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Sequential application of a cytotoxic nanoparticle and a PI3K inhibitor enhances antitumor efficacy

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Abstract

Nanomedicines that preferentially deploy cytotoxic agents to tumors, and molecular targeted therapeutics that inhibit specific aberrant oncogenic drivers are emerging as the new paradigm for the management of cancer. While combination therapies are a mainstay of cancer chemotherapy, few studies have addressed the combination of nanomedicines and molecular targeted therapeutics. Furthermore, limited knowledge exists on the impact of sequencing of such therapeutics and nanomedicines on the antitumor outcome. Here we engineered a supramolecular cis-platinum nanoparticle, which induced apoptosis in breast cancer cells but also elicited pro-survival signaling via an epidermal growth factor receptor-phosphatidylinositol 3 kinase (PI3K) pathway. A combination of mathematical modeling and *in vitro* and *in vivo* validation using a pharmacological inhibitor of PI3K, PI828, demonstrate that administration of PI828 following treatment with the supramolecular cis-platinum nanoparticle results in enhanced antitumor efficacy in breast cancer as compared with when the sequence is reversed or when the two treatments are administered simultaneously. This study addresses, for the first time, the impact of drug sequencing in the case of a combination of a nanomedicine and a targeted therapeutic. Furthermore, our results indicate that a rational combination of cis-platinum nanoparticles and a PI3K-targeted therapeutic can emerge as a potential therapy for breast cancer.

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Conflict of interest: PS, SB and SS are listed on a patent filed on the cisplatin nanoparticles by BWH. SS serves on the Scientific Advisory Board (SAB) and holds equity interest in Cerulean Pharmaceuticals, a platform-based company that develops dynamically tumor-targeted nanopharmaceuticals. In addition, SS also co-founded and holds equity with Invictus Oncology, an R&D company focused on platinum-based nanoparticles. SS serves on the board of directors and SAB of Invictus Oncology. SS's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners Healthcare in accordance with their conflict of interest policies.

Supplementary information: Text and figures included.

Supporting Information Available: Details of the mathematical model used to predict cell viability and perform parameter estimation is available free of charge via the Internet at <http://pubs.accr.org>.

Keywords

PI3K; nanoparticle; cisplatin; mathematical modeling; cancer

Quick Guide to Equations and Assumptions

Here we develop a minimal mathematical model containing three critical proteins: pAkt, XIAP, and caspase-3. The effects of cisplatin nanoparticles and PI828 treatments were also included. The basic reaction pathways are shown in Fig. 1a.

Akt phosphorylation is induced by activated PI3K. Once activated, Akt negatively regulates apoptotic pathways at a pre-mitochondrial level by blocking the actions of proteins such as Bad and Bax, which in turn inhibits the release of cytochrome *c* into the cytosol. It has also been reported that Akt directly phosphorylates and inactivates cell death protease caspase-9 (22). For simplicity and to keep the number of parameters manageable, these multiple pro-survival initiatives are considered to have the net effect of negatively impacting caspase-3 production, see (1) in Fig. 1a.

It has been also reported that active caspase-3 cleaves Akt *in vitro*, and inhibiting caspase-3 can block this effect (23). Thus, a negative feedback is assumed to represent the cleavage of Akt by caspase-3, (2) in Fig. 1a. In addition, activated Akt interacts with and phosphorylates XIAP, protecting it from auto-ubiquitination (24), (3) in Fig. 1a.

XIAP inhibits caspase-3 by the degradation of caspase-3 ((4) in Fig. 1a), and caspase-3 inhibits XIAP through ubiquitination and degradation (18, 19, 25, 26), (5) in Fig. 1a. Caspase-3 can also inhibit XIAP by inactivating XIAP through formation of a complex (18).

To include the effect of treatment, we first note that based on our experimental data and other studies (23, 27), cisplatin nanoparticles increases the production of pAkt. This activation might be mediated through EGFR and PI3K. Similar to previous studies (23), our data shows an increase in caspase-3 due to cisplatin nanoparticles. This increase could be due to activation of FasL, which in turn activates caspase-8 and subsequently caspase-3(28, 29). Another possible pathway is the release of cytochrome *c*, which subsequently activates caspase-9 and caspase-3(29, 30).

