter; $b$, largest superficial diameter.

Results

Inhibition of \textit{In vitro} Stat3 DNA-Binding Activity

A panel of novel platinum complexes was evaluated for inhibitory effects against STAT activity \textit{in vitro}, measured in terms of the level of DNA-binding activity associated with nuclear extracts prepared from EGF-stimulated mouse (NIH3T3/hEGFR) fibroblasts. Of the group of novel platinum complexes, the three (CPA-1, CPA-3, and CPA-7) that showed inhibitory effects on Stat3 DNA-binding activity \textit{in vitro} are presented in Fig. 1. The structures in Fig. 1 were confirmed by various analytic approaches, including nuclear magnetic resonance, infrared, X-ray diffraction, and elemental analysis (data not shown). Nuclear extracts containing activated Stat1, Stat3, and Stat5 were preincubated with different concentrations of platinum complexes for 30 minutes prior to incubation with radiolabeled hSIE probe that binds Stat1 and Stat3 and subjected to EMSA analysis. Preincubation of nuclear extracts with CPA-1, CPA-3, or CPA-7 results in a dose-dependent reduction in the level of DNA-binding activity of Stat3 and Stat1 (Fig. 2A), with compounds approximately two to four times more potent against Stat3 over Stat1 homodimers (IC$_{50}$ values in the low micromolar range shown in Table 1). In contrast, similar treatment of nuclear extracts with platinum compounds showed that they have a much reduced inhibitory effect on Stat5 DNA-binding activity using the MGFe probe (Fig. 2B). This suggests that CPA-1, CPA-3, and CPA-7 preferentially disrupt Stat3 and Stat1 activity \textit{in vitro}. Supershift analyses in the presence of antibodies were done, which identify the hSIE-bound complexes as Stat3:Stat3 (top band), Stat1:Stat3 (intermediate band) and Stat1:Stat1 (lower band; Fig. 2A, second and third lanes from the right), and the MGFe-bound complexes as Stat5:Stat5 (top band) and Stat1:Stat1 (lower band; Fig. 2B, the first and third lanes from the right).

Because both CPA-1 and CPA-7 are platinum (IV) complexes, we determined whether the observed effects are associated with platinum (IV) by evaluating (Pt(IV)Cl$_4$)
in in similar assays (Fig. 1). By EMSA analysis, we show that preincubation of nuclear extracts with (Pt(IV)Cl4) disrupts DNA-binding activity of Stat3 and Stat1, but not Stat5, as similarly observed for CPA-1 and CPA-7 (Fig. 2A and B). To determine the selectivity of platinum complexes for Stat3, we investigated effects on the binding of E2F1 and NF-κB to their cognate DNA-binding sequences. Analysis by EMSA shows that the DNA-binding activities of the two non–STAT-related transcription factors are not significantly altered by these platinum compounds except at the very highest concentration (Fig. 2C and D).

**Abrogation of Constitutive Stat3 Signaling in Malignant Cells by Platinum (IV)–Containing Compounds**

To extend our findings to *in vivo* conditions, we investigated the effects of platinum compounds on Stat3 signaling in malignant cells that contain aberrant Stat3 activation. Platinum complexes were first evaluated to determine their ability to inhibit the induction of Stat3 transcriptional activity using v-Src–transformed mouse fibroblasts, NIH3T3/v-Src/pLucTKS3, and NIH3T3/v-Src/pRLSRE, which stably express Stat3-dependent and Stat3-independent luciferase reporters, respectively. Results show that CPA-7 significantly suppresses the expression of the Stat3-dependent luciferase reporter pLucTKS3 (Fig. 3A), with little effect on induction of the Stat3-independent luciferase reporter pRLSRE (Fig. 3B). Although CPA-1 inhibited Stat3-dependent luciferase reporter induction, this effect was weaker than observed for CPA-7. In contrast, little inhibition of Stat3 transcriptional activity by cisplatin and platinum analogue CPA-3 was observed (Fig. 3A and B).

