Molecular Therapy
Oncology
Original Article

Targeting cancer stem cells by TPA leads to inhibition of refractory sarcoma and extended overall survival

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Refractory cancer recurrence in patients is a serious challenge in modern medicine. Tumor regrowth in a more aggressive and invasive drug-resistant form is caused by a specific subpopulation of tumor cells defined as cancer stem cells (CSCs). While the role of CSCs in cancer relapse is recognized, the signaling pathways of CSCs-driven chemoresistance are less well understood. Moreover, there are no effective therapeutic strategies that involve specific inhibition of CSCs responsible for cancer recurrence and drug resistance. There is a clinical need to develop new therapies for patients with refractory sarcomas, particularly fibrosarcoma. These aggressive tumors, with poor overall survival, do not respond to conventional therapies. Standard systemic chemotherapy for these tumors includes doxorubicin (DOX). A Tyr peptide analog (TPA), developed in our laboratory, specifically targets CSCs by drastically reducing expression of the polycomb group protein enhancer of zester (EZH2) and its downstream targets, specifically ALDH1A1 and Nanog. *In vivo* experiments demonstrated that TPA inhibited tumor growth in nu/nu mice with relapsed DOX-treated fibrosarcoma 7-fold and led to improved overall (2-fold) survival. In an experimental metastatic model, the combination of TPA with DOX treatment extended overall survival 3-fold, suggesting that targeting CSC can become an effective strategy in the treatment of refractory/relapse fibrosarcoma.

INTRODUCTION

Disease recurrence after conventional therapies is common for many cancer patients and is manifested by aggressive tumor regrowth and metastatic spread. Treatment-refractory cancer presents a significant unresolved problem in oncology[1]; connective tissue malignancies are no exception. It is thought to be associated with drug resistance that arises in a heterogeneous cellular microenvironment and driven by a small population of cancer stem cells (CSCs).[2,3] The presence of these CSCs has been reported for various tumor types.,[4–11] including sarcomas, complex mesenchymal neoplasms that do not respond well to conventional therapies and have a poor prognosis.[12–15] The current notion suggests that sarcomas arise from either primitive mesenchymal stem cells (MSCs), which direct tumorigenesis by acquired mutations, or by mutations occurring at different stages of differentiation, which leads to subsequent tumor development.[16] CSCs are one of the key components of the tumor microenvironment (TME) that play important roles in immunoregulation, drug resistance, and tumor relapse.[17,18] CSCs also exhibit phenotypic heterogeneity, and the spatial interactions with the surrounding TME confer resistance to conventional therapies. CSCs interact with immune cells to protect themselves against immune clearance by exploiting the immunosuppressive function of multiple immune checkpoint molecules, demonstrating immune evasion.[19] TME in turn dictates the CSC function that leads to cancer progression and metastasis.[20,21]

Tumor resistance to immune checkpoint inhibitors contributes to treatment failures. The immune cells are frequently suppressed by immune checkpoint molecules, such as programmed cell death receptor-1, its ligands (PD-L1/-L2), and cytotoxic T cell-associated antigen-4, which may be restored by checkpoint inhibitors.[22,23] Increased PD-L1 expression due to acquired stemness leads to dedifferentiation in metastatic cells, which in its turn contributes to drug resistance.[24] Resistance to anthracycline doxorubicin (DOX, also known as adriamycin), alone or combined with ifosfamide (IFO), standard systemic therapy for soft tissue sarcomas, is an obstacle to successful treatment because of clinical relapse, local recurrence, and metastatic spread, despite its initial strong cytotoxic effect.[25] Fibrosarcoma represents approximately 10% of musculoskeletal sarcomas. This disease responds poorly to chemotherapy and has a high rate of tumor recurrence and poor overall prognosis, with a less than 30% overall survival rate for high-grade tumors. Tumor metastases have been described in 9%–63% of patients with adult-type fibrosarcoma, with lungs and bone representing the most common sites of metastatic spread. Survival for adult patients with fibrosarcoma of all grades is ∼40% at 5 years.[26] Targeting of soft tissue

Received 18 June 2024; accepted 4 November 2024;
https://doi.org/10.1016/j.omton.2024.200905.

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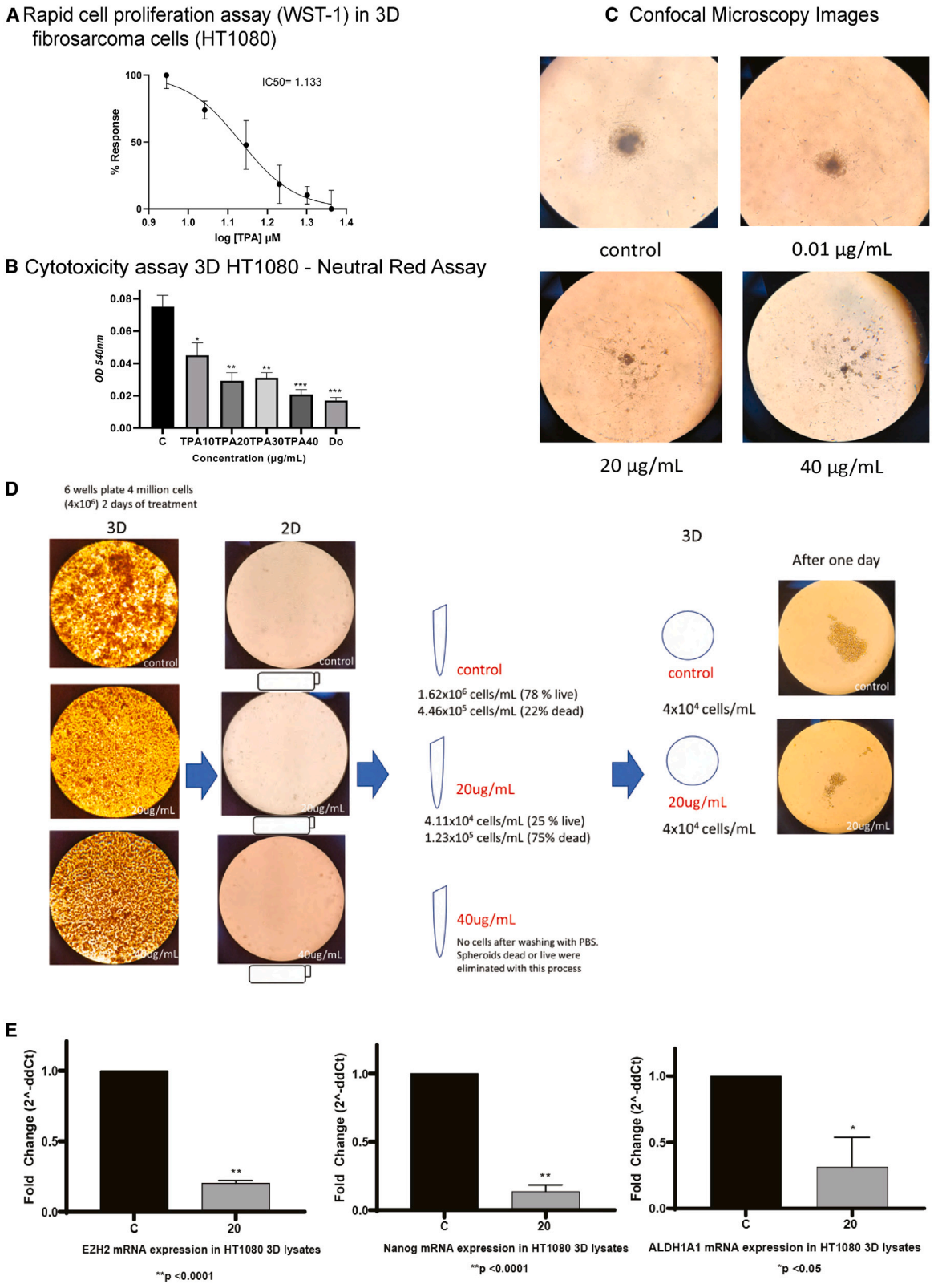

