The DNA Repair Inhibitor DT01 as a Novel Therapeutic Strategy for Chemosensitization of Colorectal Liver Metastasis

Nirmitha I. Herath1,2, Flavien Devun1,2, Marie-Christine Lienafa1,2, Aurélie Herbette1,2, Alban Denys3, Jian-Sheng Sun2, and Marie Dutreix1,4,5

Abstract

Metastatic liver disease from colorectal cancer is a significant clinical problem. This is mainly attributed to nonresectable metastases that frequently display low sensitivities to available chemotherapies and develop drug resistance partly via hyperactivation of some DNA repair functions. Combined therapies have shown some disease control; however, there is still a need for more efficient chemotherapies to achieve eradication of colorectal cancer liver metastasis. We investigated the tolerance and efficacy of a novel class of DNA repair inhibitors, Dbait, in association with conventional chemotherapy. Dbait mimics double-strand breaks and activates damage signaling, consequently inhibiting single- and double-stranded DNA repair enzyme recruitment. In vitro, Dbait treatment increases sensitivity of HT29 and HCT116 colorectal cancer cell lines. In vivo, the pharmacokinetics, biodistribution and the efficacy of the cholesterol-conjugated clinical form of Dbait, DT01, were assessed. The chemosensitizing abilities of DT01 were evaluated in association with oxaliplatin and 5-fluorouracil in intrahepatic HT29 xenografted mice used as a model for colorectal cancer liver metastasis. The high uptake of DT01 indicates that the liver is a specific target. We demonstrate significant antitumor efficacy in a liver metastasis model with DT01 treatment in combination with oxaliplatin and 5-fluorouracil (mean: 501 vs. 872 mm², \(P = 0.02\)) compared to chemotherapy alone. The decrease in tumor volume is further associated with significant histologic changes in necrosis, proliferation, angiogenesis and apoptosis. Repeated cycles of DT01 do not increase chemotherapy toxicity. Combining DT01 with conventional chemotherapy may prove to be a safe and effective therapeutic strategy in the treatment of metastatic liver cancer. Mol Cancer Ther, 15(1): 15–22. ©2015 AACR.

Introduction

Secondary hepatic tumors or liver metastases account for approximately 95% of all hepatic malignancies. A majority of liver metastases arise through primary tumors of the gastrointestinal tract (1). Liver resection remains the principle choice of treatment for early stage liver metastases (2, 3). However, most patients are diagnosed at advanced stages making these patients unsuitable for surgery. Treatment alternatives such as local ablation and transarterial therapies demonstrate a limited effect with low survival benefit (3, 4).

Chemotherapy is widely used in the treatment of metastatic colorectal carcinoma. However, patients respond poorly to chemotherapy regimens combining folinic acid, 5-fluorouracil and either oxaliplatin (FOLFOX) or irinotecan mainly due to multidrug resistance, preventing the eradication of metastatic disease (5, 6). Therefore, improvements in overcoming colorectal cancer chemoresistance are an urgent need and should be a focus of investigation. Here, we investigate how combination with the recently developed DNA repair inhibitors (Dbait) could improve efficacy of conventional chemotherapy in xenografted animal models.

Chemoresistance presents a major obstacle to the efficacy of cancer treatment. DNA repair plays a key role in chemoresistance by eliminating the damage induced on chromosomes by the chemotherapeutic agents and inhibitors of DNA repair pathways may provide novel opportunities for restoring tumor sensitivity to these treatments (7). Dbait molecules are a new class of DNA repair inhibitors triggering false DNA damage signaling in cancer cells (8). These molecules are short double-stranded DNA with a free double-strand blunt end, which target key damage signal transducers such as DNA-dependent protein kinase (DNA-PK; ref. 9) and PARP (10), triggering their activation and amplifying false damage signaling. Consequently, the recruitment of downstream DNA repair enzymes is impaired, inhibiting several DNA repair pathways such as homologous recombination (9), non-homologous end joining (8, 9), base excision repair, and single-strand break repair (10) leading to an accumulation of unrepaired damage causing cell death.

