Title: Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor pro-drug conversion

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Abstract

A major challenge in the clinical use of cytotoxic chemotherapeutics is maximizing efficacy in tumors while sparing normal tissue. Irinotecan is used for colorectal cancer treatment but the extent of its use is limited by toxic side-effects. Liposomal delivery systems offer tools to modify pharmacokinetic and safety profiles of cytotoxic drugs. In this study, we defined parameters that maximize the antitumor activity of a nanoliposomal formulation of irinotecan (nal-IRI). In a mouse xenograft model of human colon carcinoma, nal-IRI dosing could achieve higher intratumoral levels of the pro-drug irinotecan and its active metabolite SN-38 compared to free irinotecan. For example, nal-IRI administered at doses 5-fold lower than free irinotecan achieved similar intratumoral exposure of SN-38 but with superior antitumor activity. Tumor response and pharmacokinetic modeling identified the duration for which concentrations of SN-38 persisted above a critical intratumoral threshold of 120 nM as determinant for antitumor activity. We identified tumor permeability and carboxylesterase activity needed for pro-drug activation as critical factors in achieving longer duration of SN-38 in tumors. Simulations varying tumor permeability and carboxylesterase activity predicted a concave increase in tumor SN-38 duration, which was confirmed experimentally in 13 tumor xenograft models. Tumors where higher SN-38 duration was achieved displayed more robust growth inhibition compared to tumors with lower SN-38 duration, confirming the importance of this factor in drug response. Overall, our work shows how liposomal encapsulation of irinotecan can safely improve its antitumor activity in preclinical models by enhancing accumulation of its active metabolite within the tumor microenvironment.
Introduction

Liposomal carriers have become clinically accepted in cancer therapy as delivery systems that can enhance the utility of existing anticancer drugs (1). The potential benefits of these macromolecular carriers include overcoming solubility issues for certain drug classes, protecting the drug from unwanted metabolism and extending the residence time in plasma and tissue. In particular, liposomes tend to preferentially accumulate in tumors as a result of an enhanced permeability and retention (EPR) effect. The EPR effect is attributed to the abnormal tumor vasculature permitting extravasation of macromolecules, as well as impaired lymphatic drainage that promote the retention of these molecules within the tumor microenvironment, thereby providing sustained release at the tumor site mimicking a metronomic dosing (2). Increased tumor deposition via the EPR effect may also prevent drug resistance by overcoming the activity of multidrug resistant proteins (3,4) and may offer possible means of improving safety aspects by reducing systemic exposure relative to tumor exposure (5). There are potential pharmacologic advantages of the EPR effect, particularly for antineoplastic agents that have to engage their target over a longer time period or have little binding activity; an example is drugs of the camptothecin class with topoisomerase 1 enzyme (TOP1) as the primary target.

Irinotecan (CPT-11), a clinically approved camptothecin, is a pro-drug that is activated by CES enzymes, present primarily in liver and colon tissue to the active form, SN-38. The active SN-38 can be subsequently inactivated through glucuronidation by members of the UDPglucuronosyltransferase family (6). The principal mechanism of action leading to cell death is through DNA damage after replication-fork collisions with transient drug-TOP1 cleavage complexes, thus emphasizing the time of drug exposure as important driver for cytotoxicity of camptothecins (7,8). Recently, we described the development of a novel nanoliposomal formulation of irinotecan, nal-IRI (also known as MM-398 or PEP02) (9). nal-IRI features very high drug loading efficiency, a high drug payload, and marked in vivo drug retention that also stabilizes the active lactone configuration of irinotecan. The pharmacokinetic properties of the encapsulated irinotecan were dramatically altered in the plasma of female rats, with a
344x increase in the area under the curve (AUC), an 8.5x decrease in the volume of distribution and a 39.6x increase in the half-life of the total drug. Pharmacokinetic analysis in a clinical study confirmed these performance characteristics of nal-IRI in patients (10).

Plasma drug concentrations cannot readily be translated into therapeutic effect; a sufficient amount of active therapeutic agent must be transported to the tumor site of action (i.e. be available for uptake by cancer cells) in order to observe favorable drug activity (11). The transport of macro-molecules across the tumor vasculature is a complex process depending on vessel perfusion, surface area, and permeability, as well as tumor and drug characteristics. Several studies have utilized mathematical models to understand liposomal drug delivery within solid tumors (12,13). Of particular interest is work done by Hendriks et al., where the authors constructed a computational model to describe the parameters that affect the tumor delivery of pegylated liposomal doxorubicin, the first liposomal anticancer agent to receive clinical approval (14). The study concluded that liposome PK and tumor permeability to liposomes (tumor deposition) were the most important parameters controlling liposomal drug delivery to tumors.

In the case of nal-IRI, the complex metabolism (15) and mechanism of action (8,16) of free irinotecan, in addition to the above mentioned parameters, may play a role in the overall liposomal irinotecan delivery within tumors. In the present study, we describe a systems pharmacology approach to identify critical parameters that differentiate nal-IRI from free irinotecan with regard to in vivo activity. A mechanistic tumor PK model was developed and trained to describe CPT-11 and SN-38 levels observed in plasma and tumor, following administration of either nal-IRI or free irinotecan in tumor xenografts. A model sensitivity analysis was performed to identify the critical parameters driving in vivo activity, which were then experimentally confirmed by measuring these factors in multiple cell-line and patient-derived xenograft models. The findings in the present study highlight critical parameters that could serve as potential biomarkers to identify cancer indications and patient populations with an increased likelihood of nal-IRI responsiveness.
**Material & Methods**

**Materials and nal-IRI preparation**

nal-IRI was prepared as previously described (9) using a lipid composition of DSPC, Cholesterol, and PEG-DSPE (3:2:0.015, mol: mol: mol), an initial drug-to-lipid ratio of 500 g drug/mol phospholipid, and extrusion through 0.1 um polycarbonate filters. The resulting preparations displayed a particle size of 111 nm (with polydispersity index of 0.04), and a drug load of 473 mg irinotecan-HCl/mmol phospholipid. All lipids were obtained from Avanti Polar Lipids Inc. Irinotecan hydrochloride was purchased from the pharmacy. Acetic acid, methanol and acetonitrile were from EMD Chemicals Inc. Water and trifluoroacetic acid (TFA) were from J. T. Baker. Fetal Bovine Serum was from Tissue Culture Biologicals and phosphate buffered saline (PBS) was purchased from Life Technologies.