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sarcoma CSCs leading to increased and improved DOX sensitivity has been reported.[27] We have developed a novel compound that is a tyrosine peptide analog (TPA), derived from hypothalamic-proline-rich polypeptide (PRP-1), and produced by neurosecretory cells of hypothalamus (*nucleus paraventricularis* and *nucleus supraopticus*). Currently, these peptides are chemically synthetized. We have reported the properties of PRP-1 previously,[13,28–34] demonstrating its ability to target CSCs in chondrosarcoma.

The purpose of this study was to explore the CSC targeting effect of TPAs and their action on tumor growth alone and in combination with DOX *in vivo* using a human HT1080 fibrosarcoma cell line xenograft mouse model. Combining conventional chemotherapy drugs with CSC targeting agents is predicted to be more effective as it targets both CSCs and non-CSCs.[35]

RESULTS

TPA exerted its cytotoxic effect on spheroids and exhausted (diminished) their viability and maintenance pool by inhibiting polycomb EZH2 and its downstream CSC markers

To understand whether TPA inhibits CSC growth, we produced three-dimensional (3D) spheroids that mimic CSCs and performed a rapid cell colorimetric proliferation assay based on the conversion of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes (Figure 1A). TPA inhibited cell growth in a dose-dependent manner with a half-maximal inhibitory concentration (IC_{50}) of 1.13 μM. The viability neutral red cytotoxicity assay was performed to assess the dose-response effect of TPA on spheroids and to compare it with DOX. TPA inhibited the growth of human fibrosarcoma HT1080 spheroids in a dose-dependent manner and reached 70% inhibition at 40 μg/mL when compared with untreated controls—slightly less than DOX used as a positive control at 200 μM (Figure 1B). To verify inhibition morphologically, confocal microscopy was used to demonstrate drastic spheroid reduction in a dose-dependent manner (Figure 1C).

To confirm the identity of the *in vitro* cultured spheroids, a self-renewal assay was performed.[13] TPA demonstrated inhibition of spheroids in the first round in a dose-dependent manner. The cells were then dissociated, and their ability to form spheroids was tested in a second round. Moreover, TPA prevented any spheroid formation with 40 μg/mL treatment (Figure 1D).

Quantitative reverse transcription PCR =(qRT-PCR) results demonstrated that TPA specifically targets and significantly inhibits gene expression of the polycomb group protein enhancer of zester, *EZH2* (5-fold) and its downstream targets aldehyde dehydrogenase 1 (*ALDH1A1* [3.2-fold], a well-characterized CSC marker) and embryonic transcription factor *NANOG* (7.35-fold), compared with that of the controls (Figure 1E). Based on these results, TPA has the ability not only to exhaust viability and maintenance of CSCs but also to prevent their formation via the inhibition of polycomb EZH2 and downstream CSC markers.

Orthotopic and metastatic mouse model generation of human fibrosarcoma

To test the translational relevance of our *in vitro* findings, we designed a series of experiments toward setting up and validating *in vivo* models with successful tumor cell engraftments.

Both orthotopic and metastatic models were successfully generated by using HT1080 fibrosarcoma cells bearing luciferase as a reporter gene. One million HT1080 fibrosarcoma cells were injected at day 0 either in PBS in the tail vein of male nu/nu mice ($n = 5$; metastatic model) or in Matrigel:PBS (1:1) in the quadriceps of male nu/nu mice ($n = 10$; orthotopic model). Tumor growth was determined by IVIS (IVIS Bioluminescence) at days 1 and 49 in the metastatic model (Figure 2A) and at days 1 and 14 in the orthotopic model (Figure 2B). The overall survival of mice comparing the two experimental models is shown in Figure 2C. These initial experiments demonstrated that these cells efficiently engrafted orthotopically and led to fast tumor growth and short survival. While the cells injected intravenously took longer to grow, HT1080 cells showed an efficient and consistent metastatic spread after homing in the lungs.

TPA as single agent inhibited human HT1080 xenograft in immunocompromised mice

We then assessed the ability of TPA to inhibit primary tumor growth *in vivo*. TPA (0.025 mg/h) was administered subcutaneously via osmotic pumps implanted on the backs of the nu/nu mice at day −1 ($n = 10$ biological replicates). TPA dosing was chosen based on solubility in PBS; that is, the highest concentration at which the peptide would remain stable in solution at 37°C for days (50 mg/mL). PBS (vehicle)-filled pumps were used as control treatment. A total of 50×10^4 tumor (HT1080 RFP [red fluorescent protein]-Luc) cells in 30 μL Matrigel were injected in the quadriceps at day 0. The next day (day 1), DOX was administered intravenously at a dose of 15 mg/kg. The rationale for this sequence is the following. In general, in slower-growing tumors, enrollment into treatment groups is based on active tumor growth either based on volumes (e.g., reaching 100 mm^3) or IVIS signal (consecutive measurements showing signal increase). Since these tumor cells are extremely aggressive, any delay in treatment could result in an apparent lack of efficacy; therefore, we tried to compress all these events within a

