HSP90 Inhibitor-SN-38 Conjugate Strategy for Targeted Delivery of Topoisomerase I Inhibitor to Tumors

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Abstract

The clinical benefits of chemotherapy are commonly offset by insufficient drug exposures, narrow safety margins, and/or systemic toxicities. Over recent decades, a number of conjugate-based targeting approaches designed to overcome these limitations have been explored. Here, we report on an innovative strategy that utilizes HSP90 inhibitor–drug conjugates (HDC) for directed tumor targeting of chemotherapeutic agents. STA-12-8666 is an HDC that comprises an HSP90 inhibitor fused to SN-38, the active metabolite of irinotecan. Mechanistic analyses in vitro established that high-affinity HSP90 binding conferred by the inhibitor backbone could be exploited for conjugate accumulation within tumor cells. In vivo modeling showed that the HSP90 inhibitor moiety was required for selective retention of STA-12-8666, and this enrichment promoted extended release of active SN-38 within the tumor compartment. Indeed, controlled intratumoral payload release by STA-12-8666 contributed to a broad therapeutic window, sustained biomarker activity, and remarkable degree of efficacy and durability of response in multiple cell line and patient-derived xenograft models. Overall, STA-12-8666 has been developed as a unique HDC agent that employs a distinct mechanism of targeted drug delivery to achieve potent and sustained antitumor effects. These findings identify STA-12-8666 as a promising new candidate for evaluation as novel anticancer therapeutic.

Introduction

Chemotherapeutic drugs have been a mainstay of cancer therapy for many decades; however, their effectiveness is often hampered by systemic toxicities due to adverse effects on normal tissues. This lack of discrimination limits their administrable dosing in the clinic, resulting in narrow therapeutic windows and less-than-optimal efficacies. Moreover, multidrug combinations that represent the standard-of-care treatment modality for a variety of cancer types typically require further dose reductions to maintain acceptable tolerability (1). Thus, targeted delivery of cytotoxic amounts of toxic agents to tumor cells remains an ongoing challenge for cancer therapy. To address this concern, a variety of site-selective drug delivery strategies have been designed to deliver chemotherapeutics more directly to tumors (2), including coupling anticancer drugs to monoclonal antibodies, peptides, and synthetic polymers (3–7). In particular, significant translational progress has been made in the field of antibody–drug conjugates (ADC; ref. 3), evident by the recent approval of brentuximab vedotin and ado-trastuzumab emtansine (4). Such conjugate-based drug delivery systems share commonality with respect to their functional construction, comprising a tumor recognition moiety and cytotoxic payload connected via chemical linkage.

We applied these fundamentals to establish a new drug delivery system termed HSP90 inhibitor–drug conjugates, or HDC, based on the property that small-molecule inhibitors of HSP90 are preferentially retained in tumor cells in contrast to their rapid clearance from the circulation and normal tissues (8–12). HSP90 is a highly conserved molecular chaperone that plays an indispensable role in regulating the maturation and functional stability of a vast repertoire of cellular client proteins (13). HSP90 is frequently overexpressed in tumor tissues and, importantly, exists within cancer cells in an activated configuration that displays greater affinity for selective inhibitors than the resident pool found in normal cells (14). Relative to normal cells, the rate of HSP90 chaperone cycling accelerates in tumor cells in part due to increased SUMOylation, which recruits co-chaperones required to maximally stimulate HSP90 ATPase activity (15). Importantly, SUMOylation of HSP90 also facilitates the binding of ATP competitive inhibitors to HSP90, helping to explain the affinity of these agents for tumor-associated HSP90. By attaching potent chemotherapeutic drugs to HSP90 inhibitor backbones, HDC technology exploits this inherent retention property to more efficiently deliver cytotoxic payloads directly into tumor tissues and provide extended drug residency periods.

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Here we report on the preclinical characterization of a prototypical HDC compound, STA-12-8666, comprised of an HSP90 inhibitor fused to SN-38, a potent topoisomerase I inhibitor and pharmacologically active metabolite of irinotecan (16). These proof-of-concept findings, including the demonstration of extended intratumoral drug exposures and superior therapeutic indices over irinotecan therapy alone, are of considerable translational relevance for the future development of this novel class of investigational agents as cancer therapeutics.