In addition, phosphorylation of XIAP by Akt protects ubiquitination and degradation of XIAP in response to cisplatin. Since we have only considered the active form of XIAP in our model, we assume that cisplatin nanoparticles reduce XIAP, as observed in the experimental data. The reduction in XIAP could be also due to increase in caspase-3. Finally, we assume that PI828 inhibits phosphorylation of Akt by binding to PI3K.

Using all of the aforementioned considerations, a system of equations corresponding to the network shown in Fig. 1a can be formulated that includes the production, decay, inhibition and treatment effects of these proteins. Letting $P = [\text{pAkt}]/P_0$, $C = [\text{Casp3}]/C_0$, $X = [\text{XIAP}]/X_0$ denote the nondimensional concentrations of the three proteins of interest, the system of equations corresponding to the network shown in Fig. 1a is given by:

$$\frac{dP}{dt} = \frac{k_p + \lambda_p f_p(t)}{1 + \alpha_p C + \gamma_p g(t)} - \delta_p P,$$

$$\frac{dC}{dt} = \frac{k_c + \lambda_c f_c(t)}{1 + \alpha_c P} - \beta_c X C - \delta_c C,$$

$$\frac{dX}{dt} = k_x + \lambda_x P X - \beta_x C X - \delta_x X,$$

where the concentration of PI828 is denoted by $g_i(t)$ and the effects of cisplatin nanoparticle on pAkt and cleaved caspase-3 production are denoted by $f_p(t)$ and $f_c(t)$ respectively. The release of cisplatin from nanoparticles is given by a biexponential release profile that captures the rapid burst upon administration followed by a sustained release. We assume that the effect of cisplatin nanoparticle on pAkt and cleaved caspase-3 production satisfy the relations:

$$\frac{df_j}{dt} = r_{c_i} (t - T_i) - d_j f_j, \quad j=c, p,$$

where c_i is the intracellular concentration of cisplatin and T_i is an intracellular time delay. The Supplement contains additional details of the models used for nanoparticle release and transport, and subsequent effects on protein expression.

The parameters in the mathematical model were estimated from in vitro measurements of the relative expression of caspase-3, pAkt and XIAP by utilizing the Matlab toolbox PottersWheel (www.potterswheel.de). The details of the parameter estimation for protein expression and treatment effects can be found in the Supplement.

We can then consider the effects of cisplatin nanoparticle and/or PI828 therapies on tumor cells at the population level, Fig. 1b. For tumor cells, we assumed that birth rate is proportional to pAkt expression while death rate is proportional to cleaved caspase-3 expression:

$$\frac{dN}{dt} = [\lambda_N P - \delta_N C] N.$$

From this relation, we propose that the resultant cell viability of combinations of cisplatin nanoparticle and PI828 treatments can be predicted based solely on the concentrations of these two proteins; see Supplement for parameters.

Introduction

Cancer chemotherapy relies on rational drug combinations that inhibit distinct cellular targets. While the earlier generation cytotoxics were combined to target different components of the cell division machinery, the emerging paradigm is the combination of cytotoxics with molecular-targeted therapeutics. For example, in a recent study, sorafenib with idarubicin and cytarabine produced a high complete response rate in FLT3-mutated patients with acute myeloid leukemia (1). Similarly, gefitinib was shown to increase the efficacy of taxanes in breast cancer by inhibiting the EGFR-Akt signaling pathway (2). Indeed, many of these combinations are further rationalized by the feedback upregulation of these molecular targets following treatment with cytotoxic chemotherapy (3). A critical

question that therefore arises is whether the sequence of administration of the agents can potentially impact the antitumor outcome? For example, the pretreatment with the anti-angiogenic drugs was hypothesized to transiently normalize abnormal tumor vasculature and thereby improve delivery of subsequent chemotherapy (4-6). However, in a recent provocative clinical study, Van der Veldt *et al* demonstrated that pretreatment with bevacizumab reduced both perfusion and net influx rate of [^{11}C] docetaxel in non small cell lung cancer (7), highlighting the critical need to further understand drug scheduling for optimizing antitumor outcome.