We previously showed Dbait to be effective in combination with radiotherapy on several radio-resistant tumors, both in vitro and in vivo (8, 11–13). Furthermore, studies on an intestinal tumor rodent model revealed that oral administration of Dbait,
in association with the chemotherapy agents 5-fluorouracil and camptothecin leads to increased chemosensitization of intestinal tumors and to a low systemic exposure (14). To increase the efficiency of cellular uptake, the Dbait molecule was modified by covalently linking a cholesterol moiety to the 5'-end (DT01; ref. 11). We demonstrated that local administration of DT01 by intratumoral injection in association with radiotherapy increases survival of xenografted human melanoma models (13). However to date, the efficacy of systemic administration of DT01 in association with chemotherapy has not been investigated.

The aims of the current study were to firstly demonstrate the efficacy of DT01 in vitro, secondly to assess the pharmacokinetics and the distribution of DT01 in the liver, and thirdly to demonstrate the concomitant impact of systemic DT01 administration in combination with conventional chemotherapy (oxaliplatin with 5'-fluorouracil) in a colorectal cancer metastatic liver tumor model.

Materials and Methods

Cell culture, constructs, Dbait molecules, immunofluorescence, and Western blotting

Colorectal cancer cell lines: HT29 (mutated p53, ATCC: HTB-38) and HCT116 (wild-type p53, ATCC: CCL-247) were purchased directly from ATCC. These cells were authenticated by ATCC by generating human short tandem repeat profiles by simultaneously amplifying multiple STR loci and amelogenin (for gender determination) using the Promega PowerPlex Systems. These cells were cultured in the laboratory for less than 6 months from the date of purchase in DMEM medium supplemented with 10% FBS, 1% sodium pyruvate, 100 mg/mL streptomycin, and 100 mg/mL penicillin (Invitrogen), when the current study was performed. HT29 cell line stably expressing luciferase was established in-house using a pGL4.5 luciferase reporter vector (luc2/CMV/Hygro; Promega). HT29 luciferase cells were supplemented with 200 μg/mL hygromycin B. All cell lines were additionally subjected to mycoplasma testing in-house and were free of mycoplasma contamination (Biovalley).

Cells were transfected with 2.5 μg of Dbait (5'-GGTTGTGGGCACCAACCCAGCAAACAAGCCTAGA-(H) TCTAGGCTTGTTTGCTGGTCAG; Eurogentec) where H is a hexaethylene-glycol linker and underlined nucleotides are phosphorothioates. The cells were shan transfected with an 8 bp oligonucleotide control (8H) complexed with 11 kDa polyethyleneimine (PEI) as previously described (8, 9).

γH2AX immunofluorescence was performed as described previously using a monoclonal anti-φospho-Histone H2AX (Ser139) antibody, clone JBW301 (1:500 dilution; 05-636, Millipore; ref. 9).

In vitro proliferation assay

Cells were seeded at a density of 3 × 10^4 cells/60 mm dishes and transfected with Dbait. Following treatment, cells were washed and left untreated or treated with a combination of 5 μmol/L of oxaliplatin (OXA, Sigma) and 2.5 μmol/L of 5-fluorouracil (5-FU, Sigma) and live cell counts were performed on days 1, 3, 5, 6, 7, and 9.

Clonogenic assay

Cells were transfected with Dbait and left untreated or treated with 5 μmol/L of OXA and 2.5 μmol/L of 5-FU for 1 hour. The cells were diluted, allowed to grow for 14 days, and the clones were stained with crystal violet and counted.

In vivo experiments

The current study was carried out in strict accordance with the European Union guidelines for animal care. All animal experimentation was approved by the ethics committees of the Institut Curie and the French ministry. Surgical procedures were performed under anesthesia with local analgesia to minimize suffering.