**Cell culture**

Cell lines (HT-29 (colon), SK-ES-1 (Ewing’s sarcoma), A549 (lung), LoVo (colon), MDA-MB-231 (breast)) were obtained from ATCC whereas A2780 cells (ovarian) was obtained from Sigma-Aldrich. Cells from ATCC and Sigma were received in 2010. All cells were authenticated prior to receipt and were propagated for less than six months after resuscitation. Cultures are regularly tested for mycoplasma. All cell lines were cultured in humidified CO₂ atmosphere at 37°C using media recommended by the manufacturer.

**Pharmacokinetic and tissue bio-distribution study**

Five-week-old female NOD-SCID mice were purchased from Charles River Laboratory. The care and treatment of experimental animals were in accordance with Institutional Animal Care and Use Committee guidelines. Subcutaneous tumors were established by injecting 10 million HT-29 cells into the right flank of mice. When the average tumor volume reached approximately 200 mm³, mice were randomized into groups (n=4/time point) that received a single intravenous (i.v.) dose of nal-IRI at 5
mg/kg, 10 mg/kg, 20 mg/kg or 40 mg/kg. Following 1, 4, 8, 24, 48, 72 and 168 hours after a single dose, mice were sacrificed and perfused with PBS prior to harvest tumor and other normal tissues.

Antitumor activity studies

Five-week-old female NOD-SCID mice were purchased from Charles River Laboratory. Subcutaneous tumors were established by injecting 10 million HT-29 and SK-ES-1 cells or 5 million A549 cells into the right flank of mice. Tumor growth was measured twice per week by calipers and calculated with formula: width$^2 \times$ length$\times$0.52. When the average tumor volume reached approximately 200 mm$^3$, mice were randomized into treatment groups (n=5-8/group) that received weekly i.v. dose of PBS (control), free irinotecan (50 mg/kg) or nal-IRI at various doses ranging from 1.25 to 20 mg/kg.

Tumor growth inhibition (TGI) was calculated with formula:

$$\text{TGI}(\%) = \left[1 - \frac{(V_{\text{treated}(d_{\text{final}})} - V_{\text{treated}(d_0)})}{(V_{\text{control}(d_{\text{final}})} - V_{\text{control}(d_0)})}\right]$$

where, $V_{\text{treated}}$ and $V_{\text{control}}$ represent the volumes of tumor at a given time point following treatment with drug or PBS; $d_0$ and $d_{\text{final}}$ represent first day and final day of treatment, respectively.

Characterizing tumors from cell-line and patient-derived xenografts

The cell-line derived xenografts (HT-29, SK-ES-1, A549, MDA-MB-231, LoVo, AsPC-1, A2780) were established as described above. The patient-derived tumor models [CTG-0062 (colorectal), CTG-0079 (colorectal), CTG-0252 (ovarian), CTG-0288 (pancreatic), CTG-0158 (lung), CTG-0283 (pancreatic)] were established by Champions Oncology using their Champions TumorGraft® (CTG) technology. When the average tumor volume reached approximately 300 mm$^3$, mice were randomized into treatment groups (n=4/group) that received single i.v. dose of either PBS or nal-IRI at 10 mg/kg. Prior to tumor collection, intracardial perfusion was performed to remove the blood components from the tumor compartment. Briefly, a butterfly needle (23G) connected to a 10 ml syringe filled with PBS is inserted into the left ventricle. Inferior vena cava is cut and animal is perfused with 10 ml PBS (within 1-2 mins). The control tumors were harvested 24 hours after PBS administration and used for the irinotecan activation assay,
whereas the treated tumors were harvested either 24 hours or 72 hours following nal-IRI treatment and used for HPLC analysis.

**HPLC quantification of CPT-11 and SN-38**

Tumor and normal tissues were analyzed for CPT-11 and SN-38 concentrations using a modification of the method previously described (9). Briefly, tissues were weighed and homogenized for 2 minutes in 20% w/v water using a TissueLyser (Qiagen). The homogenates were extracted by mixing 0.1 ml homogenate with 0.9 ml 1% acetic acid/methanol followed by 10 s vortexing and placing at −80°C for 1 hour. The samples were centrifuged at 10,000 rpm for 10 minutes at room temperature and supernatants collected for HPLC analysis (Dionex). The samples and standards (CPT-11 and SN-38) were analyzed using a C18 reverse phase column (Synergi Polar-RP 80A 250×4.60 mm 4 μm column). The drug metabolites were eluted running a gradient from 30% acetonitrile; 70% 0.1% TFA/H₂O to 68% acetonitrile; 32% 0.1% TFA/H₂O during a 13 minutes span at a flow rate of 1.0ml/min. The initial elute composition was restored after 14 minutes and continued for 6 minutes before the next injection. The CPT-11 peak was detected at ~7.7 minutes and the SN-38 peak eluted at ~8.4 minutes, using an in-line fluorescence detector excited at 372 nm and emitting at 556 nm.
Irinotecan activation assay

Tumor tissue lysates were prepared by homogenizing the tissue in 6% w/v 0.1M Tris HCL/1% Triton X-100 solution (pH7.5) using a TissueLyser for 2-4 minutes. Protein concentration of lysates was measured using the BCA reagent (Thermo Scientific). Lysates (250 μg of protein) were mixed with an equal volume of 10 μM irinotecan and incubated at 37°C. Following 24 hours of incubation the reaction was terminated by adding an equal volume of 1% acetic acid/methanol and samples centrifuged at 10,000 rpm for 15 minutes. The supernatant was processed for HPLC quantification of CPT-11 and SN-38 as described above.