Figure 1. TPA inhibits HT1080 human fibrosarcoma 3D spheroids growth renewal by targeting EZH2

(A) Antiproliferative activity of TPA in WST-1 assay; $IC_{50} = 1.13$ μM. (B) Cytotoxicity effects of TPA and DOX alone. Neutral red assay: one-way ANOVA followed by a Dunnett's multiple comparison. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (C) Dose-response fibrosarcoma spheroid formation assay with TPA treatment, confocal microscopy analysis (Leica SP5 20×). (D) Fibrosarcoma spheroid self-renewal assay treated with TPA confocal microscope (Leica SP5 20×). (E) mRNA gene expression for *NANOG*, *EZH2*, and *ALDH1A1* in fibrosarcoma spheroid lysates treated with TPA. The results were normalized by glyceraldehyde 3-phosphate dehydrogenase mRNA and are presented as the fold difference using t test. $^*p < 0.05$; $^{**}p < 0.01$.

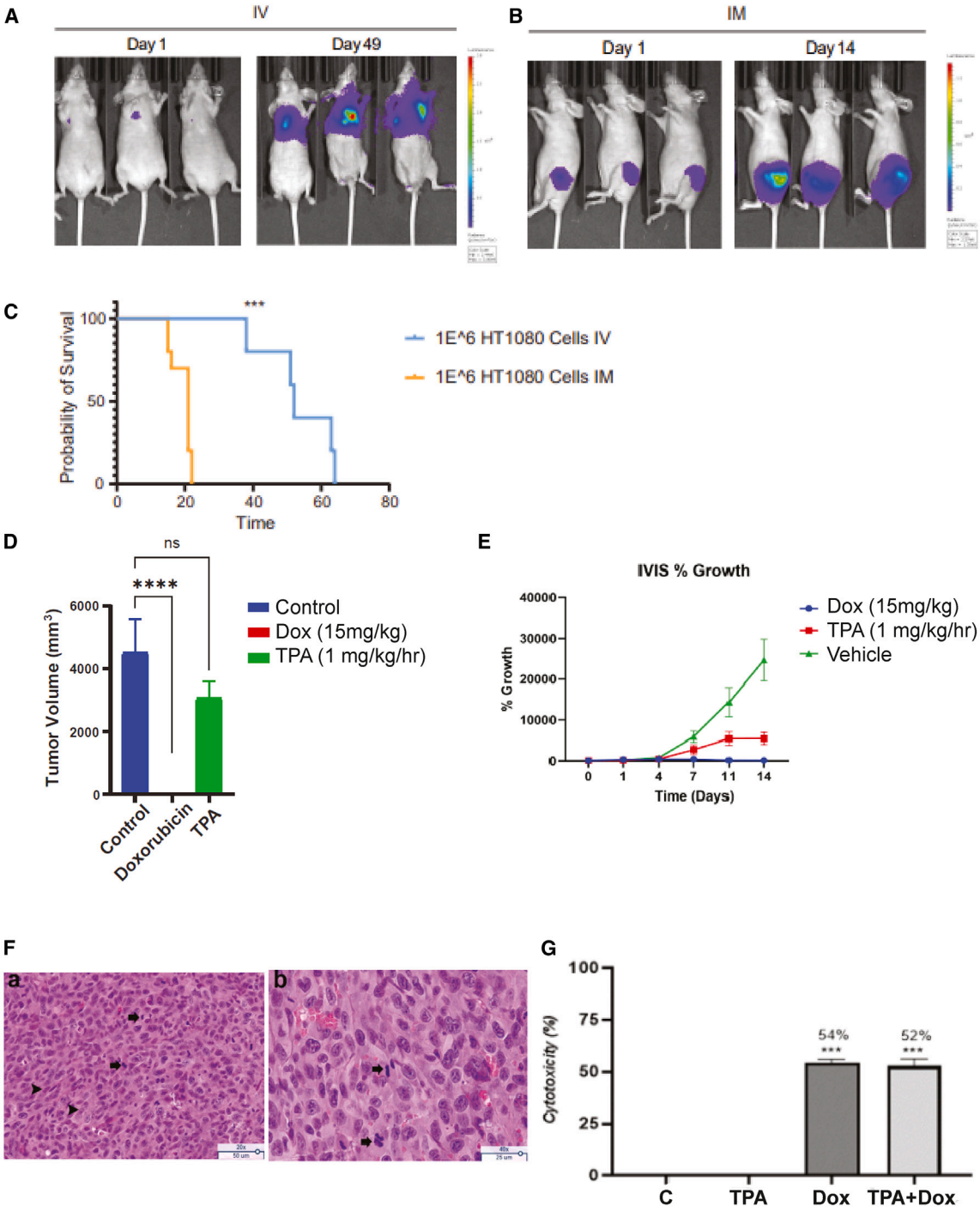

Figure 2. Growth-inhibitory activity of TPA and DOX single treatments in human orthotopic fibrosarcoma xenograft model

(A and B) Orthotopic and metastatic mouse models of human fibrosarcoma were successfully generated. Tumor growth was determined by IVIS in nu/nu mice ($n = 10$) at days 1 and 49 in the metastatic model (A) and at days 1 and 14 in the (B) orthotopic model. (C) Overall survival of mice comparing the two experimental models. (D) Caliper tumor measurements of DOX-treated tumor compared with vehicle control (PBS). (Note: DOX-treated tumors were too small to be assessed by caliper.) (E) IVIS imaging with TPA alone and TPA + DOX-treated tumor compared to the vehicle control ($p < 0.001$). (F) H&E staining of primary tumors after intramuscular injection of HT1080 cells in nu/nu mice. (G) Cardiomyocyte cytotoxicity test; statistical analysis: one-way ANOVA, followed by Dunnett's test. ***$p \leq 0.0001$.

tight schedule around tumor transplantation. In regard to the osmotic pumps, we transplanted them the day before tumor inoculation to allow some time for them to start delivering the peptide since systemic delivery and effects were not expected to be immediate and affect all tumor cells, for example, when compared to intravenous delivery. However, DOX was delivered the day after tumor inoculation to be able to confirm tumor cell viability and recovery after orthotopic inoculation and thus maximize the effects of DOX. This DOX dose is close to the maximum tolerated dose (unpublished data) in this strain and was chosen due to the aggressive nature of the tumor in the current experimental setting. DOX effectively inhibited tumor growth compared to control (PBS) mice when measured using a caliper (Figure 2D). Tumor growth was also monitored by IVIS imaging, which show earlier events like cell death, and reflect overall viability of the tumor (Figure 2E). IVIS imaging also showed tumor growth inhibition with TPA alone and DOX alone when compared to controls ($p < 0.001$).