Animals

Six-week-old female NMRPhilo®/N mice (Janvier) weighing 20 to 22 g were housed in specific pathogen-free environment on a 12-hour light and 12-hour dark schedule with food and water ad libitum. No more than 6 animals were housed per cage and they were acclimated for at least one week prior to initiating in vivo studies.

Intrahepatic HT29L, grafting. HT29 Luciferase (HT29L) cells were implanted by direct injection of cell suspensions (1 × 10⁷/10 μL of PBS) onto the upper surface of the left lobe. Tumor growth was monitored through bioluminescence analysis (IVIS, Caliper Sciences).

DT01 molecule. For in vivo studies, DT01 (Dbait with a cholesterol triethylene glycol incorporated at the 5'-end) was used (Agilent technologies; ref. 11).

Pharmacokinetics of DT01. HT29L-grafted mice were treated with a single intraperitoneal (i.p., n = 4) or intravenous (i.v., n = 3) injection of 5 mg of DT01. Blood samples were harvested prior to treatment and 1, 5, 10, 30 minutes, 1, 2, 4, and 6 hours after treatment. Plasma was recovered through centrifugation and assayed by ELISA.

Fluorescence measurement of organs. As the ELISA technique failed to produce reliable quantification in tissues, we used fluorescent imaging, a reliable technique for assessing molecule distribution (15). NMRPhilo®/N mice were injected with 1 mg of the DT01 fluorescent molecule (DT01-Cy5) through i.p. (n = 3) or i.v. (n = 3) administration. The fluorescent DT01 (DT01-Cy5) incorporates a cyanine 5 at the thymidine located immediately after the linker. Six hours after injection, fluorescent imaging was performed using a Typhoon scanner (GE Helathcare).

DT01 and chemotherapy treatment. HT29L-grafted animals (n = 49) were allocated into treatment groups and administered one cycle of treatment (Supplementary Table S1). DT01 was systemically administered through i.p. injection at a dose of 5 mg/day for 5 consecutive days starting on day 0 (D0). OXA (6 mg/kg, 1× per cycle, day 1) and 5-FU (25 mg/kg, 3× per cycle. Days 1–3) were administered 2 or 4 hours after DT01 treatment. These mice were sacrificed 22 days after treatment.

An additional group treated with DT01 and OXA/5-FU at the 4-hour interval (n = 10) were kept after treatment until the termination guidelines were met to assess the duration of treatment efficacy.

Liver function assessment. Blood samples were obtained through submandibular bleeding in lithium heparin tubes (Sarstedt) on
days 0, 4, and 18 after treatment. Plasma alanine transaminase (ALT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), amylase (AMY), and total bilirubin (TBIL) were measured using an MS-Scan II (Melet Schloesing Laboratories).

Toxicity assays. NMR[Etu/L] mice (n = 50) were treated with two cycles of DT01 at escalating doses of 3 mg/day (30 mg total), 5 mg/day (50 mg total), or 8 mg/day (80 mg total) through i.p. injection in combination with OXA or 5’-FU. OXA and 5’-FU were administered through systemic i.p. injection at doses of 1 × 6 mg/kg or 3 × 25 mg/kg, 4 hours after DT01 treatment, respectively. Animals were observed regularly for any adverse effects.

Histology
Hematoxylin, eosin, and saffron (HES)-stained tumor sections were assessed by an experienced pathologist (Dr. Huerre, Institut Curie, Paris, France) in a blinded fashion. Viable and necrotic portions (%) of the total tumor surface. Apoptosis was estimated in vitro effects. Respectively. Animals were observed regularly for any adverse effects.

Ki67 and CD31 immunohistochemistry
Immunohistochemistry was performed using rabbit anti-Ki67 (ab28364, 1/500; Abcam) and rabbit anti-CD31 (ab15580, 1/500; Abcam) antibodies. This was followed by a secondary biotinylated goat anti-rabbit IgG antibody (BA-1000; Vector) and revealed using a rabbit-specific HRP/DAB (ABC) Detection Kit. Images were captured using a fluorescence microscope (Eclipse 90i, Nikon). The average Ki67 index was scored by establishing a ratio between Ki67+ and negative cells, in five randomly selected microscopic fields per section. Average microvessel density was determined by CD31 staining. CD31+ vessels were counted in five randomly selected microscopic fields per section.

