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Suppression of migration, invasion, and metastasis of cisplatin-resistant head and neck squamous cell carcinoma through IKK β inhibition

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Abstract

We explored the role of the transcription factor, NF- κ B, and its upstream kinase IKK β in regulation of migration, invasion, and metastasis of cisplatin-resistant head and neck squamous cell carcinoma (HNSCC). We showed that cisplatin-resistant HNSCC cells have a stronger ability to migrate and invade, as well as display higher IKK β /NF- κ B activity compared to their parental partners. Importantly, we found that knockdown of IKK β , but not NF- κ B, dramatically impaired cell migration and invasion in these cells. Consistent with this, the IKK β inhibitor, CmpdA, also inhibited cell migration and invasion. Previous studies have already shown that N-Cadherin, an epithelial-mesenchymal transition (EMT) marker, and IL-6, a pro-inflammatory cytokine, play important roles in regulation of HNSCC migration, invasion, and metastasis. We found that cisplatin-resistant HNSCC expressed higher levels of N-Cadherin and IL-6, which were significantly inhibited by CmpdA. More importantly, we showed that CmpdA treatment dramatically abated cisplatin-resistant HNSCC cell metastasis to lungs in a mouse model. Our data demonstrated the crucial role of IKK β in control of migration, invasion, and metastasis, and implicated that targeting IKK β may be a potential therapy for cisplatin-resistant metastatic HNSCC.

Keywords

head and neck squamous cell carcinoma; HNSCC; IKK β ; NF- κ B; IKK β inhibitor; migration; invasion; metastasis; cisplatin resistance

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[‡]JL and ZY contributed equally to this work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

Head and neck squamous cell carcinomas (HNSCC) are currently the sixth most common cancer worldwide [1, 2]. The major factors that affect overall patient survival are due to recurrence and metastasis of cancer following standard therapies such as surgery, radiation therapy, or a combination [3–5]. Currently, cisplatin-based chemotherapy remains the primary option for patients with recurrent and metastatic HNSCC, but nearly all patients will eventually become resistant to cisplatin treatment and die within a year. Therefore, it is important to define the key signaling pathways that regulate HNSCC metastasis and discover effective inhibitors to target them and overcome metastasis [3, 6–8]. In addition, a major determinant for cancer cell metastasis is its ability to migrate and invade. Therefore, identification of the crucial proteins and signaling pathways that associate with cancer cell migration and invasion remains vital to discovery of new therapies for HNSCC metastatic cancer [8].

Multiple survival pathways involve HNSCC development, progression, metastasis, and chemotherapy resistance [4, 9–11]. Many studies have demonstrated that epidermal growth factor receptor (EGFR) plays an important role in HNSCC, since more than 90% of head and neck cancers overexpress EGFR [4, 10, 12]. EGFR overexpression correlates with poorer outcomes in HNSCC patients because it leads to the activation of several crucial pathways including phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR, MEK/MAPK/ERK, protein kinase C (PKC), JAK/STAT, and IKK/NF- κ B pathways [9–11, 13–15]. These signaling pathways control gene expression, cell proliferation, apoptosis, migration, and invasion, as well as metastasis through different mechanisms [11, 12].

The transcription factor NF- κ B plays many roles in different types of cancer, including head and neck cancer. It has been shown that NF- κ B is activated in different stages of HNSCC. NF- κ B cross-talk with multiple signaling pathways downstream of EGFR promotes cancer cell proliferation and inhibit apoptosis, as well as accelerates metastasis through transcription of its target gene. NF- κ B activity is modulated by its upstream kinases IKK α and IKK β , which cooperate to regulate proliferation and migration in HNSCC, though the mechanisms remain unclear [11, 13–16]. In this study, we explored the role of IKK β and NF- κ B in regulation of migration and invasion of cisplatin-resistant HNSCC. We showed that IKK β controls cell migration and invasion independent of NF- κ B, and CmpdA, an IKK β inhibitor, inhibits cell migration and invasion, as well as metastasis of cisplatin-resistant HNSCC.