Statistical analysis

The statistical significance of differences between groups was analyzed with one way ANOVA test. Results were considered statistically significant at p<0.05. The analysis was performed using GraphPad Prism 6.01

Model development and simulation

Pharmacokinetic profiles of metabolites in plasma and tumor from free irinotecan and nal-IRI were described by using multi-compartmental models (Fig. 1B). The model equations are explained and summarized in the supplementary materials. The models were built and implemented using Simbiology® toolbox in MATLAB® 8.2 (The MathWorks®).

Results

nal-IRI displays a prolonged exposure in both plasma and tumor compared to free irinotecan

The pharmacokinetic profiles of the pro-drug CPT-11 and its active metabolite SN-38 were measured in plasma and tumors following administration of either free irinotecan or nal-IRI (Fig. 1A). At similar doses of both free irinotecan and nal-IRI, the CPT-11 and SN-38 plasma levels cleared rapidly from circulation within 8 hours after free irinotecan injection, whereas the levels of CPT-11 and SN-38 following nal-IRI administration were persistent and remained in circulation for over 50 hours. A ~10 fold higher plasma
CPT-11 peak level was observed with nal-IRI as compared to free irinotecan. However, the plasma peak level of SN-38 achieved with nal-IRI was 10 fold lower compared to free irinotecan, probably due to the ability of the lipid bilayer to protect the conversion of pro-drug CPT-11 to SN-38 by the systemic CES enzyme present in mouse models (17). Administration of free irinotecan resulted in the clearance of greater than 90 percent of CPT-11 from tumors within 24 hours; however, following nal-IRI administration, CPT-11 levels persisted above 10,000 nM levels for 168 hours. Similar peak levels of SN-38 were achieved with both free irinotecan and nal-IRI in HT-29 tumors, though a prolonged SN-38 exposure for up to 168 hours (measured as the AUC from 0 to 168 hours) was achieved with nal-IRI as compared to less than 48 hours tumor exposure with free irinotecan. In summary, CPT-11 and SN-38 were still present in tumors at 168 hours following nal-IRI administration, though both CPT-11 and SN-38 had cleared from plasma.

Tumor SN-38 duration drives in vivo activity

We developed a mechanistic PK model to identify the determinants that may differentiate the plasma and tumor PK profiles between free irinotecan and nal-IRI (Fig. 1B). The experimental PK data were used to estimate the optimal model parameters (Table 1) fitting the model simulations within the standard deviations of in vivo PK profiles of both CPT-11 and SN-38 (Fig. 1A). As the in vitro cytotoxic effects of irinotecan on tumor cells is dependent on the concentration and the time of exposure of cells to active metabolite SN-38 (7,8), we sought to understand if the overall plasma and tumor SN-38 exposure predicts the in vivo activity of both nal-IRI and free irinotecan. The trained model determined that a five-fold higher dose of free irinotecan (50 mg/kg) was required to achieve similar SN-38 exposure in both plasma and tumor as compared to nal-IRI (10 mg/kg) (Fig. 2A). The tumor growth inhibition of HT-29 xenograft model at these equal exposure doses, was significantly greater with nal-IRI (~110%) treatment as compared to free irinotecan (~40%), despite the five-fold lower total dose administered (*p<0.05, one way ANOVA test) (Fig. 2B). In addition, other studies have shown no additional HT-29 tumor growth inhibition at the maximum tolerated dose of free irinotecan (100 mg/kg) (18). In order to identify a dose level of nal-IRI that gave comparable in vivo activity to 50 mg/kg free irinotecan, we performed a dose
escalation study in the HT-29 xenograft model (Fig. 2C). nal-IRI at 5 mg/kg showed partial inhibition of tumor growth (~40% TGI) that was comparable to 50 mg/kg free irinotecan, whereas 10 mg/kg and 20 mg/kg nal-IRI showed significant (*p<0.05, one way ANOVA test) tumor growth inhibition compared to saline (~110-130% TGI). Furthermore, we have previously tested control liposomes (that have comparable composition to nal-IRI except for the absence of irinotecan, the active pharmaceutical ingredient) and did not observe any tumor growth inhibition (data not shown).

The intratumor SN-38 concentrations achieved from 50 mg/kg and 100 mg/kg doses of free irinotecan and 1.25, 2.5, 5, 10, and 20 mg/kg doses of nal-IRI were then simulated using the trained mechanistic PK model (Fig. 2D). Although a nal-IRI dose of 5 mg/kg achieved similar TGI as 50 mg/kg free irinotecan, the tumor SN-38 AUC and peak levels were ~2-fold and ~6-fold lower respectively for nal-IRI as compared to free irinotecan. Further, we noted at these doses both drugs were able to maintain the tumor SN-38 concentration above 120 nM for the same duration of ~40 hours. In order to determine if the tumor SN-38 concentration impacts in vivo activity, we used the tumor SN-38 concentration of 120 nM as a threshold. We also determined the duration for which the various doses of nal-IRI or free irinotecan could maintain the tumor SN-38 concentration above 120 nM, hereon referred to as “tumor SN-38 duration”. A sigmoidal relationship between TGI (%) and tumor SN-38 duration (Fig 2E) was observed for both nal-IRI and free irinotecan (R²=0.62). However, when comparing TGI (%) to tumor SN-38 AUC (Fig. 2F) the relationship was less significant (R²=0.45), due to the lower TGI (%) achieved by 50 mg/kg free irinotecan compared to 10 mg/kg nal-IRI. We also observed longer SN-38 duration in tumors (> 100 hours) compared to normal tissues (< 72 hours). (Fig. 2G and Supplementary Fig. S1).