Histopathological analysis of human fibrosarcoma primary tumors in nu/nu mice was performed. H&E staining of primary tumors after intramuscular injection of HT1080 cells in nu/nu mice revealed a hypercellular tumor composed of spindle-shaped cells, cellular and nuclear pleomorphism, and noticeable anisokaryosis within large nuclei (Figure 2F, arrowheads). Multiple nucleoli were observed in the nuclei of these anaplastic tumor cells, and there were frequent mitotic figures per high-powered field (Figure 2F, a and b, arrows). Low- (a) and high-powered fields (b) of the tumor are depicted (Figure 2F).

It is known that DOX is associated with cardiotoxic side effects. To assess possible TPA-associated cardiotoxicity or to ascertain whether TPA reduces DOX-associated cardiotoxicity, we performed the same cell viability/cytotoxicity assay using AC16 human cardiomyocytes. Figure 2G shows that DOX had a significant cytotoxic effect (54%), while TPA had no observable cytotoxic effect. The combination of DOX and TPA did not diminish the cardiotoxic effect of DOX. These experiments demonstrate the inhibitory effect of TPA on the growth of human HT1080 xenograft as a single agent in comparison with vehicle control.

DOX-induced refractory sarcoma was inhibited by TPA in combination treatment

We then performed a series of experiments to understand whether the action of TPAs in combination with DOX (standard sarcoma therapy) would demonstrate any additional beneficial effect. In an initial experiment, mice were treated with DOX alone and in combination of DOX + TPA (defined as "combo"). TPA was administered via osmotic pumps from day −1 in a concentration of 50 mg/mL at a dose of 0.025 mg/h for the group of mice treated with TPA + DOX combination. DOX was given intravenously at a concentration of 15 mg/kg in both groups. A total of 50×10^4 tumor cells (HT1080 RFP-Luc in 30 μL Matrigel) was injected in the quadriceps at day 0. DOX alone or in combination treatment inhibited tumor cell growth until tumor in the DOX-treated group relapsed around day 29. TPA pumps thereafter were retransplanted on day 28 for TPA administration, and DOX was injected again on day 29. Combined treatment of TPA + DOX led to a 7-fold decrease in tumor growth compared with DOX alone (Figure 3A). IVIS imaging is shown (Figure 3B). Body weight measurements showed no difference between treatment groups (Figure 3C), arguing again for no toxicity of TPA. The experimental data showed eventual tumor growth relapse in mice treated with DOX alone, whereas DOX combined with TPA led to 7-fold inhibition of tumor growth.

TPA + DOX administration led to overall survival that was twice that of controls

To correlate the beneficial effect of TPA on DOX-treated relapsed tumors to overall survival, cells from relapsed DOX mice were transplanted in the four different cohorts of mice: control (PBS vehicle), TPA, DOX alone, and TPA+ DOX. As depicted (Figure 3D), median survival for the combination of TPA + DOX, DOX alone, and control (PBS) was 35, 28, and 21 days, respectively. TPA alone in this setting had no significant effect on survival compared to PBS control, but in combination with DOX, it led to increased overall survival (67% of mice; $^*p < 0.0005$) versus DOX-treated mice (33%; $^*p < 0.05$) compared to the control cohort. Thus, inhibition of refractory tumor growth with combination treatment led to improved (2×) overall survival compared to DOX alone treated mice.

Combined TPA + DOX led to a 3-fold greater overall survival in the experimental metastasis model

Since CSCs play an important role in metastasis,[12] we hypothesized that TPA could target these cells and improve treatment outcomes. Thus, the action of TPA alone and in TPA + DOX combination was also tested in an experimental metastatic model, where 1 million HT1080 (RFP-Luc cells) were injected intravenously via the tail vein at day 0 in nu/nu mice ($n = 10$ per group) for site-specific dissemination metastasis detection by IVIS throughout the body. TPA was administered via 28-day osmotic pumps transplanted at day −1, while DOX was injected intravenously at day 3 (15 mg/kg) to allow for tumor cell engraftment and proliferation. The median days of survival for control, TPA, DOX alone, and TPA + DOX were 50, 65, 79, and 118 days respectively.

No difference in tumor metastatic burden was observed between the DOX- and TPA + DOX-treated animals (Figure 3E). DOX and TPA alone showed survival advantages, but there was no statistically significant difference between these groups. However, the TPA + DOX combo group showed a statistically significant difference, with a 3-fold overall increase in survival compared to controls (Figure 3F).

Statistical analysis was performed for the time highlighted—that is, the time that TPA was delivered. Then, we removed the pumps, but we continued to monitor the mice, and the differences became non-significant.

Despite no differences in experimental metastasis growth between the treatment and control groups, the DOX + TPA combo group