Statistical analysis
In vitro experiments were performed with a minimum of two independent experiments. Two-sided unpaired t tests were used for comparison of cell mortality and survival. Kruskal–Wallis tests were used to compare tumor volumes, and histologic data. Error bars indicate SEM, except when specifically indicated. All statistical analyses were performed using StatEL software (adScience) and a P value of ≤0.05 was considered statistically significant.

Results
Dbait treatment increases sensitivity of colon cancer cell lines to chemotherapy
We have previously shown that Dbait acts by activating DNA-PK kinase, which phosphorylate numerous targets including the histone variant H2AX (8, 9, 14). We first confirmed the activity of Dbait in two colorectal cancer cell lines (HCT116 and HT29) by monitoring the pan-nuclear phosphorylation of H2AX (Fig. 1A). To first investigate the effects of Dbait on cell survival to chemotherapy, we determined the number of living cells, at different time points after treatment with Dbait or OXA and 5-FU or a combination of Dbait with chemotherapy (Fig. 1B). As already observed in fibroblasts (9) Dbait alone appears to have no effect on cell proliferation in both cell lines (Fig. 1B). Treatment with OXA and 5-FU resulted in a decrease in cell proliferation. The level of proliferation was significantly reduced by day 9 in cells transfected with Dbait prior to chemotherapy treatment in both HCT116 and HT29 cell lines compared with chemotherapy alone.

Dbait treatment increases sensitivity of colon cancer cell lines to chemotherapy
We have previously shown that Dbait acts by activating DNA-PK kinase, which phosphorylate numerous targets including the histone variant H2AX (8, 9, 14). We first confirmed the activity of Dbait in two colorectal cancer cell lines (HCT116 and HT29) by monitoring the pan-nuclear phosphorylation of H2AX (Fig. 1A). To first investigate the effects of Dbait on cell survival to chemotherapy, we determined the number of living cells, at different time points after treatment with Dbait or OXA and 5-FU or a combination of Dbait with chemotherapy (Fig. 1B). As already observed in fibroblasts (9) Dbait alone appears to have no effect on cell proliferation in both cell lines (Fig. 1B). Treatment with OXA and 5-FU resulted in a decrease in cell proliferation. The level of proliferation was significantly reduced by day 9 in cells transfected with Dbait prior to chemotherapy treatment in both HCT116 and HT29 cell lines compared with chemotherapy alone (P < 0.001 and P < 0.02, respectively). These differences become apparent particularly at later time points (days after treatment) indicating that the increase of efficacy with Dbait may be a slow process.
To confirm the chemosensitization effect of Dbait in combination with OXA and 5-FU, clonogenic survival assays were performed on HCT116 and HT29. HCT116 cells showed approximately 30% (P < 0.01) lethality after Dbait treatment alone (Fig. 1C) revealing their dependency on repair activity for survival, whereas no significant effect was noted in HT29. As the sensitivity of HCT116 to Dbait was not detected during the first 8 days of proliferation (Fig. 1B), this result suggests that the cells growing with Dbait accumulate lethal lesions that impair their survival later on. Treatment with chemotherapy alone (OXA/5-FU) resulted in a significant decrease in the survival of HCT116 (P < 0.001), whereas only a trend was observed with the HT29 cell line (P = 0.08). However, combination of Dbait with chemotherapy resulted in a significant reduction in survival in both cell lines (P = 0.05). HCT116 and HT29 differ by many parameters including their P53 status (HCT116 being proficient whereas HT29 is mutated). In this instance, despite some differences in their sensitivity to standalone Dbait treatment both cell lines were equally sensitive to the combination of chemotherapy with Dbait.