Identification of liposome tumor permeability and local tumor activation as critical determinants for tumor SN-38 duration

A local sensitivity analysis on the model parameters was performed to identify processes impacting the tumor SN-38 duration (Supplementary materials). In response to the administration of free irinotecan (50 mg/kg), the tumor SN-38 duration was relatively insensitive to most model parameters (Fig. 3A),
suggesting the inability of free irinotecan to modulate it. In contrast, several model parameters were found to significantly impact tumor SN-38 duration following the administration of nal-IRI (10 mg/kg) (Fig. 3B). The sensitive parameters for nal-IRI can be classified into three different categories: (i) PK, rate of breakdown of liposomes in blood (Release rate in blood, $V_{\text{max,Release,p}}$), (ii) activation of pro-drug CPT-11 to SN-38 by CES enzyme (CES activity in tumor; $V_{\text{max,CES,t}}$ and blood; $V_{\text{max,CES,p}}$), and (iii) liposome uptake within tumors i.e nal-IRI tumor deposition (nal-IRI tumor permeability, $PS_{\text{nal-IRI}}$). Among these parameters, the release rate in plasma negatively affected tumor SN-38 duration due to a decrease in the overall systemic exposure of nal-IRI. CES enzyme activity, particularly from tumor CES (local tumor activation of irinotecan) and nal-IRI permeability (tumor deposition), positively affected the tumor SN-38 duration. In order to assess the identifiability of parameter estimates, log likelihood profiling was performed for the sensitive parameters, $V_{\text{max,CES,t}}$ and $PS_{\text{nal-IRI}}$ (19). The confidence intervals suggested that both parameters were precisely estimated (Supplementary Fig. S2).

**Biological variability and simulated perturbation of nal-IRI tumor deposition and local activation**

To determine the biological relevance of these sensitive parameters towards driving tumor SN-38 duration (namely nal-IRI tumor deposition and local tumor activation of irinotecan), the parameters were measured in a panel of 13 xenograft models. We used the total CPT-11 concentrations in tumors as a surrogate for nal-IRI tumor deposition as model simulations based on nal-IRI pharmacokinetics showed that majority of CPT-11 in plasma and tumor was encapsulated and protected within the liposomes and less than 10% was available as free CPT-11 (Supplementary Fig. S3). The intra tumor concentrations of CPT-11 varied substantially across the tumor panel (Fig. 4A). The tumor models from cell-lines displayed overall higher levels of pro-drug CPT-11 deposition (from 5000 - 15000 ng/g) as compared to patient-derived tumor models (1000 - 2000 ng/g). In addition, a high degree of variability was observed between individual tumors within the same xenograft model (66% average coefficient of variation). Model simulations were used to test the effect of altering nal-IRI tumor deposition on tumor SN-38 duration (Fig. 4B). By decreasing the nal-IRI permeability parameter to zero, which simulates an impermeable
tumor microenvironment, the tumor SN-38 duration of ~100 hours achieved with 10 mg/kg nal-IRI was substantially reduced to ~50 hours and approached the levels observed with 50 mg/kg free irinotecan.

Taken together, these results suggest that the tumor deposition of nal-IRI is highly tumor specific and will dramatically impact tumor SN-38 duration.

To determine the degree to which local tumor activation of irinotecan varied in human tumors, we measured CES activity using an ex vivo assay in a panel of 80 patient-derived tumors. The tumor lysates varied in their ability to activate pro-drug irinotecan and produce SN-38 (1-25 ng/ml SN-38 produced), suggesting a high degree of variability in local tumor activation of irinotecan across indications. A significant difference in local tumor activation of irinotecan was observed between colon and lung tumors (p<0.05). However, there was no significant difference between other indications, which may be due to high variability observed within each indication (Fig. 4C). The impact of varying the tumor CES activity on tumor SN-38 duration was evaluated by simulating a knock-out of tumor CES enzyme (Fig. 4D). In the absence of local tumor activation, tumor SN-38 duration with nal-IRI (10 mg/kg) decreased from ~100 hours to ~40 hours, similar to that achieved by free irinotecan (50 mg/kg).

**nal-IRI tumor deposition and local activation collectively predict tumor SN-38 duration**

The relative contribution of nal-IRI tumor deposition and local tumor activation on tumor SN-38 duration was evaluated using model simulations. Based on the findings from the sensitivity analysis (Fig. 3B), nal-IRI permeability (PS_{nal-IRI}) and tumor CES activity (V_{max,CES,t}) values were utilized to create a map relating these parameters to tumor SN-38 duration following nal-IRI administration (Fig. 5A). Model simulations predicted a concave relationship, where the tumor SN-38 duration is dependent upon both the tumor permeability and the tumor CES activity. The tumor SN-38 duration could be increased by either increasing the PS_{nal-IRI} or V_{max,CES,t} (white arrows) and the maximum tumor SN-38 duration of 168 hours was only reached with CES activity at 0.025nmol/min and tumor permeability at 1.5E-4 L/min/kg.