Another emerging paradigm in cancer chemotherapy is the use of nanomedicines, which preferentially accumulate in the tumor through the 'enhanced permeability and retention (EPR)' effect (8). Furthermore, nanomedicines can be tailored for spatial and temporal controlled release of the payload (9). Recent clinical studies are currently underway that combine nanomedicines and molecular-targeted agents. For example, the combination of albumin-bound paclitaxel, carboplatin and trastuzumab was found to be highly active in HER2-over expressing metastatic breast cancer (10). Similarly, a combination of liposomal doxorubicin and bortezomib is approved for use in relapsed or refractory multiple myeloma (11). However, to the best of our knowledge, no studies have been conducted to elucidate the impact of sequencing the administration of a cytotoxic nanoparticle and a molecular targeted therapeutic on the antitumor outcome.

In this current study, we explored the impact of sequencing of a cisplatin-based supramolecular nanoparticle with an inhibitor of phosphatidylinositol-3-kinase (PI3K). Cisplatin is widely used, including in testicular, ovarian, cervical and lung cancer (12, 13), and is reported to be effective in triple negative breast cancer (14). The use of cisplatin is however dose-limited due to nephrotoxicity (15). As a result, in a recent study, we engineered a polymeric cisplatinum (II) nanoparticle, which preferentially accumulated in the tumor and bypassed the kidney, and exhibited an enhanced antitumor efficacy compared to cisplatin (16). However, recent studies have indicated that cisplatin can upregulate PI3K signaling, which can attenuate apoptosis via survivin (17). This suggests that a rational combination of a PI3K inhibitor and platinum-based chemotherapy can result in increased antitumor outcome. Indeed, here we demonstrate that treatment with a novel self-assembling cis-platinum nanoparticle (SACN) results in a similar activation of PI3K-Akt signaling, providing the interesting opportunity to explore the earlier raised question on the impact of drug and nanoparticle sequencing on outcome. We used mathematical modeling to predict the optimal temporal scheduling of SACNs and a PI3K inhibitor, which were then validated *in vitro* and *in vivo*. Several quantitative approaches of varying complexity have been developed to study intrinsic and extrinsic apoptosis (18-21). Here, we considered a minimal mathematical model containing three critical proteins: pAkt, XIAP, and caspase-3. Our results reveal the treatment with SACNs followed by the administration of PI828 results in greater antitumor outcome. These results indicate that the appropriate sequencing of cytotoxic nanomedicines and targeted therapeutics has to be established for optimal chemotherapeutic efficiency.

Materials and Methods

Nanoparticle synthesis

Cholesterol chloroformate was reacted with excess ethylenediamine to obtain cholesterol-ethylenediamine conjugate in near quantitative yield, which was then reacted with succinic anhydride in presence of pyridine to obtain cholesterol-ethylenediamine-succinic acid conjugate (95% yield). Cholesterol-ethylenediamine-succinic acid conjugate was reacted with aquated $\text{Pt}[(\text{NH}_3)_2(\text{OH}_2)_2]^{2+}$ in 1:1 ratio at pH = 6.5 to obtain a cholesterol-succinate-cis-platinum complex. The intermediates and the final product were characterized

by ^1H , ^{13}C and ^{195}Pt NMR spectroscopy. We engineered SACNs from the cholesterol succinate-cisplatin complex by a solvent evaporation, hydration, and extrusion method using phosphatidylcholine, cholesterol succinate-cisplatin complex and DSPE-PEG₂₀₀₀-NH₂ in 2:1:0.2 weight ratio. The SACNs were characterized using dynamic light scattering (DLS) and cryo-TEM to visualize their size distribution and ultra-structure. For release kinetics, drug loaded nanoparticles were suspended in buffer (pH = 5.5 or 7) and sealed in a dialysis membrane (molecular weight cutoff = 500 Da). The dialysis bags were incubated in 1L PBS buffer. An aliquot was collected from the dialysis bag at predetermined time intervals, and the released drug was quantified from the decrease in Pt content in the dialysis chamber over time.

In vitro cell viability studies

4T1 breast cancer cells (obtained from ATCC and used within 6 months of resuscitation of frozen stock) were seeded in 96 well plates and grown for 24 hours. Cells were treated with drugs for defined time periods and in specific sequences. Culture media was replaced and cells were washed with plain media between the two treatments so that at any given time only one drug was present in the culture media. At 48 hours of total incubation time, cell viability was measured using a CellTiter 96 Aqueous One Solution assay (Promega).