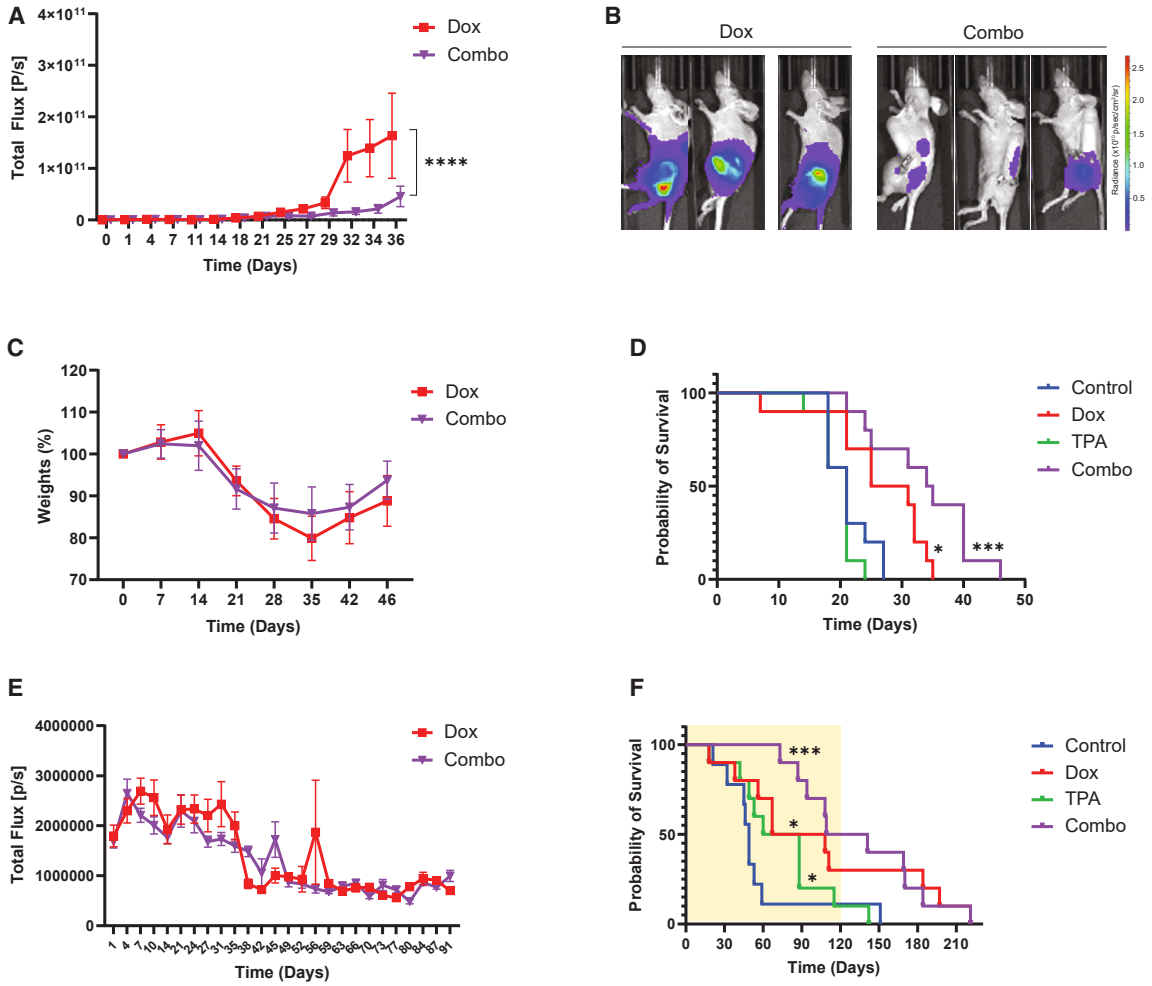

Figure 3. TPA inhibits DOX induced refractory sarcoma and extends significantly overall survival

(A) Combination treatment of TPA + DOX compared with DOX alone (***p = 0.0002; mixed-effects model with the Greenhouse-Geisser correction and α = 0.05). Data are presented as mean values ± SEM. (B) IVIS bioluminescent images of the tumors. (C) Mice body weight measurement between the DOX and combo groups (p = 0.7992; mixed-effects model with the Greenhouse-Geisser correction and α = 0.05). (D) Overall survival experiment in a refractory growth experiment in combination treatment (Log-rank (Mantel-Cox) test; p = 0.0005) versus 33% in DOX (p = 0.0146) compared to control cohort. Data are presented as mean values ± SEM. (E) Tumor metastatic burden in DOX and TPA + DOX-treated animals (p = 0.3255; mixed-effects model with the Greenhouse-Geisser correction and α = 0.05; days 1–31). Data are presented as mean values + SEMs. (F) Overall survival in experimental metastasis in TPA + DOX-treated animals compared to the control group (log rank Mantel-Cox test; ***p = 0.0008), DOX (*p = 0.0357), or TPA alone (*p = 0.0269).

demonstrated even higher overall survival (3-fold) in the experimental metastasis setting.

DISCUSSION

There is an unmet clinical need for the development of new combination therapies for patients with relapsed/refractory malignancies, especially high-grade sarcomas. These tumors are both relatively chemotherapy and radiotherapy resistant, with occasional local and frequent distant tumor relapse resulting in patient death. The standard of care for high-grade fibrosarcoma is combined radiation and surgical resection, as well as chemotherapy for patients with large tumors or metastases. Chemotherapy regimens involve DOX (adriamycin) in combination with IFO and sometimes mesna and dacarbazine in neoadjuvant settings.[36]

An overall improvement in survival of 4%–11% has been detected in sarcoma patients treated with chemotherapy. Even though multiple agents to treat soft tissue sarcomas are in the clinical trial phase,[37–43] including immunotherapy and immune checkpoint inhibitors,[38,40,41] an urgent unmet need exists to prevent tumor relapse and metastases in patients with large high-grade sarcomas. One of the causes of refractory cancer is cellular heterogeneity that is produced by a small population of CSCs.[4–8] The use of CSC targeting agents with standard systemic cytotoxic chemotherapy was shown to prevent recurrence

and extend survival.[44,45] This small-molecule strategy was implemented to target CSCs to prevent tumorigenesis[46,47]

Cytotoxic drugs, however, may spare CSCs, which are considered to be the root of cancer recurrence and drug resistance.[48,49] There is experimental evidence to suggest that CSCs not only initiate and facilitate metastatic tumor spread but also determine the organ for metastatic dissemination.[50] DOX is effective in treating tumor cells but not necessarily the CSCs, which eventually leads to relapse.[51–53]

In clinical trials small-molecule compounds have been used to target CSCs[54,55] through different signaling pathways.[56] For example, ONC201, which is in a phase 1/2 study for patients with advanced cancer (this study was registered at ClinicalTrials.gov: NCT02038699), can inhibit CSC self-renewal and the expression of CSC-related genes in prostate and glioblastoma tumors by suppressing the Wnt signaling pathway.[57,58] The stemness of CSCs is maintained by microenvironmental factors through pathways that promote self-renewal, such as the Wnt/β-catenin, Notch, and Hedgehog pathways.[59] Enhancer of Zeste Homolog 2 (EZH2) plays a very important role in tumor metastasis and is not only considered to be a CSC biomarker[60–68] but also plays a major role in CSC maintenance.[63–67] EZH2 serves as the catalytic subunit of PRC2, mediating H3K27me3 deposition and transcriptional repression. EZH2 may act in a noncanonical manner depending on cellular context, independent of PRC2 and its histone methyltransferase function, activating the downstream genes through non-histone target methylation or direct binding to proteins.[69] EZH2 is overexpressed in different malignancies, including soft tissue sarcomas.[70–75]

Tazemetostat, an (EZH2) inhibitor was approved by the US Food and Drug Administration in 2020 as the first epigenetic therapy for solid tumors.[76–78] EZH2 plays a key role in the EZH2-RUNX3-ΔNp63α-SETDB1 signaling pathway that influences an aggressive CSC phenotype of the epithelial squamous cell carcinoma.