To experimentally test the model predictions, we used the same panel of 13 xenograft models to measure the tumor concentrations of CPT-11 (as a surrogate for tumor deposition, supplementary Fig.
and CES activity (for local tumor activation of irinotecan). The experimental data supported the model simulations, confirming that the SN-38 concentration within tumors was dependent on both the tumor CPT-11 concentration and tumor CES activity (Fig 5B). All tumor models with high CPT-11 concentration (>2000 ng/g) or high CES activity (> 5 ng/ml) displayed high tumor SN-38 concentrations (“red”) ranging from 25 – 125 ng/ml (Supplementary Table 2). In certain tumor models, one of the parameters contributed predominantly towards higher SN-38 concentrations (black arrows). A2780 and SK-ES-1 tumors displayed high tumor SN-38 concentrations of 97 ng/ml and 127 ng/ml respectively (Supplementary Table 2), which was mainly due to high CPT-11 concentrations (>2000 ng/g) whereas in other tumor models (CTG-0062 and AsPC-1) the CES activity (>5 ng/ml) was the dominant factor contributing towards high tumor SN-38 concentrations. Further tumor models with the lowest tumor SN-38 concentrations ranging from 5 – 12 ng/ml (“blue”), including several patient-derived tumor models (boxed area) also displayed lower tumor CPT-11 concentrations (<2000 ng/g) and CES enzyme activity (<5 ng/ml).

**Tumor SN-38 duration correlates with nal-IRI in vivo activity**

*In vivo* tumor response studies were performed in three tumor models where different tumor SN-38 durations had been observed (as indicated by tumor SN-38 concentration at 72 hours) to determine the impact of tumor SN-38 duration on *in vivo* activity of nal-IRI. The tumor volumes observed for both HT-29 (Fig. 6A) and SK-ES-1 (Fig. 6B) models were significantly lower (p<0.05) following 10 mg/kg nal-IRI as compared to untreated tumors. In both these models, tumor regression was observed immediately after the first dose and was sustained through the course of the study. A549 tumors achieved lower SN-38 tumor levels (Fig. 4A) and did not respond to nal-IRI treatment (Fig. 6C). Interestingly, both A549 and HT-29 cells displayed similar *in vitro* sensitivity to SN-38 with IC50 values of 53 nM and 44 nM respectively (20). In summary, nal-IRI induced stronger responses (~100%TGI) in tumor models that had higher tumor SN-38 duration (> ~100 hours).
Discussion

The nal-IRI formulation dramatically alters the pharmacological properties of irinotecan as well as its active metabolite, SN-38 (9). In this study, we identified a pharmacological parameter - namely, tumor SN-38 duration - as a driver of irinotecan-based in vivo activity and propose biomarkers that can impact tumor SN-38 duration achieved by nal-IRI. Our study indicates that nal-IRI can completely inhibit tumor growth compared to free irinotecan, despite administering doses that achieve similar SN-38 exposure (measured as the AUC). Instead, the duration of prolonged exposure of SN-38 within tumors achieved by nal-IRI was shown to be a major pharmacological determinant for in vivo activity in mice.

Several studies have shown improved in vitro cytotoxic activity of SN-38 when cells are exposed to drug for longer duration (21). The in vitro cell doubling time for HT-29 cells is approximately 20 hours (21), whereas in vivo the tumor volume doubles (Fig. 2B) at a slower rate (~8 to 9 days). In addition, at a given time only 35 to 50% of cells are in the S-phase of cell cycle wherein the maximum cytotoxicity of free irinotecan has been observed (21). Thus, in order to exert maximum cytotoxic effects across different cell cycle phases, the cells have to be exposed to free irinotecan across multiple cell cycles. Our in vivo study confirms these findings as the free irinotecan is rapidly cleared from plasma and tumor tissue (tumor SN-38 duration of ~40 hours), thereby not allowing sufficient time for tumor cells to be exposed to SN-38 (for only 2 cell cycle doubling time) as compared to more than 5 cell cycle doubling times with nal-IRI (tumor SN-38 duration for >100 hours). Thus the extended exposure of tumor cells to SN-38, which is achieved by nal-IRI, can contribute towards the enhanced cytotoxicity as compared to free irinotecan.

We observed higher tumor concentrations of CPT-11 and SN-38 at 168 hours following administration of nal-IRI. In contrast, the peak plasma concentrations of SN-38 was lower with nal-IRI as compared to free irinotecan, suggesting that most of the CPT-11 from nal-IRI remains inside the liposomes and is protected from systemic conversion as described with free irinotecan (17). In addition, prolonged SN-38 duration from nal-IRI administration was observed only in tumors and much less in normal tissues,
suggesting that toxicity might not be exacerbated by nal-IRI treatment. The preferentially accumulation of nal-IRI in tumors as compared to normal tissues can be attributed to the enhanced permeability and retention (EPR) effect, where the leaky vasculature in tumor facilitates the extravasation of liposomal nanoparticles and the defective lymphatic drainage helps increase the retention within tumor (1,2). Thus, with the EPR effect, nal-IRI creates a large depot of CPT-11 only in tumors thereby prolonging tumor SN-38 duration. In contrast, free irinotecan can easily be transported in and out of the tissues with a short plasma half-life, resulting in minimal SN-38 duration in tumors.

The enhanced in vivo activity of nal-IRI as compared to free irinotecan was attributed to the ability of nal-IRI to extend the tumor SN-38 duration. Sensitivity analysis identified two key determinants that impact the ability of nal-IRI to extend tumor SN-38 duration - (i) nal-IRI tumor deposition, as measured by the extent of pro-drug CPT-11 deposition within tumors and (ii) nal-IRI local activation, from pro-drug CPT-11 to SN-38 facilitated by the local tumor CES enzyme. The experimental data, in this study supported the importance of each of these determinants. We observed high degree of variability in the overall nal-IRI tumor deposition across the 13 xenograft models that were tested. Several studies have highlighted a role for tumor permeability, tumor perfusion, and stromal matrix in limiting the delivery of therapeutic agents into tumors (22). In our model simulations, when the nal-IRI tumor permeability was decreased to zero, the benefit of higher tumor SN-38 duration with nal-IRI was negatively impacted and reduced to levels simulated for free irinotecan. We also observed that the tumors with lower nal-IRI deposition had considerable lower SN-38 tumor levels. This data is consistent with other findings suggesting that a dense tumor stroma can impede drug permeability and limit drug delivery within tumors (23,24).