We then determined the effects of platinum compounds on constitutive activation of Stat3 in v-Src–transformed mouse fibroblasts (NIH3T3/v-Src), human breast carcinoma cells (MDA-MB-231, MDA-MB-435, and MDA-MB-468), colon carcinoma (CT26), and melanoma cells (M2 and Cl10). Cells were treated with or without compounds for 24 to 48 hours prior to preparing nuclear extracts for DNA-binding assay. EMSA analysis shows strong inhibition by CPA-1 and CPA-7 of constitutive Stat3 activation (Fig. 4A and C, (iii, iv)), in contrast to cisplatin and CPA-3 that show minimal or no effect on Stat3 activation (Fig. 4A). Altogether, these findings indicate that both CPA-1 and CPA-7 are strong inhibitors of Stat3 activation in intact cells.

The kinetics of inhibition of constitutive Stat3 activation by platinum complexes were determined in MDA-MB-435. Analysis by EMSA of nuclear extracts prepared from treated cells shows that CPA-1, CPA-7, and Pt(IV)Cl4 significantly inhibit Stat3 activation and DNA-binding activity by as early as 6 hours, with no recovery for up to 48 hours (Fig. 4B, (i)). To determine if this inhibition was accompanied by changes in Stat3 tyrosine phosphorylation, whole-cell lysates were prepared and analyzed by SDS-PAGE and Western blot. Except for treatment with CPA-7 for 12 hours, treatment with platinum complexes for short durations of 12 hours or less induced weak or no significant effect on pTyrStat3, whereas prolonged treatments (24–48 h) resulted in relatively stronger or complete reduction in pTyrStat3 (Fig. 4B, (ii)), without altering total Stat3 protein levels, suggesting that platinum (IV) compounds disrupt phosphotyrosine levels of Stat3 on prolonged treatment. Altogether, our findings suggest two mechanisms by which platinum complexes disrupt Stat3 DNA-binding activity in cells. One mechanism occurs early, which is consistent with the effects of these compounds on Stat3 DNA-binding activity *in vitro* (Fig. 2), and a second mechanism involves a reduction in the phosphotyrosine levels of Stat3. In the context of both modes of disruption, results show that CPA-7 is the most potent.

Because platinum compounds were observed to disrupt Stat1 DNA-binding activity *in vitro* (Fig. 1A and B), we examined whether Stat1 activation in cells could be affected by compounds. Normal NIH3T3 or NIH3T3/hEGFR fibroblasts were first treated with platinum compounds

| Table 1. IC50 values for disruption of STAT DNA-binding activity *in vitro* (μmol/L) |
|---------------------------------|------------------|------------------|
| CPA-1                           | 5.0              | 9.3              | 20.0            |
| CPA-3                           | 5.8              | 27.0             | 8.3             |
| CPA-7                           | 1.5              | 3.5              | 4.0             |

Because platinum compounds were observed to disrupt Stat1 DNA-binding activity, we examined whether Stat1 activation in cells could be affected by compounds. Normal NIH3T3 or NIH3T3/hEGFR fibroblasts were first treated with platinum compounds.

![Figure 3](image-url)
for 24 hours, stimulated with IFN-γ or EGF, and then nuclear extracts prepared for DNA-binding assay and EMSA analysis. Stimulation of cells by IFN-γ induces predominantly Stat1:Stat1 (lower band) and to a lesser extent, Stat1:Stat3 (intermediate band), and Stat3:Stat3 (top band), whereas treatment with EGF induces all three complexes (Fig. 4C, (i)). Pretreatment of cells with CPA-1, CPA-7, or Pt(IV)Cl₄ has no significant effect on the activation of Stat1:Stat1 DNA-binding activity by IFN-γ or EGF (Fig. 4C, (i) and (ii)); lower band). In contrast, Stat1:Stat3 (intermediate band) is partially blocked, consistent with strong inhibition of Stat3:Stat3 (top band) in both cell lines (Fig. 4C, (i) and (ii)), and in mouse colon, CT26, or melanoma, C110 and M2 cell lines. Positions of STAT-DNA complex, pTyrStat3, and total Stat3 are shown. β-Actin is shown for normalization of total protein.