Immunoblotting

4T1 breast cancer cells (1×10^5 /well) were grown in 6 well plates for 24 hours. Cells were then treated as described earlier. The cells were lysed post-incubation with $3\times$ RIPA buffer containing $1\times$ HALT phosphatase/protease inhibitor (ThermoFisher). Proteins were electrophoretically resolved on a SDS/PAGE gel, transferred onto polyvinylidene difluoride membranes, blocked for 1 hour with 7% nonfat dry milk and subsequently incubated overnight at 4°C with primary antibody directed against the desired protein. Proteins were detected with HRP conjugated secondary antibodies and Lumi-LightPLUS western blotting substrate. The blots were developed using GeneSnap and optical density of protein bands was quantified using GeneTools.

In vivo tumor growth study

4T1 Breast cancer cells (3×10^5) were implanted subcutaneously in the flanks of 4-week-old BALB/c female mice (weighing 20 g, Charles River Laboratories). The drug therapy was started when the tumors reached approximately 100 cc in volume. SACNs were administered via tail vein injection while PI828 was administered intraperitoneally. Treatments were administered on alternate days and a total of 3 doses were given for each drug. In animals groups receiving sequential treatment with 2 different drugs (SACNs and PI828), the administration of second drug was started only after completion of 3 doses of the first drug. The tumor volumes were calculated using formula $L \times B^2$. Tumor volume was then used to calculate the % increase in tumor volume using the formula $V_t/V_0 \times 100$. Plotting the % increase in tumor volume vs time gives the rate of tumor growth. The animals were sacrificed when the average tumor size of the control exceeded 1500 mm³ in the control group. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

Results

Synthesis of SACNs

In a previous study, we had defined a novel Pt chelation via a monocarboxylato and an O→Pt coordinate bond, which releases Pt in a pH-dependent manner and can undergo aquation more efficiently than when the Pt is chelated using dicarboxylato bonds or via a

monocarboxylato and an N→Pt coordinate bond [16]. In this study, we harnessed this Pt coordination environment via the design of a cholesterol-succinate leaving group (Fig. 2a). This enabled the supramolecular assembly of a nanoparticle arising from hydrophobic hydrophilic arrangements of cholesterol-succinic acid-platinum (II) molecule, phosphatidylcholine (PC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG) (Fig. 2a). The ultrastructure analysis using cryo-transmission electron microscopy (cryo-TEM) (Fig. 2b) revealed the formation of predominantly unilamellar structures less than 200 nm in diameter, although $29.9 \pm 5.47\%$ of the nanoparticles formed were bi-lamellar. Dynamic light scattering (DLS) further confirmed the size distribution of SACNs (Fig. 2c). Loading efficiency was calculated to be $48 \pm 15\%$. Furthermore, as shown in Fig. 2d, SACNs exhibited a pH-dependent release of platinum (II). Interestingly, during the study period, we observed a plateauing of the amount of drug released, suggesting that the nanoparticles enable sustained-release, which could potentially arise from the insertion of the hydrophobic cholesterol component in the lipid bilayer while the Pt-succinate coordination end could be oriented both on the external and internal surfaces of the lipid bilayer. We observed a maximal 60% (and not 100%) of platinum (II) release from the SACNs at pH 5.5 over the study period, which could arise due to an dissociation equilibrium that exists between Pt and the leaving group, which is further supported by the fact that we only observe 35% of the aquated platinum being released at pH7 (acidic pH can drive the equilibrium towards dissociation or greater release of Pt (II) from the SACNs). A similar release kinetics pattern has been reported earlier (16).

Cisplatin nanoparticle causes activation of PI3 kinase pathway by downstream EGFR signaling