Pharmacokinetic and biodistribution analyses of i.p. versus i.v. administration of DT01

To avoid transfec tant adjuvant toxicity, all in vitro studies were performed with DT01, a Dbait–cholesterol conjugate facilitating the cellular uptake of these molecules without added toxicity (11). To determine the best route for systemic administration of DT01 mice were treated with either a single i.p. or an i.v. dose of 5 mg of DT01. I.p. administration resulted in a C\textsubscript{max} of 578 µg/mL, a t\textsubscript{max} of 1 hour and an AUC\textsubscript{0–6} of 799, whereas i.v. administration led to a C\textsubscript{max} of 1,917 µg/mL, a t\textsubscript{max} of 0.08 hours and an AUC\textsubscript{0–6} of 799 (Fig. 2A). Pharmacokinetic analyses revealed that after i.p. injection, the plasmatic exposure of DT01 was longer than that of i.v. bolus injection with an AUC corresponding to approximately 70% of the AUC with i.v. administration (Fig. 2A).

We used a fluorescent-labeled cy5-DT01 molecule to monitor the biodistribution in excised whole organs (Fig. 2B). Both cy5-DT01 and DT01 have similar properties in terms of pharmacokinetics and DNA-PK activation (11). The maximal DT01 fluorescence was observed in the liver, intestines, and the kidneys by both routes with the highest intensities observed in the liver and intestines following i.p. administration. The high fluorescence emitted by the kidneys and urine observed in mice suggest that DT01 is preferentially eliminated by the kidneys. Although there was no measurable DT01 in the blood 6 hours after injection (Fig. 2A), significant amounts of DT01 were still detectable in the liver indicating a specific retention in this organ (Fig. 2C).

As already demonstrated in vitro, DT01 activation of DNA-PK in tissue can be revealed by the phosphorylation of the histone H2AX (9). We monitored DT01 activity by analyzing distribution of H2AX phosphorylation in livers bearing HT29-grafted tumors. Interestingly, a high level of γH2AX was specifically observed in

Figure 2. Pharmacokinetics and bio-distribution of DT01. A, DT01 pharmacokinetics. Intrahepatic tumor bearing NMuMG mice were treated with a dose of 5 mg of DT01 through a single i.p. (black) or i.v. (gray) bolus administration. DT01 concentration in plasma was measured at 1, 5, 10, 30 minutes and 1, 2, 4, or 6 hours post treatment using an ELISA assay. B, DT01 biodistribution with fluorescent DT01-cy5. Mice were injected with 1 mg of DT01-cy5 through i.p. or i.v. administration. Six hours after injection, the animals were sacrificed, organs were excised, and scanned using a Typhoon scanner. The organs from left to right include lung (L), liver (Li), spleen (S), intestine (I), kidney (K), bladder (Bl), heart (H), brain (B), pancreas (P), and muscle (M). C, DT01-cy5 fluorescence quantitation of organs scanned using a Typhoon scanner. The relative fluorescence measurements were recorded and expressed in arbitrary units (AU), and the results are expressed as an average ± STD. The organs from left to right include liver (Li), intestine (I), kidney (K), lung (L), and spleen (S). D, DT01 induction of H2AX phosphorylation in liver tumor cells. Intrahepatic tumor bearing mice were treated with 1 mg of DT01 and the tumors were harvested 6 hours after treatment and subjected to immunolabeling with a γH2AX antibody (green) and DAPI (in blue).
the tumor and not in the surrounding healthy tissues (Fig. 2D) indicating a preferential uptake or activity of the DT01 molecules in the tumor cells of the liver.

**DT01 significantly increases sensitivity to OXA and 5-FU in vivo**

To explore the interest of associating DT01 with the front-line treatment for metastatic colorectal cancer, we used a HT29 xenografted liver tumor model, as previous reports and our in vitro data demonstrate this line to be highly chemoresistant mainly due to the V600E BRAF mutation (16, 17). The animals were treated with OXA and 5-FU, a treatment close to the traditional FOLFOX protocol for patients, using two different schedules based on biodistribution data (Fig. 3A). The two schedules consisted of either 2 or 4-hour intervals between the two treatments, as the maximum DT01 levels in the liver were observed at 1 and 3 hours after treatment (Fig. 2C). In previous studies we established that DT01 must be administered prior to the genotoxic treatment in order to act as a chemosensitizer.