Materials and Methods

Cell culture

Cal 27 cells were obtained from ATCC and the UMSCC74B cell lines were purchased from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI, USA). All cell lines were authenticated by Short Tandem Repeat (STR) assay and tested for mycoplasma contamination in the Genomics and Translational Core Facility of the University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center (UMGCCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/mL penicillin and streptomycin (Gibco).

Antibodies and reagents

The antibodies were purchased from Cell Signaling Technology: phospho-IKK α (S176)/ β (S177) (CST-2697 and CST-2078), IKK α (CST-2682), IKK β (CST-8943), phospho-p65-S536 (CST-3033), p65 (CST-6956), phospho-STST3-Y705 (CST-9113), STST3 (CST-4904), E-Cadherin (CST-3195), N-Cadherin (CST-4061), β -actin (CST-4967) and GAPDH (CST-5174). Protease and phosphatase inhibitors were from Roche. The IKK β inhibitor CmpdA (Bay65–1942) was provided by Dr. Albert Baldwin at the University of North Carolina at Chapel Hill [17].

Cell lysis and Western blot analysis

Cells were lysed in ice-cold M-PER lysis buffer purchased from Thermo Fisher Scientific (78501). Cell lysates were spun down by centrifugation for 15 minutes at $15,000 \times g$ at 4 °C and protein concentration was measured with the BCA protein assay (Thermo Fisher Scientific). 20–50 μ g of protein were separated by SDS-PAGE. The gel was transferred to a PVDF membrane, blocked in 5% nonfat milk, and blotted with the indicated antibodies.

siRNA transfection

siRNA SMARTpool IKK β (catalog #M-003503) and NF- κ B (p65) (catalog #M-003533) were from Dharmacon. Each siRNA represents four pooled SMART-selected siRNA duplexes that target the indicated mRNA. Cells were transfected with indicated SMARTpool siRNA or nonspecific control pool using (D-001810) Lipofectamine® RNAiMAX™ Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were recovered in full serum. Cells were harvested 48–72 hours post-siRNA transfection.

Colony focus assay

Cells (1000/well) transfected with siRNA control, IKK β , or NF- κ B for 24 hours were seeded in 12-well plates and grown in normal media for 10 days, washed once with 1x PBS, fixed with methanol, and stained with crystal violet.

Measurement of cell migration and invasion

xCELLigence real-time migration and invasion experiments were conducted as described previously [18].

Generation of luciferase-Yellow fluorescent protein expressing cells

CL20IM-luc-IYFP lentiviral supernatant (yellow fluorescent protein, YFP, and luciferase controlled by the same promotor) was the generous gift of the St. Jude Children's Research Hospital Vector Core. Cal27 cells were harvested and plated into a 24 well plate, and the following day lentiviral supernatant was added to the cells. After 72 hours, cells were harvested and re-plated for expansion. YFP-luciferase positive cells were sorted on the Aria II platform (BD Biosciences) in UMGCCC's flow cytometry core. YFP-luciferase positive

cells were then expanded, frozen viably and re-tested by STR analysis for cell line authentication prior to *in vivo* studies.

Tumor metastasis in lungs in mice

Six-week old female NSG (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ*) or NRG (NOD.Cg-*Rag1^{tm1Mom} Il2rg^{tm1Wjl}/SzJ*) mice were obtained from and bred by University of Maryland Baltimore's Comparative Medicine Department under a license from Jackson Labs (Maine). All *in vivo* experiments were carried out in compliance with institutional and NIH guidelines and the Institutional Animal Care and Use Committee regulations for care and use of experimental animals. In the metastasis model, 1×10^6 YFP/luc-Cal 27 cells were injected intravenously into 6-week old, female NRG or NSG mice. Within hours of the IV injection, mice were imaged for bioluminescence on Perkin Elmer's IVIS Xenogen system following intraperitoneal injection with 150 mg/kg luciferin. At the termination of the experiment, mice were euthanized and lungs excised and imaged for YFP.

Statistics

In vitro experiments were expressed as mean $\pm$ SD using 3 independent experiments. Comparisons between groups were carried out by 2-way ANOVA or Student's *t*-test. For mouse studies, the two-tailed *t* test was used to compare tumor numbers between control and treatment groups. *P* values < 0.05 were considered significant.