As expected with platinum-based chemotherapeutics, 4T1 cells treated with SACNs exhibited a dose-dependent increase in the levels of cleaved caspase 3, indicating the onset of apoptosis with time. Interestingly, treatments with SACNs also resulted in a time-dependent increase in the expression of phospho-AKT on Western blot analysis, indicating an activation of the PI3 kinase signaling pathway (Figs. 3a, b,e-h). We also observed a time-dependent decrease in expression of XIAP with SACN-treatment (Fig. 3c, i,j). Furthermore, SACN-treatment also induced a time-dependent increase in the phosphorylation of EGFR at Tyr1086 (Fig. 3d), but not at Tyr1173. To confirm the involvement of EGFR, co-incubation of the EGFR-specific inhibitor, erlotinib (1 μ M), with SACN (1 μ M) led to ablation of the enhanced phospho-AKT signaling (Fig. 3k). To confirm the importance of Akt phosphorylation as a survival advantage, we next isolated a subset of 4T1 cells which escaped SACN-induced cell death. Specifically, the generation of SACN survivors (SACN-S) was achieved by incubation of 4T1 breast cancer cells with 5 μ M SACN for 48 hours. Subsequently, adherent cells were trypsinized, replated and cultured for an additional 24 hours. The adherent cells were lysed and analyzed by Western blot, which confirmed that SACN-S retain a significantly enhanced phosphorylated Akt signature, consistent with activation of this pathway (Fig. 3l). Additionally, as shown in Figs. 4a, b treatment with PI828 inhibited baseline PI3-kinase signaling in the 4T1 cells in a time-dependent manner, with significant reduction in the phospho-Akt evident by 6 hours. No effect was observed in the levels of total Akt, confirming the effect of PI828 in inhibiting PI3K.

Mathematical modeling predicts optimal temporal combination

Our mathematical model captured the rising cleaved caspase-3 and phospho-Akt levels, as can be seen by the accurate fits in Figs. 4c, d along with decreasing XIAP levels after treatment with cisplatin nanoparticle (Fig. 4e). The potent inhibition of pAkt after PI828 administration was also facilitated by the model (Fig. 4f). Based on our assumptions and the above data, the model predicted the desired synergistic effects of the combination treatments. Relative to the control case, the mathematical model predicted synergistic

increase in caspase-3 due to treatment with SACNs along with the phospho-Akt inhibition provided by PI828 (Figs. 4g, h respectively). Additionally, as shown in Fig. 5a, PI828 administered after SACNs treatment was predicted to result in lower cell viability compared with pre-treatment with PI828 followed by SACNs treatment or with SACNs alone. We then sought to find the most efficient time for the administration of PI828 after cisplatin nanoparticle treatment. It was determined that administration of PI828 approximately 24 hours after cisplatin would yield lower cell viability as compared to 36 hours or earlier times (Fig. 5b).

Validation of predicted model *In vitro* and *in vivo*

To evaluate the efficacy of combination therapy regimen we performed cell viability assay using 4T1 cell line. Cell viability was quantified by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, assay. As shown in Fig. 5c, a significant shift in the cell viability curve to the left with combination regimen of SACNs followed by PI828 post-treatment compared to SACNs alone ($IC_{50}=7.87 \mu M$) and combination regimen of pretreatment with PI828 followed by SACNs ($IC_{50}=14.93 \mu M$). Indeed, in the 4T1 cells pre incubated with SACNs for 24 hours, PI828 resulted in marked suppression of the PI3 kinase activity as early as 6 hours post-treatment (Figs. 5d, e, g) and was maintained until 30 hours. Additionally, post-treatment with PI828 also significantly increased the levels of cleaved caspase 3 levels in a temporal manner when combined with SACNs, indicating synergistic pro-apoptotic effects (Fig. 5d, f). Interestingly, while post-treatment with PI828 abolished SACN-induced phosphorylation of Akt to baseline, pre-treatment with PI828 only had a minimal effect (Fig. 5g).

Antitumor efficacy of the proposed temporal combination regimens was evaluated *in vivo* using 4T1 syngeneic tumor model. BALB/c mice bearing subcutaneous 4T1 breast tumors were treated with the following drug combinations: (1) vehicle only, (2) PI828 (10.0 mg/kg $\times$ 3 doses), SACNs (0.5 mg/kg $\times$ 3 doses), (3) pretreatment with PI828 (10 mg/kg $\times$ 3 doses) followed by SACNs (0.5 mg/kg $\times$ 3 doses) and (4) SACNs (0.5 mg/kg $\times$ 3 doses) followed by PI828 (10 mg/kg $\times$ 3 doses). We selected a sub-optimal dose of SACNs and a therapeutically active dose of PI828 such that we can dissect any synergistic effect that would otherwise get masked by higher doses of SACNs. While our predictive modeling based on *in vitro* data suggested that the maximal efficacy will be attained where the PI3K inhibitor is administered 24 hours post treatment with SACNs, in the *in vivo* studies such a temporal sequence was not followed as PI828 administered after the first dose of SACN would have served a pre-treatment for the next dose of SACN, thereby confounding the results. As the goal of the study was to test whether sequencing of administration of a molecularly targeted therapeutic and a cytotoxic nanoparticle has any effect on the antitumor outcome, we used a modified regimen. We rationalized that the repeated dosing will enable enhanced drug concentration in the tumor as a result of sustained drug release as observed in our previous study (16), leading to the activation of Akt and enabling us to test the hypothesis. Indeed, as seen in Fig. 5h, animals that received PI828 post-treatment with SACNs exhibited greater inhibition in the rate of tumor growth than pre-treatment with PI828 in combination with SACNs, consistent with the *in vitro* studies and mathematical predictions. To elucidate the mechanism behind the synergistic effect on tumor growth reduction, Western blot analysis was performed on tumor samples which showed a significantly increased expression of cleaved caspase-3 in tumors treated with PI828 post-treatment with SACNs (Fig. 5i).