Materials and Methods

Cell lines, antibodies, and reagents

The H1975, SK-NP-E, and SW780 cell lines were obtained from ATCC between March and November 2013; the MDA-MB-231 line was purchased in October 2007. All were authenticated by routine company DNA typing, maintained according to the suppliers’ instructions, and never continuously cultured for more than 3 months. No further cell line authentication has been performed by the authors. The human small-cell lung cancer (SCLC) cell line SR2 was a gift from Dr. Niramol Savaraj (University of Miami, FL) in January 2011. No authentication of the gifted line was carried out by the authors. All antibodies were purchased from Cell Signaling Technology with the exception of the HSP70 (Enzo Life Sciences), KAP1 and phospho-KAP1 (Abcam), and GAPDH (Santa Cruz Biotechnology Inc.). Iринotecan was purchased from LC Laboratories. Ganetespib, STA-12-8666, STA-12-9432, STA-12-8663, STA-12-9455, and STA-12-9467 were synthesized by Synta Pharmaceuticals Corp.

Immunofluorescent staining

H1975 cells were treated with vehicle (DMSO), or 1 μmol/L ganetespib, SN-38, or STA-12-8666 for 1 hour. Following drug washout, cells were cultured for an additional hour prior to fixation and permeabilization. Cells were stained with p-KAP1 antibody (1/1000 in PBS + 0.1% Triton X-100) overnight at 4°C before incubation with Alexa Fluor S94 Goat Anti-Rabbit IgG (Life Technologies) for 1 hour at room temperature. Slides were mounted using DAPI-containing medium (VECTASHIELD) and images obtained with an EVOS-FL fluorescent microscope (Life Technologies).

Western blotting

Following in vitro drug assays with SN-38, ganetespib, STA-12-9432, or STA-12-8666, H1975 tumor cells were disrupted in lysis buffer (Cell Signaling Technology) on ice for 10 minutes. Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before transfer to nitrocellulose membranes (Invitrogen). Membranes were blocked with StartingBlock T20 blocking buffer (Thermo Scientific) and immunoblotted with the indicated antibodies. Antibody–antigen complexes were visualized using an Odyssey system (LI-COR).

In vivo xenograft tumor models

Female CB.17 (SCID) mice (Charles River Laboratories) at 7 to 12 weeks of age were maintained in a pathogen-free environment and all in vivo procedures were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Synta Pharmaceuticals Corp. Institutional Animal Care and Use Committee. H1975, MDA-MB-231, SK-NP-E, SW780 (all 5 × 10³), or SR2 cells (7.5 × 10⁶) were subcutaneously implanted into SCID mice. Mice bearing established tumors were allocated into treatment groups of 6 to 8 exhibiting similar average tumor volumes (100–200 mm³) and intravenously dosed with vehicle, STA-12-9432, STA-12-8666, and/or irinotecan, using the doses and schedules indicated. For the large tumor H1975 study, xenograft tumors were permitted to grow to an average volume of 750 mm³ (upper limit, 900 mm³) before initiation of therapy. In the combination study, mice bearing SR2 tumors (n = 8/group) were dosed with vehicle, STA-12-8666 (150 mg/kg), ganetespib (100 mg/kg), irinotecan (50 mg/kg), or the combination of irinotecan plus ganetespib, once weekly for a total of 4 doses. All compounds were formulated in DRD [10% dimethyl sulfoxide (DMSO), 18% Cremophor RH 40, 3.6% dextrose].

For the extended patient-derived xenograft (PDX) study, anonymous surgical pancreatic tumor tissues were procured at Fox Chase Cancer Center under an IRB-approved protocol after receiving informed patient consent. DMSO-cryopreserved PDX tumors from passage 3 were subcutaneously implanted into both flanks of fifteen 5- to 8-week-old ICR-C.B17-scid mice. When tumors reached an average 250 mm³, mice (n = 5 per group) were randomized to receive weekly dosing with vehicle [20% Cremophor RH 40/80%5W (dextrose 5% in water)], irinotecan (50 mg/kg), or STA-12-8666 (150 mg/kg). Following an initial 3-week treatment cycle, drug treatment was discontinued and animals monitored for recurrence out to 150 days. Upon progression, tumors were re-treated with 150 mg/kg STA-12-8666 using a second weekly dosing schedule. Tumor growth inhibition was determined as described previously (17). Statistical analyses were conducted using two-way ANOVA followed by Bonferroni post tests.