Results

Cisplatin-resistant HNSCC cells show elevated IKK β /NF- κ B signaling and have stronger abilities to migrate and invade

CAL 27 is a frequently used oral squamous cell carcinoma cell line for HNSCC studies, including those that involve cisplatin resistance [19]. We recently established a cisplatin-resistant Cal27CP cell line by treatment of parental Cal27 cells with 0.5 μ M to 5 μ M of cisplatin for 6 months. The IC₅₀ of Cal27 and Cal27CP to cisplatin were 3 μ M and 15 μ M, respectively. In the Western blot analysis, increased levels of IKK α / β phosphorylation, especially IKK β , were detected in Cal27CP cells. Consistently, phosphorylation of NF- κ B (p65), the downstream target of IKK, was higher in Cal27CP cells than in parental cells (Figure 1A). These results indicated that IKK/NF- κ B signaling was up-regulated in cisplatin-resistant Cal27 (Cal27CP) cells. Next, the xCELLigence real-time cell system was used to monitor the migration ability of Cal27 and Cal27CP cells. Cal27CP cells showed an increase in migration over time (Figure 1B). In addition, Cal27CP cells had a stronger ability to invade compared to their parental partners (Figure 1C). These data are consistent with the previous report that the epithelial to mesenchymal transition (EMT) increased in cisplatin-resistant Cal27CP cells [19].

Knockdown of IKK β , but not NF- κ B, impairs migration and invasion of cisplatin-resistant HNSCC

Next, we determined the roles of IKK β and NF- κ B in the regulation of Cal27CP cell migration and invasion. siRNA against IKK β and NF- κ B (p65) was used to knock down IKK β and NF- κ B, respectively, in Cal27CP cells. Western blot analysis showed that IKK β

and NF- κ B were effectively knocked down 48 hours post-transfection (Figure 2A). In a parallel experiment, cell migration and invasion were monitored after knockdown of either IKK β or NF- κ B. Knockdown of IKK β , but not NF- κ B, dramatically impaired cell migration (Figure 2B) and significantly decreased cell invasion (Figure 2C). It should be noted that we observed significant difference in cell proliferation between cells treated with control siRNA and IKK β siRNA in 96 hours (Supplementary Figures 1B), rather than 48 hours (Supplementary Figures 1A). Thus, depletion of IKK β -induced inhibition of cell migration and invasion did not occur through inhibition of cell proliferation. Interestingly, colony assay experiments showed that NF- κ B knockdown more significantly decreased cell proliferation compared to IKK β knockdown (Figure 2D). Our data indicate that IKK β regulates cell migration and invasion independent of NF- κ B, whereas it may regulate cell proliferation through NF- κ B (Figure 2E).

IKK β inhibitor, CmpdA, inhibits EMT and cell migration and invasion

CmpdA is a selective IKK β inhibitor [17, 18, 20]. To test whether IKK β /NF- κ B inhibition suppresses migration and invasion of cisplatin-resistant HNSCC, Cal27CP cells were treated with CmpdA (2 μ M), and we subsequently monitored cell migration and invasion. CmpdA treatment significantly inhibited cell migration (Figure 3A) and invasion (Figure 3B). The UMSCC74B cell line was derived from a patient treated with chemotherapy [21]. Our data demonstrated that these cells were resistant to cisplatin with an IC₅₀ of 20 μ M. The xCELLigence real-time cell system also demonstrated that CmpdA dramatically inhibited UMSCC74B cell invasion (Figure 3C).

In a separate experiment, we treated Cal27CP cells with 2 or 5 μ M of CmpdA for 48 hours and found that there was no significant inhibition of cell proliferation (Supplementary Figures 2). These results indicated that CmpdA inhibition of cell migration and invasion is independent of inhibition of cell proliferation. However, treatment of Cal27CP Cells with 1 μ M of CmpdA significantly decreased colony formation in 10 days (Supplementary Figures 3). These data are consistent with the results shown in Figure 2, which demonstrates that IKK β depletion decreased cell proliferation over a longer period of time. Thus, IKK β plays roles in regulation of cell proliferation, migration, and invasion. Our data demonstrate that IKK β targeting may inhibit EMT in cisplatin-resistant HNSCC.