In a separate experiment, we next compared a traditional combination of the SACNs and PI828 administered simultaneously to a sequenced dosing where PI828 was administered following treatment with SACNs (adapted from the previous experimental design which

demonstrated that this sequence was superior compared to pre-treatment with PI828). As shown in Fig. 5j, the sequenced dosing was observed to be superior to treatments administered simultaneously.

Discussion

Triple negative breast cancer (TNBC) is characterized by a considerably more aggressive course than other subtypes (31). Currently, about 15% of all breast cancer patients are diagnosed as TNBC, and cytotoxic chemotherapy regimens remain the standard of care for these patients (32). Preclinical studies have revealed that basal-like TNBC, which constitutes approximately 75% of all TNBCs, is sensitive to cisplatin, and indeed cisplatin is an emerging treatment modality for this indication (33). Cisplatin is a widely used cytotoxic agent with very high efficacy but is limited by its dose-limiting nephrotoxicity (15). In a recent study, we overcame this limitation by engineering a supramolecular nanoparticle (SACN), which preferentially accumulated in tumors and bypassed the kidney (34). Indeed, consistent with the clinical data demonstrating efficacy of platinates in TNBC, our results indicate that the SACNs does exert a potent apoptotic effect on the 4T1 breast cancer cells as evident by increased temporal expression of cleaved caspases. Interestingly, treatment with the SACNs simultaneously induced a statistically significant temporal upregulation of phospho-Akt, indicating a pro-survival signaling program initiated by the cell in response to chemotherapy. This is consistent with previous observations where PI3K-Akt signaling was reported to contribute to platinum drug resistance (35, 36). Our results indicate that a cisplatin-based nanoparticle will face a similar limitation in the clinics, and may need to be combined with PI3K inhibitors. Indeed, combinations of drugs that inhibit the PI3K signaling axis and platinum-based chemotherapy are currently in early phase clinical trials for TNBC (37).

Up-regulation of the PI3K pathway, either upstream or through mutation-based activation, is one of the most frequently altered pathways in breast cancer (38). The class I PI3K family (p110 α , p110 β , p110 Δ , p110 γ) are activated by tyrosine kinases or G protein-coupled receptors to generate PIP3, which activates downstream effectors such as Akt (39). While our results demonstrated a temporal expression of phosphorylated Akt following treatment with SACN thereby confirming the activation of PI3K signaling, we also observed a similar temporal increase in the phosphorylation of EGFR at Y1086. The increased auto-phosphorylation was site-specific as there was no significant increase at Y1173, another EGFR phosphorylation site involved in downstream ERK signaling (40). The Y1086 residue is known to be involved in downstream PI3K signaling (41), thus strengthening the case for EGFR downstream signaling to be responsible for the observed phospho-Akt levels following SACN-treatment. This observation correlates with prior studies, where cisplatin resulted in EGFR activation (27, 42). In a separate study, we have observed that cytotoxic chemotherapeutics can enable the selection of a subset cells that exhibit enhanced PI3K signaling (43). The current results are consistent with our previous observation, suggesting that the cytotoxic nanoparticles will also be limited by adaptive resistance, and therefore will need to be combined with molecularly targeted therapeutics. Indeed, combination of cisplatin along with EGFR or PI3K inhibitors has shown promise in prior studies (44, 45). However, a number of phase 3 trials evaluating the combination of cisplatin with gefitinib have failed, and indications for their failure include poor drug scheduling (46, 47). We rationalized that developing an optimal algorithm for temporally sequencing a PI3K inhibitor and SACNs could potentially lead to superior outcomes.