Use of tumor CES activity as a cellular parameter for predicting free irinotecan response had limited success both in preclinical (25,26) and clinical studies (27). Through the sensitivity analysis performed in this study, we identified CES activity as a critical parameter for nal-IRI activity. Tumor models that displayed high ability to activate CPT-11, achieved high tumor SN-38 concentrations despite limited
deposition of CPT-11, thus suggesting the importance of local tumor CES enzyme expression in facilitating longer SN-38 exposure following nal-IRI administration. In fact, others have shown that in vitro and in vivo activity of free irinotecan can be enhanced by overexpressing of CES enzyme in tumor cells (28,29). In addition to tumor cells expressing CES enzyme (30), other components of the intracellular matrix such as tumor-associated macrophages (TAMs) express CES1 enzyme and play a role in CPT-11 activation (31). In fact, we performed in vitro studies that confirmed the ability of TAMs to hydrolyze CPT-11 to SN-38 (Supplementary Fig. S5). Thus our data suggests, the extended tumor PK achieved by nal-IRI provides high local depot of pro-drug CPT-11 for prolonged time thus allowing for activation by tumor CES enzymes. Collectively our data provides rational for investigating tumor CES enzyme activity as potential marker for nal-IRI activity.

Pharmacogenetic and pharmacodynamics markers such as Topo1 have shown limited correlations with free irinotecan response (6,32–34). In addition to the intrinsic sensitivity of tumor cells to SN-38, our data indicates that the duration for which tumor cells are exposed to SN-38 (tumor SN-38 duration) also plays a critical role in driving treatment response to irinotecan. Tumor models with extended SN-38 duration (HT-29, SK-ES-1) showed robust in vivo response to nal-IRI, whereas A549 with shorter tumor SN-38 duration did not respond to therapy. The fact that in vitro sensitivity of both HT-29 and A549 to SN-38 is very similar (20) corroborates the finding that the duration of SN-38 is driving the tumor response.

In conclusion, our data demonstrate that nal-IRI enhances the pharmacokinetic profile of tumor SN-38, prolonging tumor exposure to SN-38 compared with free irinotecan, and therefore has the potential for therapeutic effect in human cancers. Liposome permeability and CES activity were the critical factors that emerged from model simulation of tumor SN-38 duration, which were experimentally shown to vary across and within tumor indications. Thus, translational research exploring the utility of tumor liposome permeability and local activation of irinotecan as biomarkers for nal-IRI clinical activity is warranted.
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Footnote

In the manuscript, CPT-11 is used when referring to the pro-drug levels in plasma or tumor samples following either free irinotecan or nal-IRI administration. SN-38 is used when referring to the active metabolite of CPT-11.