EZH2 is upstream of both SETDB1 and ΔNp63α, activating these targets via repression of the tumor suppressor RUNX3. It was shown that targeting EZH2-RUNX3-ΔNp63α-SETDB1 signaling pathway with inhibitors of EZH2 results in the activation of RUNX3 and suppression of both SET domain bifurcated 1 (SETDB1, also known as ESET) and truncated P63 protein ΔNp63α, antagonizing, impairing CSC population.[79] EZH2 expression, along with other histone modifying enzymes, is thought to control the differentiation lineage process in MSCs, which are considered the source of sarcoma stem cells and, therefore, determine disease progression and outcome.[68] EZH2 is upstream from ALDH1A1,[80,81] and is an established CSC biomarker that is unique to sarcomas, both soft tissue and chondrosarcoma.[13,14,82–84] Inhibition of ALDH1A1 has been linked to a beneficial effect in confronting drug resistance and poor survival.[85–87] Embryonic stem cell transcription factor NANOG is downstream of EZH2 and is involved in drug resistance and tumor progression.[88–91] TPA inhibits the enhancer of EZH2, along with its downstream targets. In our experiments, EZH2 activity was not linked to its methyltransferase function, and synergistic inhibition of EZH2 and its downstream targets, CSC biomarkers ALDH1A1 and NANOG, was observed to be associated with TPA treatment. We assume, therefore, that EZH2 functions in HT1080 as a transcriptional co-activator independent from PRC2. As previously mentioned, CSCs are thought to be responsible for drug resistance and relapse.[92–96] For this reason, therapies that combine cytotoxic agents and CSC targeting agents are attracting more attention and maybe a preferred approach to address malignant disease progression and extend survival.[97–99] TPA was not toxic to normal cells while targeting tumor CSCs and tumor cells. In combination with DOX, it substantially inhibited refractory cancer cells after DOX therapy and led to statistically significant overall survival in our mouse model as well as in an experimental metastasis setting.[100] The potential benefit of this peptide might extend to other high-grade sarcomas as well. Interestingly, HT1080 is also considered to be an aggressive, highly malignant dedifferentiated chondrosarcoma with fibroblastic features due to the presence of IDH1 mutations.[101] In our *in vitro* preliminary data (not shown), TPA manifested an inhibitory effect on other malignancies, such as primary undifferentiated pleomorphic sarcoma, select osteosarcoma, and even triple (estrogen, progesterone, and $Her2^{-}$)-negative breast cancer MDA-MB 231 cell lines. The ability of TPA to prevent spheroid formation might be linked to the inhibition of tumor metastases initiation. With this knowledge, future investigations will involve testing this molecule in spontaneous metastatic models, not only for fibrosarcomas and dedifferentiated chondrosarcomas but also for other sarcoma types,[102] especially epithelial sarcomas, since EZH2 was shown to be a driver of those tumors.[72,77]

While targeting CSCs enhanced the effects of DOX in clinical settings, the existing combination treatments did not show any improvement in survival.[103,104] To better mimic human cancer and evaluate treatment efficacy, we will analyze the effect of TPA on human refractory patient-derived orthotopic xenografts for both fibrosarcoma and dedifferentiated chondrosarcoma.[105–107] Xenotransplantation to the orthotopic site that matches the patient's tumor site will more closely mimic the patient's disease to better assess treatment efficacy.[106,107]

Outcomes from a CSC-targeted therapy may look different from those of traditional treatments; for example, bulk tumor cells may not be reduced immediately.[44] Thus, CSC-targeting strategies will need to be combined with other cytotoxic standard regimens. Ideally, anti-CSC therapy could also be used as maintenance therapy to prevent recurrence and prolong survival. While plasticity and heterogeneity of CSCs present a challenge for precision oncology as future tumor therapy, more research is needed to develop strategies to eradicate CSCs.

To achieve durable therapeutic outcomes, overcoming drug resistance will require different strategies to target CSCs. Dendritic cells (DCs) serve as a potent antigen presenting tool for motivating antitumor responses to eradicate CSCs effectively. DC-pulsed vaccines are under clinical investigation and have shown promising therapeutic modality.[108,109] Metabolic reprogramming plays an important role in regulating the growth and activity of CSCs.[110] Blocking mitochondrial

oxidative phosphorylation therapeutically suppresses CSC growth, including sphere and tumor formation potential.[111–113] Evidence has suggested that the plasticity of CSCs can be exploited by differentiation therapy to achieve their eradication, based on the principle to convert undifferentiated cells with high malignancy into differentiated cells with low malignancy.[114] Targeting CSC biomarkers to eradicate CSCs selectively may be an effective strategy to inhibit cancer progression and reduce the risk of tumor relapse.[115–117] Some of the approaches combine radioimmunotherapy and biomarkers.[118–120] Aptamers, short, single-stranded DNA or RNA molecules that bind to specific targets with high affinity and selectivity, may be used to carry biomarkers.[121,122] Another approach to target CSCs is via signaling pathways that regulate CSC self-renewal capabilities.[123] Some possibilities include neurogenic locus notch homolog protein1/protein kinase B,[124] wingless-related integration site (WNT/β-catenin) transforming growth factor β signaling pathway,[125,126] and Salvador-Warts-Hippo (Hippo signaling).[127]

In this work, we show that the ability of TPA to eradicate and deplete the CSC pool by targeting its stemness factors and biomarkers leads to the inhibition of DOX-induced refractory cancer and significantly increases overall survival both in primary tumor growth and metastatic models.

Treatment outcomes might be affected by the timing and sequence of treatments. In this work, we chose to continuously deliver TPA and initiate early TPA treatment to maximize the effectiveness while targeting CSCs in the early stages of tumor development. We did not expect single TPA treatment to have a major impact on tumor burden initially (although as a single agent, TPA inhibits cancer growth in comparison with control in a statistically significant manner), but in combination with DOX, which targets the bulk of the tumor by affecting proliferating cells, we hypothesized that will result in longer-term remission, prevent relapse, and prevent metastatic spread. Delay in TPA treatment will most probably result in less-effective response, since metastatic spread is likely to have occurred, but also because DOX treatment would be less efficient as the tumor grows, and the bioavailability of the drugs might also be affected by tumor size. However, it is likely that in a clinically relevant setting, when the primary tumor is resected, the combination of TPA and DOX could be an effective treatment regimen, not only to inhibit refractory cancer but also to prevent the spread in still undetectable metastatic foci or in the early dissemination stage due to the ability of TPA to prevent spheroid (CSC) formation, as described in this work. In addition, the extension of overall survival that we observed is the most relevant clinical indication of the benefit this treatment could have.