In a recent study, Lee et al demonstrated pretreatment, but not post-treatment or co-treatment, of a subset of TNBC with EGFR inhibitors can enhance their apoptotic response to DNA-damaging cytotoxic agents (48). However, given the redundancies in signaling

downstream of tyrosine kinases such as EGFR, we focused on the inhibition of PI3K using a highly potent PI3 kinase inhibitor, PI828 (49), which was found to suppress both basal and SACN-induced activation of PI3K-Akt signaling resulting in increased cleaved-caspase expression, highlighting the balance between survival signaling and apoptosis. However, both our mathematical model as well as *in vitro* and *in vivo* data indicated that unlike the results in the Lee *et al* study, post-treatment, and not pre-treatment, with PI828 synergized with SACNs. A likely explanation is the temporally-sustained release of aquated platinate (II) from the SACNs, which was found to be pH-dependent, and was factored into the model. Alternatively, the relative hierarchy in the signaling pathway, i.e. PI3K being downstream of EGFR, could potentially translate into distinct temporal requirements for optimal inhibition of the signaling cascade with pharmacological inhibitors when combined with SACNs.

The subcutaneous 4T1 *in vivo* model employed in this study is an extremely aggressive and spontaneously metastasizing syngeneic model, and is representative of late stage metastatic triple negative breast cancer (50). The enhanced efficacy displayed in this model with PI828 post-treatment following a SACNs therapy is therefore encouraging, and could potentially be clinically significant as combinations of nanomedicines and molecular-targeted therapeutics are increasingly used. However, while it well established that drug ratio is critical to determine whether a drug combination is additive or synergistic, in the case of a combination of a nanoparticle with a targeted therapeutic it becomes more complicated since in all likelihood the nanoparticle slowly releases the cytotoxic before and after PI828 is administered. As a result the tumor cell is actually exposed to varying ratios of these two drugs over the course of treatment. *In vivo*, one could assume that the repeated cycles of the cytotoxic nanoparticle, combined with the EPR effect (resulting in preferential accumulation in the tumor) and the sustained release of the active drug, could translate into steady state concentration of the cytotoxic in the tumor. Future studies are required to dissect this out. Furthermore, the strategy of combining signaling inhibitors with cytotoxic nanoparticles to achieve temporally synergistic combinations while reducing peripheral side effects can be refined even further by potentially combining both agents in a single nanoparticle. Indeed, in a previous study, we have demonstrated that a nanoparticle model system enabling combination therapy with temporal release can exert a superior anti-tumor outcome (9). The current study opens up the possibility of engineering next-generation nanoparticles, harnessing mathematical modeling in nanoparticle design such that multiple payloads are released at a differential but defined rate thereby producing optimal synergy between the active agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (A) The simplified pathway including pAkt, XIAP and caspase-3. (B) Tumor cells proliferate with rate proportional to pAkt. The death rate is assumed to be proportional to caspase-3.

Fig. 2. Synthesis and characterization of SACNs. (a) Schematic representation of Self Assembled Cisplatin Nanoparticles (SACNs). Nanoparticles were characterized by (b) cryo-TEM and (c) the size distribution measured by dynamic light scattering. (d) Release of platinum from the SACNs was determined by a release kinetics assay where nanoparticles were allowed to homogenize with acidic and basic conditions (pH 5.5 and 8.5) inside sealed 500 mwco dialysis bag. The leached out platinum was measured at different time points and expressed as % of total loading (n=3 independent experiments).

Fig. 3.