**Table 1. Summary of model parameters for plasma PK and tumor deposition models**

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<tr>
<td><strong>Plasma PK Model Parameters</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>For free irinotecan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT-11 clearance rate</td>
<td>1.222e-4</td>
<td>L/min</td>
<td>Plasma clearance rate of CPT-11</td>
<td>Estimated</td>
</tr>
<tr>
<td>SN-38 clearance rate</td>
<td>2.138e-5</td>
<td>L/min</td>
<td>Plasma clearance rate of SN-38</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of CPT-11 transport</td>
<td>8.444e-5</td>
<td>1/min</td>
<td>from plasma to peripheral compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of CPT-11 transport</td>
<td>4.213e-2</td>
<td>1/min</td>
<td>from peripheral to plasma compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of SN-38 transport</td>
<td>2.656e-5</td>
<td>1/min</td>
<td>from plasma to peripheral compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of SN-38 transport</td>
<td>3.44e-4</td>
<td>1/min</td>
<td>from peripheral to plasma compartment</td>
<td>Estimated</td>
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<tr>
<td>Maximum rate coefficient</td>
<td>2.263e-1</td>
<td>nmol/min</td>
<td>for CES enzyme in plasma compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Michaelis-Menten constant</td>
<td>2.67e5</td>
<td>nM</td>
<td>for CES enzyme in plasma compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Volume of plasma</td>
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<td>L</td>
<td>compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Volume of peripheral</td>
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<td>L</td>
<td>compartment</td>
<td>Fixed (14)</td>
</tr>
<tr>
<td><strong>For nival-IRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPT-11 clearance rate</td>
<td>1.87e-7</td>
<td>L/min</td>
<td>Plasma clearance rate of nival-IRI</td>
<td>Estimated</td>
</tr>
<tr>
<td>SN-38 clearance rate</td>
<td>1.634e-5</td>
<td>L/min</td>
<td>Plasma clearance rate of SN-38</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of CPT-11 transport</td>
<td>2.957e-6</td>
<td>L/min</td>
<td>from plasma to peripheral compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Rate of CPT-11 transport</td>
<td>5.349e-7</td>
<td>L/min</td>
<td>from peripheral to plasma compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Maximum rate coefficient</td>
<td>2.263e-1</td>
<td>nmol/min</td>
<td>for CPT-11 release from nival-IRI in plasma compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Michaelis-Menten constant</td>
<td>5.943e-2</td>
<td>nmol/min</td>
<td>for CPT-11 release from nival-IRI in plasma compartment</td>
<td>Estimated</td>
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<td>Volume of plasma</td>
<td>1.198e5</td>
<td>nM</td>
<td>compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Volume of peripheral</td>
<td>0.02</td>
<td>L</td>
<td>compartment</td>
<td>Fixed (14)</td>
</tr>
<tr>
<td><strong>Tumor Deposition Model Parameters</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Blood flow rate to tumor</td>
<td>2.119e-6</td>
<td>L/min</td>
<td></td>
<td>(14)</td>
</tr>
<tr>
<td>Tissue permeability</td>
<td>7.858e-5</td>
<td>L/min/kg</td>
<td>coefficient of nival-IRI</td>
<td>Estimated</td>
</tr>
<tr>
<td>Tissue permeability</td>
<td>1.851e-3</td>
<td>L/min/kg</td>
<td>coefficient of CPT-11</td>
<td>Estimated</td>
</tr>
<tr>
<td>Tissue permeability</td>
<td>2.687e-2</td>
<td>L/min/kg</td>
<td>coefficient of SN-38</td>
<td>Estimated</td>
</tr>
<tr>
<td>Tissue-capillary partition coefficient of nival-IRI</td>
<td>3.181e-3</td>
<td>L/min</td>
<td>Estimated</td>
<td></td>
</tr>
<tr>
<td>Tissue-capillary partition coefficient of CPT-11</td>
<td>5.24e-1</td>
<td>L/min</td>
<td>Estimated</td>
<td></td>
</tr>
<tr>
<td>Tissue-capillary partition coefficient of SN-38</td>
<td>2.109e-1</td>
<td>L/min</td>
<td>Estimated</td>
<td></td>
</tr>
<tr>
<td>Rate coefficient for CPT-11 release from nival-IRI in tumor tissue compartment</td>
<td>1.681e-4</td>
<td>1/min</td>
<td>Estimated</td>
<td></td>
</tr>
<tr>
<td>Maximum rate coefficient</td>
<td>2.17e-2</td>
<td>nmol/min</td>
<td>for CES enzyme in tumor tissue compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Michaelis-Menten constant</td>
<td>2.36e9</td>
<td>nM</td>
<td>for CES enzyme in tumor tissue compartment</td>
<td>Estimated</td>
</tr>
<tr>
<td>Volume of tumor capillary compartment</td>
<td>7e-7</td>
<td>L</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>Volume of tumor tissue compartment</td>
<td>1e-5</td>
<td>L</td>
<td>Fixed</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1.** Pharmacokinetic profile of nal-IRI and free irinotecan. (A) Plasma and tumor PK of nal-IRI were compared to free irinotecan in HT-29 xenograft bearing mice. NOD SCID mice bearing HT-29 tumors were treated with single intravenous dose of free irinotecan or nal-IRI. Plasma and tumors were collected at various intervals and the CPT-11 and SN-38 were measured by HPLC analysis (n=4 animals/time point). Plasma PK data for free irinotecan were taken from Kaneda et al. (35). **Solid lines**, represent the model simulations for nal-IRI, whereas **dashed lines** represent the model simulations for free irinotecan. (B) Diagram of the mechanistic tumor pharmacokinetic model developed to describe the various steps in metabolism, pharmacokinetics and tumor deposition of nal-IRI.

**Figure 2.** Relation of nal-IRI in vivo activity to tumor SN-38 duration. (A) Model predictions for similar SN-38 AUC in plasma and tumor following free irinotecan (50 mg/kg) and nal-IRI (10 mg/kg) administration. (B) Tumor response observed in HT-29 xenograft following weekly administration (arrows) of 50 mg/kg free irinotecan and 10 mg/kg nal-IRI (n=8/group). The tumor volumes for nal-IRI (10 mg/kg) were significantly lower (*p<0.05) compared to saline and irinotecan groups (one way ANOVA test) (C) Tumor response in HT-29 xenografts following weekly administration (arrows) of various nal-IRI doses (n=5/group). The tumor volumes for nal-IRI (10 mg/kg) and nal-IRI (20 mg/kg) groups was significantly lower (*p<0.05) compared to saline tumors on Day 25 and Day 28 (one way ANOVA test). (D) Model simulations were used to compare tumor SN-38 concentration following the administration of varying doses of free irinotecan or nal-IRI. **Black dashed line** represents threshold concentration of 120 nM to determine tumor SN-38 duration. (E) TGI(%) achieved by nal-IRI and free irinotecan treatment in HT-29 xenografts were compared to the tumor SN-38 duration above 120 nM (E) and SN-38 AUC (F) at varying doses of nal-IRI or free irinotecan. **Solid lines** represent non-linear regression lines based on five parameter logistic curve fitting. (G) The SN-38 duration over a threshold of 120 nM was computed from the pharmacokinetic profiles of SN-38 in tumor and normal tissues following 20 mg/kg of nal-IRI.
Figure 3. Model parameters impacting tumor SN-38 duration. Sensitive analyses for free irinotecan (A) and nal-IRI (B) were performed on key model parameters which are responsible for plasma clearance, tissue deposition and metabolic reactions. Parameters whose values were not estimated in this study including compartment volumes and tumor blood flow were excluded from the analysis. The doses of free irinotecan (50 mg/kg) and nal-IRI (10 mg/kg) that achieved similar SN-38 plasma and tumor exposure were used for sensitivity analysis. The model parameters were modulated by 10% and their effect on tumor SN-38 duration was determined as a sensitivity index (Equation S6).