In the results section, we demonstrated the efficacy of TPA in combination with DOX, which led to the inhibition of refractory cancer progression. Moreover, we observed statistically significant increases of overall survival (2-fold) in a primary tumor growth model and (3-fold) in an experimental metastasis setting. Combination treatment is recommended, since drug resistance to a single agent is likely to be developed.[128,129] Simultaneous administration of TPA along with standard chemotherapy, as well as implementation of potential maintenance therapy, is possible. Recently, the correlation between patient survival and CSCs was highlighted in selected publications.[130–132]

Future studies will shed light on the ability of TPAs to inhibit/prevent metastasis formation in a clinically relevant setting. In summary, TPA addresses an urgent clinical need and holds promise as a future treatment option in this lethal cancer, insofar as we observed increased survival and inhibition of refractory cancer inhibition via CSC suppression.

MATERIALS AND METHODS

Cell culture

The human HT1080 fibrosarcoma cells (CCL-121, American Type Culture Collection [ATCC]) were maintained in a culture medium containing Eagle's minimal essential medium, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and periodically checked for mycoplasma per the manufacturer's instruction using the MycoAlert Mycoplasma Detection Kit (LT07–118, InvivoGen).

For the *in vivo* experiments, we used human fibrosarcoma HT1080, RFP-Luc. Cytomegalovirus stably transfected cells (SC-1482, Cellomics Technology) were xenografted into mice. For the cardiotoxicity assay, the AC16 human cardiomyocyte cell line was used (SCC109, Millipore Sigma). The complete medium was DMEM:F12 (ATCC) and 12.5% FBS (ATCC).

TPA

TPA is a chemically synthesized 15-amino acid peptide with a molecular weight of 1709.75 g/mol.

Rapid cell proliferation assay

The procedure was done following the manufacturer's instructions (rapid cell proliferation assay; 2210M, Millipore Sigma). In brief, cell suspensions were seeded onto 96-well plates (100 μL/well) with or without the test compounds and were incubated at 37°C in a humidified incubator with 5% CO_2 for the indicated treatment time. Then, 10 μL WST-1 reagent solution was added to each well, and the plate was incubated at 37°C for 2 h. After incubation, the absorbance was measured at 450 nm with a multiplate reader.

Cell viability/cytotoxicity assay

Neutral Red cell viability assay was used to detect cell viability/cytotoxicity according manufacturer's instructions (cytotoxicity kit no. 234049, Abcam). In brief, 150 μL of neutral red dye (100 μg/mL) was dissolved in the serum-free (pH 6.4) culture medium and incubated for 3 h at 37°C. Cells were washed with PBS, and 150 μL elution medium (EtOH/AcCOOH, 50%/1%) was added, followed by gentle shaking for 60 min. Absorbance was recorded at 540 nm using a microtiter plate reader.

Cardiotoxicity assay

The cell viability/cytotoxicity assay described above was applied to determine the cytotoxicity of cardiomyocytes using the AC16 human cell line.

Establishing 2D and 3D cultures (spheroids) of human HT1080 fibrosarcoma cell line

These procedures were previously described by our group for human chondrosarcoma cell cultures.[13] Two-dimensional (2D) cell cultures of HT1080 fibrosarcoma cells were grown in T175 flasks (no. 353112, Falcon) in a 37°C, 5% CO_2 incubator for 2 days until confluency. Absence of mycoplasma contamination was confirmed for the cell lines used in all experiments. After trypsinization, a 40-μm cell strainer (431750, Corning) was used to create a single-cell suspension. The 3D cultures were established by seeding cells at an optimized density of 500,000 cells/well in 6-well low-attachment plates (3471, Corning) in Advanced DMEM/F12 media (ATCC) with reduced FBS (12634028, Thermo Fisher Scientific), including 10 ng/mL basic fibroblast growth factor (FGF; AF10018C, Peprotech), 10 ng/mL epidermal growth factor (EGF; AF10015, Peprotech), and 10 μL/mL N_2 supplement (17502048, Thermo Fisher Scientific). The human EGF (10 ng/mL) and human basic FGF (10 ng/mL) were added to each well every other day. After 5–6 days, sarcosphere growth was observed. The images of the colonies were taken on day 6 on a Leica DMI3000 B inverted microscope.

Self-renewal assay

To confirm that we had generated sarcospheres, we performed a sarcosphere self-renewal assay. Cultured 3D sarcospheres were dissociated into single-cell suspensions and inoculated into medium without serum in T175 flasks (353112, Falcon). Only cells from spheroids would propagate in a serum-deprived condition. Once cells reached confluency they were harvested, reseeded as single cells, and plated at a density of 500,000 cells/well in 6-well ultralow attachment plates (3471, Corning) and in 150 × 15-mm Petri dishes (351058, Falcon). We then used the same media and supplement content described above for 3D cultures. Sarcosphere growth was monitored every day for 56 days using a confocal Leica TCS SP5 microscope with 10× and 20× objective lenses.

Spheroids lysates

Spheroids were cultivated, and treatment was added every day for 3 days. After 4 days, cells were washed with ice-cold PBS, and protease inhibitor was added to the CelLytic M cell lysis reagent (Sigma-Aldrich, Merck KGaA). Following the collection of cells and cell membrane lysis with an 18G needle, the cells were centrifuged, and the supernatant was collected. The protein content was measured using a NanoDrop spectrophotometer.