A temporal expression of total Akt, phospho-Akt, cleaved caspase-3 expression after treatment of 4T1 cells with SACNs at (a) 3 μ M and (b) 5 μ M cisplatin-equivalent concentrations respectively. (c) Representative Western blot shows the temporal dose-dependent decrease in XIAP expression with SACN treatment. (d) Expression of total and phospho EGFR after similar treatment course with SACNs (equivalent to 5 μ M of cisplatin) shows a time-dependent increase in Tyr1086 phosphorylated EGFR expression but not in Tyr1173 phosphorylated EGFR. Densitometry quantification of western blot bands showing time dependent increase in expression of cleaved caspase-3 with SACN at (e) 3 μ M and (f) 5 μ M (Pt equivalent concentrations) respectively. Similar quantification of Western blot bands shows a time dependent increase in phospho Akt expression with (g) 3 μ M and (h) 5 μ M cisplatin-equivalent concentrations, and a concurrent decrease in XIAP expression (i,j) respectively (*p<0.05; #p<0.01, ANOVA followed by Newman Keul's posthoc test). (k) 4T1 cells were incubated with 1 μ M SACN in the presence or absence of 1 μ M erlotinib, which diminishes Akt signal. (l) Following incubation with 5 μ M SACN for 48 hours, adherent cells were trypsinized and replated for acute population outgrowth (24h) to isolate an adherent, surviving population of cells (SACN-S). 4T1 parent cells or SACN-S were analyzed for phosphorylated Akt expression by western blot.

Fig. 4. (a) A representative Western blot showing the temporal basal expression of total and phospho-Akt expression in 4T1 cells after treatment with PI828 (5 μ M). (b) Densitometric quantification of Western blot bands showing suppression of phospho-Akt with PI828 within 6 hours of treatment. (c-h) Graphs represent mathematical prediction of expression of cellular proteins after treatment with SACN alone and in temporal sequence with PI828. Mathematically predicted expression of (c) caspase, (d) pAkt and (e) XIAP in presence of different concentrations of SACN; the inhibition of (f) pAkt by PI828 alone and (g) inhibition of pAkt by PI828 post-treatment with SACN and the (h) synergistic increase in caspase expression as compared to SACN alone-treated controls.

Fig. 5.

(a-b) Mathematical model-based prediction of cell viability using different dosing schedules of SACNs and PI828. (c) Graph shows *in vitro* validation of the mathematical model, where the effect of different drug combinations on 4T1 cell viability was quantified using MTS assay. *PI828*-cells were incubated with PI828 containing media in an increasing concentration gradient (0.5 μM to 50 μM) for 12 hours after which it was replaced with control media for 36 hours. *SACN*-treated cells were incubated with SACN containing media in an increasing concentration gradient (0.5 μM to 50 μM) for 36 hours after which it was replaced with control media for 12hr. *PI828 Pretreatment*-cells were pretreated with 5 μM PI828 for 12 hours after which the culture media was removed and cells were incubated with SACN in an increasing concentration gradient (0.5 μM to 50 μM) for 36 hours. *PI828 post-treatment*-cells were incubated with SACN containing media in a increasing concentration gradient (0.5 μM to 50 μM) for 36 hours after which it was replaced with PI828-containing

media at 5 μ M concentration for 12 hours. All readings were taken at 48 hours. (Data shown are mean $\pm$ SEM, n=3). (d) Western blot shows a time course of total Akt, phospho-Akt and cleaved caspase-3 expressions in PI828 (5.0 μ M) treatment in cells pretreated with 24 hours of SACN (3.0 μ M). (e, f) Densitometric quantification of western blot bands showing significantly decreased phospho-Akt expression and increase cleaved caspase-3 expression in a time dependent fashion when compared to the control group. (*p<0.05; #p<0.01, ANOVA followed by Newman Keul's PostHoc test). (g) Immunocytochemistry showing differential expression of phospho-AKT (green) in control, SACN (5.0 μ M), PI828 pre-treatment and PI828 post-treatment groups. (h) Graph shows effect of combination therapy with PI828 and SACN on tumor growth rate in a murine syngenic 4T1 breast model. BALB/c mice bearing 8 day old subcutaneous tumors were subjected to drug treatments as follows (n=5 for each group). The arrows show the scheduling of treatment in each group. Tumors sizes were measured every day from the day of first drug treatment. The graph represents the rate of tumor growth, where Day 0 was the day of start of treatment. (*p<0.05, ANOVA) (i) Western blot based estimation of cleaved caspase-3 expression in animal tissue samples showing significantly increased cleaved caspase-3 expression in the PI828 post treatment model. (j) Graph shows tumor growth rate in animals treated with SACN (0.5 mg/kg of Pt equivalent) and PI828 (10 mg/kg) administered simultaneously or sequenced (PI828 administered as post-treatment to SACN administration using schedule described in h. Data shown are mean $\pm$ SEM (n=5) (*P<0.05, ANOVA).