Bioanalysis

STA-12-8666 (150 mg/kg) and irinotecan (50 mg/kg) were administered as a single bolus injection to MDA-MB-231 xenograft-bearing mice (n = 3). Tumor samples were collected from euthanized animals at 0.25, 1, 3, 5, 7, 11, and 15 days after dosing, snap-frozen in liquid nitrogen, and stored at −80°C. Prior to analysis, tumor tissues were weighed and homogenized in a 3× volume of PBS (containing 40 mg/mL sodium fluoride). Homogenized tissue samples were extracted by protein precipitation and analyzed by LC/MS-MS. A Waters SymmetryShield RP18 column (5 μm, 2.1 × 100 mm) was used with a run time of 5 minute per sample.

Immunostaining

Tumor tissues were harvested from MDA-MB-231 xenograft-bearing mice 6 hours to 15 days following a single administration of vehicle, 50 mg/kg irinotecan, or 100 mg/kg STA-12-8666. Samples were fixed in neutral buffered formalin overnight and transferred to 70% ethanol. Fixed tissues were paraffin-embedded, sectioned, and stained by Reveal Biosciences. Briefly, immunohistochemical staining for γH2AX (Cell Signaling Technologies) and hematostatin counterstaining was performed using a Leica Bond automated immunostainer. A 3DHistech Pannoramic SCAN digital scanner (Perkin Elmer) was used for whole bright-field imaging. Quantitative image analysis was performed using ImmunoRatio (18), which calculated the percentage of positively stained nuclei area for γH2AX expression (labeling index) through segmentation of diaminobenzidine (DAB)-stained and hematostatin-stained nuclei regions.
Results
Validation of the HDC mechanism of action
In HDC design, the primary function of the HSP90 inhibitor moiety is to serve as a delivery vehicle. Initial proof-of-concept studies utilized an analogous pair of HSP90 inhibitor–attached fluorescent probes, STA-12-9455 and STA-12-9467 (Supplementary Fig. S1A). Each consisted of a resorcinol-based HSP90 inhibitor backbone, structurally similar to the small-molecule inhibitor ganetespib (19). Replacement of a key hydroxyl with a methoxy in STA-12-9467 abrogated HSP90 binding and thus this compound served as a control for STA-12-9455. BT-20 breast cancer cells were treated with 5 μmol/L STA-12-9467, STA-12-9455, or BODIPYFL for 30 minutes, prior to drug wash-out and subsequent imaging over 24 hours (Supplementary Fig. S1B). All three compounds entered the cells, however, both the naked fluorophore and nonbinding STA-12-9467 displayed only weak cytoplasmic staining after 1 minute and the fluorescent signal for each was lost by 30 minutes. In stark contrast, strong fluorescence of STA-12-9455 was observed for 24 hours, demonstrating that the HSP90-binding conjugate was retained within the cell for this extended time period. Similar effects were seen in multiple tumor lines (unpublished observations). Importantly, competitive pretreatment with ganetespib was sufficient to prevent intracellular accumulation of STA-12-9455 (Supplementary Fig. S1C), confirming that compound retention was HSP90-dependent.

Evaluation of the chemotherapeutic activity of STA-12-8666, an SN-38-containing HDC
A schematic representation of the structural features of HDC, incorporating an HSP90 inhibitor targeting construct, cleavable linker, and anticancer payload, is presented in Fig. 1A. For the prototypical cytotoxic payload we selected SN-38, a potent topoisomerase I inhibitor and metabolite of irinotecan (20). STA-12-8666 comprised a similar resorcinol-based HSP90 inhibitor scaffold to which SN-38 was attached via a carbamyl linker (Fig. 1B). In this manner, SN-38 is transported in an inactive state until intracellular cleavage regenerates active chemotherapeutic agent within tumors. Enzymatic linker cleavage is primarily achieved through carboxylesterase (CES) activity, also involved in prodrug conversion of irinotecan to SN-38 (20). CES are a multigene family of serine-dependent esterases involved in the metabolism of endogenous lipids and drugs (21). When assayed in the presence of purified recombinant CES1b, CES1c, and CES2 proteins, STA-12-8666 was similarly cleaved by all three isoenzymes to release SN-38 (Supplementary Fig. S2). As expected, CES2 showed the highest catalytic efficiency for irinotecan activation (22). In addition to tumors, CES are present in the blood as well as