Figure 4. Evaluation of effects of platinum complexes on Stat3 activation in cells analyzed by EMSA and Western blot. Nuclear extracts and whole-cell lysates were prepared from ligand-stimulated normal cells or malignant cells that contain constitutively activated Stat3 and were treated with or without platinum complexes for the indicated times. A and B (i), EMSA analysis of in vitro DNA-binding activity of Stat3 that is associated with extracts prepared from v-Src–transformed NIH3T3/v-Src, human breast carcinoma MDA-MB-231, MDA-MB-435, and MDA-MB-468; (ii), SDS-PAGE and Western blot analysis of whole-cell lysates from MDA-MB-435 that probes for pTyrStat3, and total Stat3. C, EMSA analysis of in vitro DNA-binding activity of Stat3 that is associated with extracts prepared from NIH3T3 treated with IFN-γ, NIH3T3/hEGFR treated with EGF, mouse colon carcinoma CT26, and mouse melanoma C110 and M2 cell lines. Positions of STAT-DNA complex, pTyrStat3, and total Stat3 are shown. β-Actin is shown for normalization of total protein.

CPA-1, CPA-7, and (Pt(IV)Cl₄) Are Selective Inhibitors of Stat3 Signal Transduction

To determine the possibility of widespread nonspecific effects in vivo, we studied changes in other signaling pathways that could be induced by platinum complexes, including changes in MAPK family, Erks, p38mapk and JNK, as well as phosphatidylinositol 3-kinase/Akt, and Jak5. Viral Src-transformed fibroblasts (NIH 3T3/v-Src) were used in these studies to take advantage of the extensive signal transduction pathways that are induced due to the presence of the Src oncoprotein. In NIH3T3/v-Src or NIH3T3/hEGFR cells stimulated with EGF, pretreatment with CPA-1, CPA-7, or Pt(IV)Cl₄ does not significantly alter the levels of total cellular tyrosine phosphorylated proteins, as analyzed by Western blot using antiphosphotyrosine antibody, clone 4G10 (Fig. 5A). As a positive control, treatment with the Src kinase inhibitor SU6656 (51) suppressed phosphotyrosine levels in the NIH3T3/v-Src cells (Fig. 5A). Consistent with no change in tyrosine phosphorylated proteins (Fig. 5A), the induction by v-Src of p38mapk (Fig. 5B; ref. 42), Akt (Fig. 5C; ref. 52), JNK (Fig. 5D; ref. 45), and Jak1 (Fig. 5E; ref. 45) was not affected by treatment with platinum compounds. Similarly, the
induction by EGF of Erk1 and Erk2 was not significantly altered by the same treatment (Fig. 5F). These findings together indicate that CPA-1, CPA-7, and Pt(IV)Cl4 are selective and potent inhibitors of constitutive activation of Stat3 DNA-binding and Stat3-mediated gene expression in malignant cells.

Inhibition of Cell Proliferation by Platinum Complexes

Based on previous studies that Stat3 has a key role in cell growth (11–17), we investigated whether platinum complexes have effects on cell proliferation, which were measured by trypan blue counting of viable cells and [3H]-thymidine incorporation. Treatment with CPA-1, CPA-7, or Pt(IV)Cl4 of human breast cancer cell lines (MDA-MB-231 and MDA-MB-435), human prostate cancer cell line (DU145), human non–small cell lung cancer line (A549), mouse colon cancer cell line (CT26), mouse melanoma cell line (M2), or v-Src–transformed mouse fibroblasts, all of which harbor persistently activated Stat3, strongly inhibited growth as measured by trypan blue exclusion (Fig. 6, and data not shown) and [3H]-thymidine incorporation (Fig. 7, and data not shown). In the tumor cell lines, HCT116p53−/− (human colon), and Cl10 (mouse melanoma), which have low levels of constitutively active Stat3 (Fig. 4C, (iv), and data not shown), treatment with platinum compounds weakly diminished viability (Fig. 6). Similarly, normal or tumor cell lines harboring no constitutive Stat3 activity (normal mouse fibroblasts, NIH3T3, and human breast carcinoma, MCF-7 or MDA-MB-453) are less sensitive to these platinum complexes and are partially inhibited in their growth (Figs. 6 and 7, and data not shown). Moreover, treatment of mouse NIH3T3 and NIH3T3/v-Src fibroblasts with cisplatin has no effect on growth of these cells (data not shown). Together, these data suggest that malignant cells that contain constitutively activated Stat3 signaling are more sensitive to platinum complexes in terms of cell proliferation.