IKK β inhibitor, CmpdA, inhibits epithelial-mesenchymal transition (EMT) marker, N-cadherin, expression in cisplatin-resistant HNSCC cells

EMT plays a crucial role in regulation of cancer cell migration and invasion. N-cadherin and E-cadherin are important biomarkers for EMT [8]. Sun, *et. al.*, previously reported that cisplatin-resistant Cal27 cells showed higher levels of N-cadherin, but lower levels of E-cadherin in comparison to the parental cells [19]. A similar result was found in our Cal27CP cells compared to Cal27 cells (Figure 4A). Next, we treated Cal27CP and UMSCC74B cells with CmpdA for 72 hours to test its effects on the expression of N- and E-cadherin expression. The phosphorylation of p65, an IKK β substrate, significantly decreased in response to CmpdA treatment, which suggested that CmpdA treatment inhibited IKK β /NF- κ B signaling (Figure 4A). Importantly, the expression of N-cadherin was also downregulated by CmpdA treatment, while there were no changes to E-cadherin levels (Figure 4B). These

data demonstrate that CmpdA inhibition of migration and invasion of cisplatin-resistant HNSCC may involve N-Cadherin inhibition.

IL-6 expression in cisplatin-resistant HNSCC cells is significantly elevated and is also inhibited by CmpdA

Previous reports demonstrated that IL-6, a pro-inflammatory cytokine, played a crucial role in regulation of migration, invasion, and metastasis through the IL-6/STAT3 signaling pathway in HNSCC [22–27]. Here, we found that IL-6 expression in Cal27CP cells was much higher than that in Cal27 cells, whereas the phosphorylation of STAT3 in Cal27CP cells was lower (Figure 5A). Furthermore, we showed that CmpdA treatment dramatically decreased IL-6 expression and led to increased STAT3 phosphorylation in Cal27CP but not UMSSC74B cells (Figure 5B). These results suggest that CmpdA can inhibit migration and invasion through IL-6, independent of STAT3.

CmpdA abates Cal27CP cell metastasis to lungs in mice

To better monitor tumor metastasis *in vivo*, we transduced Cal27 and Cal27CP cells with a lentivirus that expressed yellow fluorescent protein (YFP) and luciferase (luc) in order to visualize these cells in mice and in tissue. YFP expression was confirmed under fluorescence microscopy in both Cal27 (YFP/luc-Cal27) and Cal27CP (YFP/luc-Cal27CP) cells (Supplementary Figure 4A). Proliferation assays showed that the IC₅₀ of YFP/luc-Cal27CP cells was 15 μ M, whereas that in YFP/luc-Cal27 cells was around 4 μ M. Thus, the IC₅₀ of YFP/luc-Cal27CP cells remains consistent with prior studies (Supplementary Figure 4B). YFP/luc-Cal27CP cells were intravenously injected into mice and luminescence was measured. Significant luminescence signaling, which correlates to disease burden, gradually increased from days 4 to 36 (Supplementary Figures 4C and 4D). These results demonstrate that YFP/luc-Cal27CP cells metastasize to the lungs, making this a good model to study cisplatin-resistant, metastatic HNSCC.

Next, we determined whether CmpdA could suppress metastatic cisplatin-resistant HNSCC cell growth in lungs. YFP/luc-Cal27CP cells treated with vehicle control or CmpdA (2.5 μ M) for 48 hours were injected into NSG mice and eleven mice with similar levels of luminescence signaling in their lungs were divided into two groups: Group 1 (vehicle control) contained five mice, whereas Group 2 (CmpdA treatment) had six. The mice were treated with vehicle control or 10 mg/kg CmpdA starting from day 3 for three weeks (twice/week) and luminescence signaling in lungs was monitored at days 1, 8, 14, and 22 (Figure 6A). There were no significant differences in photo intensity of luminescence between treatment and control groups after 24 hours (Figure 6A). However, the bioluminescence intensity in the treatment group was weaker than the control group mice on days 8 and 14, and that was further confirmed on day 22 (Figures 6A and B). Furthermore, when the mice were sacrificed, lungs removed, and luciferase activity measured, a significant decrease in luciferase signaling was found in the CmpdA-treated group (Figures 6C and D). In addition, there was no difference in mice body weight between the treatment and control groups (Figure 6E). Furthermore, we found that metastatic lesions (numbers) in the mice treated with CmpdA were significantly less than those in the mice treated with vehicle control (Figure 6F), which suggested that CmpdA treatment could decrease tumor numbers (Figure