Figure 1.
Design, structure, and activity of the SN-38-containing HDC STA-12-8666. A, schematic showing design features of HDC, incorporating an HSP90 inhibitor targeting moiety, cleavable linker, and anticancer payload. B, chemical structure of STA-12-8666. The carbamyl linker is shaded in red. C, representative images of H1975 NSCLC cells treated with 1 μmol/L ganetespib, SN-38, STA-12-8666, or vehicle (DMSO). Cells were exposed to drug for 1 hour; following washout, cells were cultured for an additional hour prior to staining. Immunofluorescence was performed for p-KAP1 induction (red) and cells counterstained with DAPI (blue). Scale bar, 200 μm. D, H1975 NSCLC cells were exposed to graded concentrations (10—1,000 nmol/L) of SN-38, STA-12-8666, or ganetespib for 24 hours and cell lysates immunoblotted for HSP70, phosphorylated KAPI (p-KAPI), and γ-H2AX as indicated. GAPDH was included as a loading control.
normal tissues. Moreover, linkages that depend on esterase activity for payload release, such as the one in STA-12-8666, may also show species-related differences in activity (23). Therefore, STA-12-8666 and irinotecan stability were assessed by monitoring their disappearance in plasma samples from mice, dogs, and humans (Supplementary Table S1). In mouse plasma, STA-12-8666 levels remained at 81% and 43% of original after 15 and 30 minutes, respectively, and were further reduced to 24% by 1 hour. Of translational relevance, STA-12-8666 was found to be relatively more stable in human plasma compared to mouse (i.e., 91% vs. 24% at 1 hour). Similar findings were observed for irinotecan (Supplementary Table S1). These data suggest that STA-12-8666 may exhibit favorable pharmacology in humans, sufficient to allow tumor accumulation and site-specific processing by intracellular esterases.

Phosphorylation of KAP1 (KRAB-ZFP-associated protein 1), a protein involved in DNA repair, has been identified as a readout for topoisomerase I inhibition (24). As shown in Fig. 1C, treatment of H1975 non-small cell lung cancer (NSCLC) cells with either SN-38 or STA-12-8666 induced robust p-KAP1 expression. Importantly, HSP90 inhibition alone using ganetespib did not, thus p-KAP1 provided a selective marker for STA-12-8666 payload activity. These results were confirmed by immunoblotting (Fig. 1D). In addition, γ-H2AX is a sensitive indicator of DNA double-strand break formation and additional marker of SN-38 activity (25). Consistent with this, γ-H2AX was induced by both SN-38 and STA-12-8666 at equivalent concentrations to those that increased p-KAP1 levels.

γ-H2AX expression can also be indirectly impacted by HSP90 inhibition and ganetespib treatment elevated levels of this protein (Fig. 1D). However, ganetespib also more potently upregulated the stress-inducible chaperone HSP70, a commonly used biomarker of HSP90 blockade (26), than STA-12-8666. These data suggested that, despite robust chaperone binding affinity, the intrinsic HSP90 inhibitory activity of the HDC compound was relatively weak. This premise was additionally supported by the in vitro antiproliferative activities of the two agents in a panel of NSCLC cell lines (Table 1).

**HSP90 binding is required for HDC activity**

As with the fluorophore experiments above, we generated a corresponding ‘mock’ HDC, STA-12-9432, via modification of the 2-hydroxy group in the resorcinol branch necessary for interaction with the Asp93 residue within the HSP90 N-terminus (Fig. 2A). Apart from compromised HSP90-binding capacity, STA-12-9432 remained similar to STA-12-8666 in terms of overall structure and physicochemical profile (Supplementary Table S2).

Molecular characterization of the comparative cellular effects of STA-12-8666 and STA-12-9432 were then investigated using H1975 cells (Fig. 2B). As expected, dose-dependent increases in HSP70 expression were observed following STA-12-8666 (albeit at high concentrations) but not STA-12-9432 exposure. This differential response was confirmed by HDC-induced destabilization of EGFR (EGFR), the mutant form of which expressed by this line is a highly sensitive HSP90 client protein. When p-KAP1 levels were assessed as a surrogate marker for SN-38 effects, STA-12-8666 and STA-12-9432 promoted comparable, robust p-KAP1 expression (≥300 nmol/L) suggesting that both compounds underwent effective intracellular cleavage and payload release (Fig. 2B).