Figure 4. *In vivo* variability in nal-IRI tumor deposition and local activation. (A) Intra tumor CPT-11 concentrations were measured across cell-line derived (HT-29, SK-ES-1, A549, MDA-MB-231, LoVo, AsPC-1, A2780) and patient-derived (CTG-0062, CTG-0079, CTG-0252, CTG-0288, CTG-0158, CTG-0283) tumor models. Tumor bearing mice were administered a single intravenous dose of 10 mg/kg nal-IRI and tumors excised 24 hr later. CPT-11 concentrations were determined in the tumor lysates using HPLC analysis as described in methods (n=4-8 tumors/model). (B) The effect of nal-IRI permeability on tumor SN-38 concentrations was simulated by reducing the nal-IRI permeability parameter, $PS_{nal-IRI}$ to zero. The simulated tumor SN-38 concentrations were compared at the equal exposure doses of 10 mg/kg nal-IRI and 50 mg/kg free irinotecan. Black solid line: nal-IRI (10 mg/kg) with base $PS_{nal-IRI}$. Gray solid line: nal-IRI (10 mg/kg) with zero $PS_{nal-IRI}$. Black dashed line: free irinotecan (50 mg/kg). Dotted line represents threshold concentration of 120 nM (C) Carboxylesterase (CES) activity for 80 patient-derived xenograft tumors across different indications was determined using *ex vivo* irinotecan activation assay. Tumor lysates (250 µg of protein) from untreated mice was incubated with free irinotecan (5 µM) for 24 hr at 37°C and the amount of SN-38 produced was measured with HPLC analysis (*p<0.05; t-test). (D) The effect of knocking out tumor CES activity on tumor SN-38 duration was simulated by reducing the tumor CES parameter, $V_{max,CES,t}$ to zero. The simulated tumor SN-38 concentrations were compared at the equal exposure doses of 10mg/kg nal-IRI and 50 mg/kg free irinotecan. Black solid line: nal-IRI 10 mg/kg with base $V_{max,CES,t}$. Gray solid lines: nal-IRI 10 mg/kg with zero $V_{max,CES,t}$. Black dashed line: free irinotecan 50 mg/kg with base $V_{max,CES,t}$. Dotted line represents threshold concentration of 120 nM.
Figure 5. nal-IRI tumor deposition and local activation impacts tumor SN-38 duration. (A) The effect of changing tumor CES activity and nal-IRI permeability parameters (arrows) on tumor SN-38 duration (color-coded in hours) in tumors was simulated. The optimal parameter values for HT-29 were marked with the symbol ‘*’. (B) Experimental data in tumor xenograft models showing the impact of tumor CPT-11 and CES activity on tumor SN-38 concentrations. Tumor CES activity (as surrogate for local tumor activation of irinotecan) and tumor CPT-11 concentration at 72 hr (as surrogate for tumor deposition) for different xenograft models were plotted and color-coded based on their SN-38 concentrations in the tumor 72 hr after nal-IRI (each data point represents median of n=4-8 tumors). Dotted arrows indicate dependence of tumor SN-38 concentrations on tumor CPT-11 concentration and CES activity.

Figure 6. In vivo tumor response for nal-IRI. NOD SCID mice were inoculated with HT-29; colon (A), SK-ES-1; ewings (B) and A549; lung (C) cell lines. Tumor bearing mice were randomized when the tumor volume was approximately 200 mm³. Each group received weekly intravenous dose (arrows) of either saline (●) or 10mg/kg dose of nal-IRI (○). Tumor volumes were measured twice per week (n=5-10 animals/group).
Figure 1A

Figure 1B

Peripheral Tissue

Central

CES_{plasma}

Tumor Capillary

CES_{tumor}

Tumor Tissue
Figure 2

(A) SN-38 AUC (nM/min) for Irinotecan at 50mg/kg and nal-IRI at 10mg/kg.

(B) Tumor volume (mm$^3$) over time (Days) for saline, Irinotecan at 60mg/kg, and nal-IRI at 10mg/kg.

(C) Tumor volume (mm$^3$) over time (Days) for saline, Irinotecan at 50mg/kg, and nal-IRI at various concentrations (1.25, 2.5, 5, 10, 20mg/kg).

(D) SN-38 concentration (nM) over time (hr) for different treatments.

(E) TGI (%) over SN-38 Duration (hr) for SN-38 AUC values.

(F) SN-38 AUC (μM*hr) over time (Days) for different concentrations of nal-IRI.

(G) SN-38 Duration over 120nM for different organs (Colon, Duct, Kidney, Liver, Lung, Skin, Tumor).

Figure 2
Figure 3

A. Free Irinotecan
- Liposome release rate in tumor
- Liposome release rate in blood
- CES activity in tumor
- CES activity in blood
- nal-IRI permeability
- SN-38 permeability
- CPT-11 permeability
- nal-IRI clearance rate in plasma
- SN-38 clearance rate in plasma
- CPT-11 clearance rate in plasma

B. nal-IRI
- Liposome release rate in tumor
- Liposome release rate in blood
- CES activity in tumor
- CES activity in blood
- nal-IRI permeability
- SN-38 permeability
- CPT-11 permeability
- nal-IRI clearance rate in plasma
- SN-38 clearance rate in plasma
- CPT-11 clearance rate in plasma

Sensitivity
Figure 4

A

B

C

D

Tumor CPT-11 (ng/g)

Tumor SN-38 (nM)

CES activity

(SN-38 present, ng/mL)

Time (hr)

Time (hr)

Time (hr)

Time (hr)
Figure 6

A

HT-29

Tumor Volume (mm³)

Days

12 14 16 18 20 22 24 26 28 30

0 100 200 300 400 500 600 700

B

SK-ES-1

Tumor Volume (mm³)

Days

14 16 18 20 22 24 26 28 30

0 200 400 600 800 1000

C

A549

Tumor Volume (mm³)

Days

26 28 30 32 34 36 38 40 42 44 46

0 400 800 1200 1600 2000
Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor pro-drug conversion


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