6F). Furthermore, we found that metastatic lesions (sizes) in the mice treated with CmpdA were significantly smaller than those in the mice treated with vehicle control (Figure 6G). In summary, CmpdA inhibits growth of metastatic cisplatin-resistant HNSCC cells through inhibition of both metastasis and colony formation.

Discussion

In this study, we established a cisplatin-resistant HNSCC cell line derived from oral squamous cell carcinoma Cal27 cells and found that IKK β /NF- κ B signaling was upregulated in these cells. Consistent with previous reports [19], we showed that Cal27CP cells have a stronger ability to migrate and invade compared to the parental Cal27 cells. Importantly, we found that knockdown of IKK β dramatically slowed cell migration and invasion in Cal27CP cells. Furthermore, the IKK β inhibitor, CmpdA, decreased N-cadherin expression and inhibited cell migration and invasion in Cal27CP cells, as well as in UMSCC7B cells, a patient-derived, chemotherapy-resistant HNSCC cells. CmpdA also decreased Cal27CP cell metastasis and invasion in the lungs of mice. Our data demonstrated the crucial role that IKK β plays to control cell invasion and metastasis in cisplatin-resistant HNSCC and indicated that use of IKK β as a target could be a potential strategy to treat cisplatin-resistant, metastatic HNSCC.

Many studies, including ours, have already shown that NF- κ B plays important roles in cell proliferation, chemotherapy resistance, migration, invasion, and metastasis in many cancer types, including HNSCC [16, 28–30]. The study by Wu, *et. al.*, demonstrated that NF- κ B regulated breast cancer metastasis through transcriptional regulation of Snail [31]. Li, *et. al.*, showed that NF- κ B regulated Twist to control cancer invasion and metastasis [32]. We previously reported that NF- κ B promoted prostate cancer proliferation, migration, and invasion, and that the IKK β inhibitor, CmpdA, inhibited these processes through NF- κ B inhibition [18]. In the current study, we found that IKK β regulated cell migration and invasion, independent of NF- κ B. It should be noted that this regulation may be cell-type specific.

The most common biochemical change associated with epithelial-mesenchymal transition (EMT) is the over-expression of N-cadherin and the decrease of E-cadherin expression. N-cadherin and E-cadherin are transcriptionally regulated by multiple proteins such as Snail (SNAIL), Slug (SNAIL2), and ZEB-1 [33]. Down-regulation of N-cadherin and increases of E-cadherin expression leads to inhibition of migration and invasion [33]. Interestingly, the IKK β inhibitor, CmpdA, inhibited only the expression of N-cadherin without any effects on E-cadherin. We are currently attempting to ascertain the precise mechanism(s) by which IKK β regulates N-cadherin.

While many studies have already demonstrated that NF- κ B controls IL-6/STAT3 signaling to promote EMT and tumor metastasis [22–27], we have shown that the IKK β inhibitor CmpdA inhibits NF- κ B and IL-6, while still causing elevated STAT3 phosphorylation. It will be interesting to elucidate the detailed molecular links among IKK β /NF- κ B, IL-6, and STAT3 in more cisplatin-resistant HNSCC models.

In our study, CmpdA targeting of IKK β caused dramatic inhibition of migration and invasion *in vitro* and significant inhibition of metastasis *in vivo*. These data suggest that the inhibition of IKK β alone, or in combination with other targeted therapies, could be a potential regimen for treatment of cisplatin-resistant, metastatic HNSCC. We are currently investigating new potential crucial pathways associated with IKK/NF- κ B activation in order to identify the most effective multiple-targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HNSCC	head and neck squamous cell carcinoma
EMT	epithelial-mesenchymal transition
IKKβ	inhibitor of nuclear factor kappa-B kinase subunit beta
NF-κB	nuclear factor kappa-B

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Figure 1. Cisplatin-resistant HNSCC cells have elevated IKK β /NF- κ B signaling and stronger abilities to migrate and invade.