Next, SN-38 drug concentrations were measured in H1975 xenograft tumors following treatment with either STA-12-8666 or STA-12-9432 (Fig. 2C). Initial post-dosing SN-38 levels were comparable for each compound; however, the degree of accumulated SN-38 derived from STA-12-8666 remained relatively constant over time, unlike the steady decline observed with STA-12-9432. To functionally discriminate between the HDC and mock compounds in vivo, the comparative efficacies of irinotecan, STA-12-9432, and STA-12-8666 treatment were evaluated in H1975 xenografts (Fig. 2D). Weekly dosing with STA-12-9432 had only minimal effects on tumor growth, comparable with irinotecan, consistent with STA-12-9432 acting as a non-HSP90–binding SN-38 prodrug. In contrast, STA-12-8666 treatment completely suppressed tumor growth over the course of the study.

**Sustained tumor SN-38 exposure following STA-12-8666 treatment confers superior growth control to irinotecan**

Pharmacokinetic analyses were performed in MDA-MB-231 breast cancer xenograft-bearing mice to evaluate payload drug exposures that resulted from STA-12-8666 treatment. Following a single bolus injection of STA-12-8666 at its highest nonseverely toxic dose (HNSTD) of 150 mg/kg, HDC levels within the tumor compartment declined slowly with time, showing retention out to 15 days (Fig. 3A). Confirming expectations, STA-12-8666 treatment resulted in extended SN-38 payload release and nanomolar levels of liberated SN-38 were detectable over this same period (Fig. 3A). The appearance of the cleaved HSP90 inhibitor fragment, STA-12-8663, provided further evidence that effective HDC cleavage occurred in vivo. This profile was in sharp contrast to that observed in irinotecan-treated animals, where comparable tumor concentrations of SN-38 were initially achieved with a 50 mg/kg dose but levels rapidly declined to below quantifiable limits by 48 hours (Fig. 3B).

In agreement with these data, STA-12-8666 demonstrated long-term growth suppression (~2 weeks) following cessation of drug treatment in mice bearing MDA-MB-231 tumors, whereas the growth inhibitory effects conferred by irinotecan treatment were rapidly lost once drug administration was discontinued (Fig. 3C). When temporal changes in the DNA damage response signature were assessed by γ-H2AX immunostaining (Fig. 3D), irinotecan treatment promoted only transient γ-H2AX expression that peaked 1 to 3 days following dosing. This contrasted to a more amplified and protracted induction that occurred for 10 to 15 days following STA-12-8666 exposure. Expression changes in the labeling index for each treatment regimen are quantitated in Fig. 3E. A similar kinetic profile was observed when tissues were stained for p-KAP1 expression (Supplementary Fig. S3), and administration of the HSP90 inhibitor fragment STA-12-8663 alone to MDA-MB-231 xenograft-bearing animals had minimal DNA-damaging effects (Supplementary Fig. S4). Taken together, the strong correlation...
between SN-38 exposure, augmented DNA damage response, and durable efficacy suggest that topoisomerase inhibition, as a consequence of sustained intratumoral release of SN-38, serves as the primary antitumor mechanism of STA-12-8666.

STA-12-8666 exhibits a favorable therapeutic window of activity
Next, a direct comparison of the therapeutic windows for STA-12-8666 and irinotecan were undertaken using a clinically relevant SCLC model. Mice bearing SR2 xenografts were treated with increasing doses of both agents on a weekly dosing regimen for 3 weeks (Fig. 4A and B). All regimens were well tolerated with no significant changes in body weight observed (Supplementary Fig. S5). Irinotecan treatment at its HNSTD of 60 mg/kg resulted in disease stabilization (Fig. 4A). Comparable antitumor activity with STA-12-8666 was observed at 30 mg/kg, a dose representing approximately 20% of its HNSTD, and higher doses promoted robust tumor regressions (Fig. 4B). When efficacy was plotted as a function of tolerability, i.e., percentage of maximally tolerated dose (MTD) for each agent (67 mg/kg for irinotecan; 200 mg/kg for STA-12-8666), the curve shift to the right for STA-12-8666 underscored a substantially broader window of therapeutic opportunity versus the narrower margin between effective and toxic dosing seen with irinotecan (Fig. 4C).