RNA extraction and cDNA synthesis

The procedure was performed as described.[15] Frozen HT1080 spheroids lysates were prepared with 500 μL Trizol reagent (15596018, Thermo Fisher Scientific), homogenized, and incubated for 5 min at room temperature. After adding 100 μL chloroform (CX1058-6, Sigma-Aldrich), the samples were incubated for 10 min at room temperature, vortexed, and centrifuged. The aqueous phase was transferred to a new microfuge tube with 500 μL 2-propanol (I9516, Sigma-Aldrich), vortexed, and incubated for 10 min at room temperature. The supernatant was removed after centrifugation, and the remaining RNA pellet was washed with cold 70% ethanol (V001229, Sigma-Aldrich). The samples were centrifuged, and the RNA pellet was dried and then dissolved in 20 μL RNase-free water (P1193, Promega). RNA was quantified, and the quality of RNA was analyzed using NanoDrop (ND-1000, Thermo Fisher Scientific). Reverse transcription was performed using 2,500 ng total RNA and 4 μL reagents (SuperScript IV VILO Master Mix). Primers were annealed for 10 min at 25°C. They were incubated for 10 min at 50°C and then at 95°C for 5 min. The volume of cDNA was diluted to obtain 100 μL of 20 ng/μL cDNA.

qPCR

Forward/reverse primers, TaqMan Fast Advanced Master Mix for qPCR (4444557, Applied Biosystems), and cDNA were mixed in PCR tubes. The qPCR was performed in triplicate wells in a 384-QuantStudio TM 6 Flex Systems. Primers were purchased from OriGeneTechnologies: HP215086, *NANOG*; HP207764, *EZH2*; and HP200638, *ALDH1A1*. The nucleotide sequences for corresponding primers were h*NANOG(forward)*:*5'CTCCAACATCCTGAACCTC AGC3'*; h*NANOG*(*reverse*):5'CGTCACACCATTGCTATTCTTCG3'; h*EZH2(forward)*:5'GACCTCTGTCTTACTTGTGGAGC3'; h*EZH2 (reverse)*:5'CGTCAGATGGTGCCAGCAATAG3'; h*ALDH1A1*(*forward*):5'CGGGAAAAGCAATCTGAAGAGGG3'; hALDH1A1(reverse):5'GATGCGGCTATACAACACTGGC3'. Relative mRNA gene expression was calculated using the fold change ΔCT analysis method, and expression was normalized by the housekeeping gene. Values were expressed as fold change compared to the control condition.

In vivo testing of TPA peptide in human xenograft of soft tissue sarcomas

All animal procedures and experiments were performed in compliance with University of Miami Institutional Animal Care and Use Committee-approved protocol no. 19-079. Human HT1080 (RFP-Luc) was injected into nu/nu mice quadriceps. This study was approved by the ethical review and human subject research review board of the University of Miami, under University of Miami institutional review board protocol 20100167, "Primary cell cultures generation from sarcoma patients tissues and biopsies." HT1080 is considered to be dedifferentiated chondrosarcoma with fibroblastic features due to the presence of the IDH1 mutation.[101]

Experimental metastasis

The experimental metastasis model was performed with the fibrosarcoma HT1080 (RFP-Luc) cell line. One million cells were injected intravenously through the tail vein to determine the survival advantage after metastatic growth of the tumor and treatment in four groups of nu/nu immunocompromised mice (control, TPA, DOX alone, and TPA + DOX; 10 mice per group). Metastatic growth was

assessed *in vivo* by IVIS (IVIS Spectrum, Revvity). When feasible, metastatic growth was confirmed *ex vivo* using IVIS and by histology after organ isolation. DOX (15 mg/kg) was injected intravenously once, while TPA was delivered through an osmotic pump implanted subcutaneously for 14 days (0.025 mg/h). In all *in vivo* experiments, control animals were also transplanted subcutaneously with osmotic pumps filled with vehicle solution (PBS) at the same time point and monitored (tumor burden and health status) in the same manner.

Relapse experiments

Relapse experimental setup was done with HT1080 (RFP-Luc) tumor cells that relapsed after DOX treatment *in vivo*. Cells obtained from orthotopic tumors that have relapsed after two rounds of DOX were isolated, expanded *in vitro* and reinjected in mice orthotopically. In an initial round of treatments, mice were enrolled in four distinct groups (control, TPA, DOX alone, TPA + DOX; 10 mice per group). DOX (15 mg/kg) was injected intravenously once, while TPA was delivered continuously through an osmotic pump implanted subcutaneously for 28 days (0.0125 mg/h). Control animals were also transplanted subcutaneously with osmotic pumps filled with vehicle solution (PBS) at the same time point and monitored (tumor burden and health status) in the same manner.

Statistical analyses

All *in vitro* experiments were performed in triplicate, and $p < 0.05$ was considered statistically significant. One-way ANOVA followed by a Dunnett's multiple comparison was used for the cytotoxicity (neutral red) assay to compare treatment groups with the control. Gene expression data from the qRT-PCR methods were analyzed using one-way unpaired t tests. Regarding animal size, for comparisons of different treatment regimens with corresponding controls, groups with 8–10 mice will provide $\geq$85% power to detect an effect size of 1.6 between groups with two-sided type I error of 5%. The effect size was defined as mean tumor burden or luminosity difference between groups/pooled SD. We began treatment groups with 20% excess animals to account for failed engraftments. Survival was estimated with the Kaplan-Meier survival curve method, and differences in survival were calculated by log rank (Mantel-Cox) test. For group comparisons in efficacy studies, a mixed-effects model with the Greenhouse-Geisser correction and $\alpha = 0.05$ was used. Statistical tests were performed using GraphPad Prism 6.0.

DATA AND CODE AVAILABILITY

- Nucleotide sequences for primers were obtained from the NCBI.
- The composition of matter, chemical structure of TPA peptide is available and is under University of Miami patent PCT/US2023/073277, published as WO2024050501A2.
- Tyr peptide composition and methods for use: it is not under another protein database.

ACKNOWLEDGMENTS

The present study was supported by Miami Center of Orthopaedic Research and Education and the Department of Orthopedics, University of Miami, Miller School of Medicine. We thank the Cancer Modeling Shared Resource (RRID: SCR_022891) of the Sylvester Comprehensive Cancer Center for support with the *in vivo* modeling, efficacy studies, noninvasive imaging and histology work. This work was also supported by funds from 1P30CA240139-01 from the National Cancer Institute.

AUTHOR CONTRIBUTIONS

K.G. directed this work and contributed to the experimental design and planning of this study from the inception to data interpretation. D.B., E.R., and N.C.G. performed the *in vivo* animal studies. D.B. led that effort and crucially contributed to experimental design and data interpretation. C.D. and D.M. performed the tissue culture and *in vitro* experiments. D.M. and C.D. were involved in data processing and statistical analyses contributions. H.T.T. performed the orthopedic surgeries and was responsible for providing tissue biopsies from which the primary cultures were generated for histiocytoma. He was also involved in data interpretation and manuscript editing for this study.

DECLARATION OF INTERESTS

K.G. is the inventor on the PCT patent of the University of Miami PCT/US23/73277.

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