Induction of Apoptosis by Platinum Complexes

Numerous studies have established that constitutive Stat3 signaling is critical for the enhanced survival of malignant cells (15, 19, 20, 51, 53, 54). We therefore evaluated the effects of platinum complexes on the viability and survival of malignant cells that harbor constitutive Stat3 activity compared with those that do not. Normal mouse (NIH3T3) and viral Src-transformed (NIH3T3/v-Src) fibroblasts, as well as human breast cancer cell lines and mouse colon, were treated with the platinum compounds and subjected to TUNEL analysis for evidence of DNA damage and apoptosis. Compared to vehicle (DMSO)–treated cells, there is significant apoptosis observed in NIH3T3/v-Src, MDA-MB-435, and CT26 cell lines that harbor high constitutively active Stat3 (12, 21, 40, 41) and that are treated with CPA-1, CPA-7, or Pt(IV)Cl4 (Fig. 8). In contrast, minimal to no significant apoptosis was detected in similarly treated normal mouse fibroblasts (NIH3T3), human breast cancer cells (MDA-MB-453 and MCF-7) that lack activated Stat3. Similarly, in the human colon cancer cell line, HCT116p53−/− harboring...
low constitutively active Stat3, which is insensitive to Stat3 inhibition by dominant-negative Stat3, no significant apoptosis is observed. These findings parallel the minimal effects of platinum compounds on proliferation of MCF-7 cells (Fig. 6), and together show that CPA-1, CPA-7, and Pt(IV)Cl4 preferentially inhibit the survival of malignant cells that are dependent on constitutively active Stat3. Our findings have strong implications for treatment of tumors derived from malignant cells that harbor constitutively active Stat3, suggesting that such tumors might have greater sensitivity to platinum (IV) compounds due to blockade of aberrant Stat3 activity.

Platinum (IV)–Containing Compounds Induce Regression of Colon Tumors in a Mouse Model

We extended our studies to evaluate the antitumor efficacy of CPA-7 using mouse model colon tumors harboring persistently active Stat3. Mouse colon tumor–bearing mice were given i.v. injection with CPA-7 on days 1, 4, 7, 10, and 12 following implantation of tumor, and tumor sizes measured every 3 days. Compared to control (vehicle-treated) tumors, we observed strong or complete regression in colon CT26 tumors following the i.v. administration of CPA-7 (Fig. 9, top). Furthermore, significant to complete inhibition of constitutively active Stat3 is observed in the majority of treated tumors (Fig. 9, bottom), which correlates with tumor regression. In the tumor that only partially regressed following treatment, there was significant Stat3 activity, suggesting that failure to regress is likely due to lack of inhibition of Stat3. These findings indicate that platinum (IV) complexes induce the regression of colon tumors, accompanied by inhibition of Stat3 activity. Together with cell-based studies in Figs. 6, 7, and 8, the findings here provide support for platinum (IV) compound–induced tumor regression in part due to loss of viability and apoptosis of tumor cells through inhibition of constitutively active Stat3.

Discussion

Current application of cisplatin and its analogues as anticancer agents is predominantly based on their DNA-damaging effects which contribute to cell death. Although initial evidence indicates that cisplatin modulates MAPK signaling pathway (36, 37), the effects of platinum complexes on signal transduction events have not been extensively studied. We provide evidence that novel platinum (IV) compounds, CPA-1, CPA-7, and (Pt(IV)Cl4) are potent disruptors of STAT activity. Of the three STAT family members (Stat1, Stat3, and Stat5) examined here, Stat3 is preferentially disrupted. In addition, CPA-7 is the most potent compound in vitro, in whole cells and in animal models of cancer. The disruption of STAT activity by platinum compounds in part depends on their interaction with the proteins, as suggested by the direct inhibition of DNA-binding activity in vitro, and by effects on cellular phosphotyrosine levels of Stat3. It remains to be determined how prolonged treatment of cells with platinum compounds alters phosphotyrosine Stat3 levels without changes in the levels of Stat3 protein.