HDC exposure is therapeutically superior to combination irinotecan plus ganetespib treatment
To directly compare STA-12-8666 with combination irinotecan plus HSP90 inhibitor treatment, SR2 tumor-bearing mice were administered 4 weekly doses of ganetespib (100 mg/kg) or irinotecan (50 mg/kg), both alone and in combination, or STA-12-8666 (150 mg/kg; Fig. 4D). The addition of ganetespib to irinotecan conferred a modest yet nonsignificant improvement in tumor growth inhibition over irinotecan monotherapy alone. In stark contrast, STA-12-8666 was significantly more efficacious than either irinotecan or combination therapy and promoted tumor regressions. In terms of tolerability, 3 of 8 mice died while on-study in the irinotecan arm, while only one animal was removed from study due to low body weight in the STA-12-8666–treated group.
STA-12-8666 is efficacious in tumor models independent of irinotecan sensitivity

The comparative efficacies of STA-12-8666 and irinotecan therapy were then evaluated in models of high and low irinotecan sensitivity. As expected, irinotecan treatment induced significant tumor regressions in the highly sensitive SK-NEP-1 Ewing sarcoma xenograft model; an identical response was achieved with STA-12-8666 (Fig. 5A). Weekly dosing of irinotecan at the same level exerted minimal antitumor activity against SW780 bladder cancer xenografts, a model that we recently demonstrated is also

Figure 3.

STA-12-8666 treatment provides prolonged intratumoral SN-38 exposure and superior growth control to irinotecan. A, SCID mice bearing established MDA-MB-231 tumors were administered a single 150 mg/kg dose of STA-12-8666 and tumor concentrations of the intact HDC, HSP90 inhibitor fragment (STA-12-8663), and released SN-38 payload were assessed over a 15 day time period. B, mice were treated with a single bolus injection of irinotecan (50 mg/kg) and tumor concentrations of the prodrug and its metabolite SN-38 were determined for 15 days. C, mice bearing established MDA-MB-231 tumors (n = 8 per group) were intravenously dosed with vehicle, 50 mg/kg irinotecan, or 100 mg/kg STA-12-8666 once weekly for 3 weeks. Tumor growth was monitored for an additional 15 days following the final drug administration. Error bars, ± SEM. (*, P < 0.0001). D, kinetic analysis of γ-H2AX induction in MDA-MB-231 xenograft tumors harvested at the indicated time points ranging from 6 hours to 15 days following a single dose of vehicle, 50 mg/kg irinotecan, or 100 mg/kg STA-12-8666. Representative immunostaining patterns for γ-H2AX expression from each regimen are presented. Scale bar, 100 μm. E, quantification of γ-H2AX labeling index for each group, calculated as the percentage of DAB-stained nuclear area (i.e., γ-H2AX positive) over total nuclear area. (STA-12-8666 vs. irinotecan: *, P = 0.0226; **, P = 0.0015; ***, P = 0.0002; ****, P < 0.0001; ns, not significant).
Insensitive to resorcinol-based HSP90 inhibitors due to endogenous glucoronidation (27). STA-12-8666 treatment significantly suppressed SW780 tumor growth (Fig. 5B), suggesting that conjugate stability was less affected by tumor metabolism in this model. This was likely due to differential substrate sensitivity and/or expression of UDP-glucuronosyltransferases that are...
STA-12-8666 induces durable tumor regressions in aggressive xenograft models. A, pancreatic cancer PDXs were established in SCID mice and animals administered an initial 3-week cycle of either irinotecan (50 mg/kg) or STA-12-8666 (550 mg/kg). Irinotecan-treated mice (average tumor volumes presented) rapidly progressed and were removed from study after 1 month. The first 2 HDC-treated mice to progress were administered a second 5-week cycle of STA-12-8666 beginning at day 110; the next 2 animals with recurrent tumors were re-treated on days 124 and 131. All recurrent PDX tumors to receive a second round of STA-12-8666 dosing underwent further, complete regressions. B, H1975 xenografts were allowed to grow to an average volume >750 mm^3 prior to initiation of drug treatment. Dosing on either a weekly (days 0, 7, 14, 21, 28) or every other week schedule (days 0, 14, 28) with STA-12-8666 induced significant tumor regressions.

STA-12-8666 treatment induces durable regressions and controls tumor growth with intermittent dosing in aggressive tumor models

Extended xenograft modeling of aggressive human disease was used to evaluate the durability of response to STA-12-8666 (Fig. 6A). Mice bearing pancreatic cancer PDXs were administered 3 once-weekly doses of vehicle, STA-12-8666, or irinotecan and tumor growth monitored out to 150 days. Vehicle and irinotecan-treated animals quickly progressed and were removed from study after 1 month. Complete regressions occurred in all five animals bearing nine tumors that were administered STA-12-8666 (or a nonresorcinol inhibitor control) remained present in the media over the same time period (unpublished observations).