A: Cal27 and Cal27CP cells were lysed and the phosphorylation status and total protein levels of IKK α / β and p65, as well as β -actin, were analyzed by Western blot. **B** and **C:** Cal27 and Cal27CP cell migration (**B**) and invasion (**C**) were monitored by xCELLigence system. SFM: serum-free medium.

Figure 2. IKKβ knockdown impairs cisplatin-resistant HNSCC cell migration and invasion.

A: Cal27CP cells were transfected with non-target siRNA, siRNA IKKβ, or siRNA p65 for 48 hours and the expression of IKKβ, p65 and β-Actin was tested by Western blot. **B and C:** Cal27CP cells were transfected with non-target siRNA, siRNA IKKβ, or siRNA p65 for 48 hours and cell migration (**B**) and invasion (**C**) were monitored by xCELLigence system. SFM: serum-free medium. **D:** Cal27CP cells transfected with non-target siRNA, siRNA IKKβ, or siRNA p65 for 24 hours were seeded in 12 well plates (800/well) and colony formation was observed after 10 days. **E:** IKKβ regulates cell migration and invasion independent of NF-κB, whereas it may regulate cell proliferation through NF-κB migration in Cal27CP cells.

Figure 3. IKK β inhibitor, CmpdA, inhibits cisplatin-resistant HNSCC cell migration and invasion.

A and B: Cal27CP cells were treated with IKK β inhibitor, CmpdA (2 μ M) and cell migration (**A**) and invasion (**B**) were monitored. **C:** UMSCC74B cells were treated with increasing doses of CmpdA and cell invasion was monitored.

A **B**

Figure 4. CmpdA inhibits N-Cadherin expression in cisplatin-resistant HNSCC cells.

A: Cal27 and Cal27CP cells were lysed and the expression of E-Cadherin, N-Cadherin, and β -actin was detected by Western blot. **B:** Cal27CP and UMSCC74B cells were treated with increasing doses of CmpdA for 24 hours and the levels of phospho-p65, p65, E-Cadherin, N-Cadherin, and β -actin were detected by Western blot.

Figure 5. CmpdA inhibits IL-6 expression in cisplatin-resistant HNSCC cells.

A: Cal27 and Cal27CP cells were lysed and the levels of IL-6, phospho-STAT3, Stat3, and GAPDH were detected by Western blot. **B:** Cal27CP and UMSCC74B cells were treated with increasing concentrations of CmpdA for 24 hours and the levels of IL-6, phospho-STAT3, Stat3, and GAPDH were detected by Western blot.

Figure 6. CmpdA abates Cal27CP cell metastasis to lungs in mice.

A: 1×10^6 YFP/luc-Cal27CP cells pre-treated for 72 hours with vehicle control or $2.5 \mu\text{M}$ CmpdA were injected intravenously into NRG mice. On day of injection, mice were imaged and grouped into the control group (Group 1; $n=5$) and the treatment group (Group 2; $n=6$) so that mean intensity of luminescence signaling in lungs was similar. Mice were then treated intraperitoneally (IP) three times per week with 10% DMSO (Group 1) or 10 mg/kg CmpdA (Group 2), for three weeks. Luminescence signaling (photon intensity) in lungs was monitored. **B:** Intensity of luminescence signaling in lungs at days 1 (start) and 22 (end) were compared ($*P < 0.05$). **C:** 11 mice labeled from 1 to 11 (control group: 1–5; and treatment group: 6–11) were euthanized at day 22, lungs were washed and collected, and fluorescence images (red) were taken. **D:** Fluorescence images on lungs were measured ($**P < 0.01$). **E:** Percent body weight changes in mice treated with vehicle control or CmpdA were recorded from days 1 to 22. **F:** Lung lesions in mice treated with vehicle control or CmpdA (mice 1–11 in **Figure D**) were counted and compared. **G:** Hematoxylin and eosin (H&E) staining of paraffin sections of lungs from control and CmpdA treated mice are shown at the same magnification. Yellow arrows indicate metastatic lung tumors.