The biological effects of CPA-1, CPA-7, and Pt(IV)Cl4 in our study include growth inhibition and apoptosis of malignant cells that harbor constitutively active Stat3. These effects are stronger and more selective against malignant cells that contain persistently active Stat3 than those that do not, reflecting in part the inhibition of constitutive activation of Stat3 signaling, and providing further support for a critical role for abnormal Stat3 activity in the growth control and survival of transformed cells (22–29). In contrast, the absence of consistent cellular effects of cisplatin or CPA-3 in malignant cells harboring...
persistent Stat3 signaling parallels their lack of ability to significantly inhibit Stat3 activity at the concentrations used, which raises the possibility of different modes of action for platinum complexes inside cells. Moreover, CPA-1 and CPA-7 are platinum (IV) complexes, in contrast to cisplatin and CPA-3, which are platinum (II)–containing complexes. It remains to be determined how inhibitory effect against Stat3 activity and biological functions are influenced by the oxidative state of platinum in CPA-1 and CPA-7, an issue that requires evaluation of a series of novel platinum (IV) complexes to resolve.

Although current studies indicate that disruption of constitutive Stat3 signaling might not be an important factor in the induction of biological effects to cisplatin and CPA-3, they as well as others (36, 37) strongly support modulation of signal transduction pathways as one of the key mechanisms underlying the biological effects of platinum-containing complexes. Thus, in addition to alkylation of DNA, the effects on signal transduction pathways owing to cisplatin (36, 37), carboplatin, and other platinum complexes may be part of a general molecular mechanism for their antitumor efficacy. On the basis of the present data, we can conclude that inhibition of constitutive Stat3 signaling is a key component of the mechanism whereby the biological effects of the three platinum (IV) complexes, CPA-1, CPA-7, and Pt(IV)Cl₄, are manifested.

Our studies also provide the rationale for evaluating the antitumor effects of these platinum (IV)–containing complexes in patients with tumors harboring aberrant Stat3

Figure 8. Induction of apoptosis by platinum complexes. Normal NIH3T3 fibroblasts and their v-Src–transformed counterparts, human breast carcinoma cells (MDA-MB-453, MCF-7, and MDA-MB-435), as well as colon tumor cell lines HCT116p53⁻/⁻ (human) and CT26 (mouse) were treated with platinum complexes for 48 hours and analyzed for evidence of DNA damage using TUNEL staining kit.

Figure 9. Tumor regression induced by platinum complex. Mouse colon CT26 tumor–bearing mice were given CPA-7 (5 mg/kg) i.v. on days 1, 4, 9, 12, and 15. Tumor sizes were monitored every 3 to 4 days and plotted (top). Extracted tumor tissues following treatment were investigated for Stat3 activity in in vitro DNA-binding assays with EMSA analysis (bottom). Broken lines, control tumors (50% DMSO); solid lines, treated tumors.
signaling. Compared to cells that do not contain aberrant Stat3 activity, malignant cells harboring constitutively active Stat3 are more sensitive to the platinum IV complexes, CPA-1, CPA-7, and Pt(IV)Cl4. This observation indicates that tumors showing evidence of persistently active Stat3 are more likely to be targeted by these platinum (IV) compounds. Indeed, our initial observation of the regression of mouse colon tumors harboring constitutively active Stat3 upon platinum (IV) compound administration provides a proof-of-concept for their antitumor efficacy that is based on anti-Stat3 activity, as supported by the abrogation of constitutive Stat3 activity in the regressed tumors. These studies have to be extended to other tumor types that harbor constitutively active Stat3 for evaluation of sensitivity to novel platinum (IV) complexes.

We do not exclude other modes of action for platinum (IV) complexes, including possible interactions with other signaling proteins, which might contribute to their antitumor cell activity. Contrary to other reports on cisplatin (37, 38, 55–58), no significant effects of these novel platinum (IV) complexes are observed on ERK1/2, p38, JNK, Akt and Jak signaling proteins, which might contribute to their antitumor activity. Contrary to other reports on cisplatin (4), no significant effects of these novel platinum (IV) compounds are observed on ERK1/2, p38, JNK, Akt and Jak signaling proteins, which might contribute to their antitumor activity.

In summary, our findings reported here strongly support the potential of platinum compounds to modulate signaling proteins, and the importance of these interactions for their biological properties, including their overall therapeutic outcomes. Modulation of Stat3 signaling pathway by certain platinum (IV) compounds might be part of their general mode of action. These findings provide the rationale for further evaluation of the therapeutic potential of platinum (IV) compounds as inhibitors of Stat3 with antitumor activity.

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Stat3 Inhibition by Novel Platinum Compounds


Molecular Cancer Therapeutics

Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent antitumor activity

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