STA-12-8666 is an HDC carrying SN-38, prototypical of a new class of therapeutic agents designed to provide selective drug accumulation and durable chemotherapeutic exposures within tumors. A unique aspect of HDC technology is that it employs one small-molecule entity (i.e., HSP90 inhibitor) as a targeting moiety for tumor-directed delivery of another that serves as a cytotoxic molecule inhibitors of HSP90 and, while the underlying basis of this effect remains to be fully elucidated (14, 15), this feature is also currently being exploited in the development of new functional imaging and therapeutic monitoring approaches (28–30). Here we report that the HSP90-binding capacity of STA-12-8666 was both necessary and sufficient to promote intratumoral localization and retention of the HDC molecule, resulting in prolonged SN-38 exposure and optimized therapeutic indices for the topoisomerase I inhibitor. While we cannot rule out a contribution of direct chaperone inhibitory effects by STA-12-8666, these were unlikely exerting a major influence as evidenced by the compounds' weak intrinsic HSP90 inhibitory activity, as well as the superior efficacy achieved when compared with irinotecan plus ganetespib combination therapy. Further evidence was provided by the comparatively weaker antiproliferative effects of STA-12-8666 on tumor lines in vitro relative to ganetespib, yet the robust tumor regressions seen in xenograft lung tumors in vivo responsible for glucuronidation in these cells. Furthermore, treatment of SW780 cells in culture with ganetespib resulted in complete metabolism of the HSP90 inhibitor within 24 hours, whereas significant levels of intact STA-12-8666 (or a nonresorcinol inhibitor control) remained present in the media over the same time period (unpublished observations).

**Discussion**

STA-12-8666 is an HDC carrying SN-38, prototypical of a new class of therapeutic agents designed to provide selective drug accumulation and durable chemotherapeutic exposures within tumors. A unique aspect of HDC technology is that it employs one small-molecule entity (i.e., HSP90 inhibitor) as a targeting moiety for tumor-directed delivery of another that serves as a cytotoxic payload. Preferential tumor retention is characteristic of small-molecule inhibitors of HSP90 and, while the underlying basis of this effect remains to be fully elucidated (14, 15), this feature is also currently being exploited in the development of new functional imaging and therapeutic monitoring approaches (28–30). Here we report that the HSP90-binding capacity of STA-12-8666 was both necessary and sufficient to promote intratumoral localization and retention of the HDC molecule, resulting in prolonged SN-38 exposure and optimized therapeutic indices for the topoisomerase I inhibitor. While we cannot rule out a contribution of direct chaperone inhibitory effects by STA-12-8666, these were unlikely exerting a major influence as evidenced by the compounds' weak intrinsic HSP90 inhibitory activity, as well as the superior efficacy achieved when compared with irinotecan plus ganetespib combination therapy. Further evidence was provided by the comparatively weaker antiproliferative effects of STA-12-8666 on tumor lines in vitro relative to ganetespib, yet the robust tumor regressions seen in xenograft lung tumors in vivo.
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exceeded ganetespib’s previously published antitumor activity in the same models (31, 32). Thus, our findings clearly demonstrate that HDC-mediated delivery of an established cytotoxic agent can achieve dramatic improvements in efficacy over traditional clinical formulations for these agents and that the approach is not complicated by effects due to loss of HSP90 function. Interestingly, even modest, nonproteotoxic effects on HSP90 inhibition have been shown to limit the emergence of resistance to anticancer chemotherapeutics (33); in this regard, STA-12-8666 may retain an inherent capacity to augment SN-38 activity via restricting potential development of topoisomerase inhibitor resistance. Overall, the HSP90 inhibitor backbone in STA-12-8666 functioned as an effective targeting construct with the principal cellular impacts arising from sustained SN-38 release.

SN-38 was chosen as an appropriate payload due to its broad clinical potential, well-established pharmacologic profile, and relative ease of synthetic incorporation into the HDC construct. A number of issues, including restricted active drug bioavailability and poor solubility (34, 35), have stimulated considerable interest in utilizing irinotecan and/or SN-38 in alternate delivery formulations intended to improve drug pharmacokinetics and therapeutic activity. Such investigational agents include the ADCs IMMU132 and epratuzumab-SN-38 (36–38); polymer–drug conjugates (PDC) EZN-2208 (39) and etirinotecan pegol (40); and polymer-based nanoparticle therapeutics like NK012 (41). A number of unique characteristics differentiate the HDC modality from these approaches. For example, unlike ADCs, HDCs do not require recognition of unique cell surface antigens (typically with restricted tumor distributions) and subsequent endocytosis for cellular uptake. Instead, tumor-expressed HSP90 provides a ubiquitous intracellular "sink" to attract the conjugate compound and, as small molecules, cellular entry and accumulation is achieved through either passive diffusion or common active transport mechanisms. In addition, highly potent "ultratoxic" chemotherapeutic payloads are typically required to achieve sufficient ADC antitumor activity (42) and HDC construction is not restricted by such constraints. Furthermore, nanoparticle and PDC macromolecular therapeutics are acutely reliant on the enhanced permeation retention effect (EPR) for passive accumulation into solid tumors (43) and optimal benefit is often mitigated by a variety of physiologic obstacles (44). HDC-based therapy thus represents an alternative strategy to provide site-specific accumulation and durable exposure of chemotherapeutic agents to tumors but with wider applicability and without existing limitations of ADC or PDC delivery.

Confiming expectations, STA-12-8666 showed a favorable biodistribution and activity profile, with tumor-selective accumulation of the compound acting as a reservoir for sustained intracellular cleavage and SN-38 payload release. This was validated by persistent drug activity and DNA damage following STA-12-8666 dosing, in contrast to the more restricted kinetics obtained with irinotecan treatment. This characteristic was directly related to the degree of tumor growth control exhibited by STA-12-8666; accordingly, the extended tumor residency periods and maintenance of SN-38 at therapeutic levels provided robust and durable antitumor activity in vivo. Indeed, STA-12-8666 demonstrated remarkable efficacy in suppressing aggressive xenograft tumor growth and promoting durable regressions across a variety of cancer types, including tumors refractory to irinotecan treatment. Irinotecan is indicated for use in metastatic colorectal cancer (16), and adjuvant activity has been observed in a range of other malignancies (45). This profile suggests quite broad-spectrum clinical activity for the topoisomerase inhibitor; in practical terms, however, a number of factors have prevented widespread use of this agent, including disappointing response rates, transient clinical responses, major dose-limiting toxicities, and drug resistance (46). Importantly, the antitumor effects of topoisomerase I inhibition appear more proportionate to length of drug exposure rather than the concentration of the inhibitor used (47). Thus, the controlled release of cytotoxic levels of SN-38 within the tumor compartment for an extended period following HDC administration was sufficient to account for the superior antitumor activity and broader therapeutic window achieved with STA-12-8666 exposure compared with irinotecan therapy. In light of these considerations, it is reasonable to suggest that HDC-mediated delivery represents an innovative strategy for extending the clinical applicability of this type of agent into additional indications which, due to either insufficient drug exposures and/or narrow safety margins, were previously not considered possible and the feasibility of this approach warrants further evaluation.

Two further considerations with direct translational relevance emerged from this study. First, robust tumor regressions in NSCLC xenograft tumors permitted to grow to large volumes before treatment was initiated were achieved with infrequent dosing of STA-12-8666, a finding that not only underscores a profound response to HDC therapy but also supports the potential use of intermittent dosing schedules within the clinical setting. Second, in a highly aggressive human pancreatic PDX model, STA-12-8666 therapy significantly outperformed irinotecan to induce durable tumor regressions following one cycle of treatment. More importantly, recurrent tumors remained sensitive to subsequent therapeutic challenge with STA-12-8666, indicating that tumor regrowth was not due to the emergence of selected clones with acquired resistance to the HDC compound. It is reasonable to suggest, therefore, that HDC delivery of SN-38 may represent an effective means to circumvent common mechanisms of resistance that frequently develop in response to irinotecan therapy (16).

In summary, we have developed and characterized a unique HDC agent that employs a distinct mechanism of targeted drug delivery to achieve potent and sustained antitumor effects. STA-12-8666 displays optimal pharmacologic properties, including high tumor retention and controlled cytotoxic payload release, which combine to produce a remarkable therapeutic index. These findings thus identify STA-12-8666 as a promising new therapeutic candidate for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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