As previously observed in vitro, the tumors were highly resistant to chemotherapy alone and DT01 had only a moderate effect when administered alone (Fig. 3B and C). Interestingly, the association of DT01 to OXA and 5-FU significantly decreased the liver tumor size in both combination treated groups compared with chemotherapy alone when administered at 2 (mean volume: 525.80 vs. 872.01 mm², \( P = 0.03 \)) and 4 hours (mean volume: 501.05 vs. 872.01 mm², \( P = 0.02 \)) before chemotherapy (Fig. 3B and C). This effect was not observed when DT01 was associated with a single chemotherapy agent, either OXA or 5-FU (Supplementary Fig. S1). Detailed blinded histologic analyses including measures of the viable tumor area, necrosis, and apoptosis were assessed in hematoxylin–eosin–saffron (HES)-stained sections, by an experienced pathologist. Both groups with DT01 and chemotherapy combined treatment showed higher treatment efficacy than the groups receiving single treatment, with a marked increase in necrosis in the group treated with a 4-hour interval between chemotherapy and DT01 (\( P < 0.0001 \)) than

---

**Figure 3.**

DT01 significantly increases sensitivity to chemotherapy in a colorectal cancer (HT29) liver metastatic model. Intrahepatic tumor-bearing NMuMGKO/KO mice were treated as described in the Materials and Methods, and sacrificed 22 days after treatment. Livers were sampled for macroscopic and microscopic examination. A, sequence of DT01 and CT therapy. Chemotherapy was administered 2 or 4 hours after DT01 treatment. B, mean liver tumor volume (mm³) in each treatment group. C, representative macroscopic and HES sections of liver tumors in each group. D, mean liver tumor volume (mm³) in each treatment group. E, tumor necrotic component assessed through HES staining. Necrosis is expressed as a proportion (%) of the total tumor surface of the tissue section analyzed. F, immunohistochemistry. Proliferation of the viable tumor component and the average microvessel density were determined by Ki67 (E) and CD31 (F) staining, respectively. Results are expressed as an average \( \pm \) SEM.
2 hours ($P < 0.01$), compared with chemotherapy alone (Fig. 3D). Furthermore, a high apoptotic index was apparent in both groups treated with DT01 and chemotherapy (Supplementary Fig. S2). Similar to other histologic parameters, the extent of apoptosis was elevated in animals treated with a 4-hour delay ($P < 0.0001$). These histologic findings were not apparent in the DT01 or chemotherapy alone–treated groups.

For many solid tumors, proliferation and microvascularization are indispensable prerequisites for tumor development and metastasis. To further investigate these parameters, immunostaining for Ki67 and CD31, markers of cell proliferation and angiogenesis respectively, were performed in the viable tumor component (Fig. 3E and F). Ki67 immunoreactivity indicated that tumors treated with either DT01 or chemotherapy alone were densely packed with a high degree of proliferation. Treatment with a 2-hour interval between DT01 and chemotherapy resulted in a moderate decrease in proliferating cells ($P = 0.02$; Fig. 3E). Strikingly, immunoreactivity of Ki67 was 10-fold reduced in the group treated with a 4-hour interval between DT01 and chemotherapy ($P < 0.001$; Fig. 3E). In this group, immunoreactivity was detected only in the tumor rim due to the high degree of necrosis observed in the center core region of the tumor. In addition, diminished intratumoral vessel densities were detected in groups treated with a combination of DT01 and chemotherapy, compared with chemotherapy alone (Fig. 3F). However, the mean microvessel density was even more notably reduced in the group treated with a 4-hour interval ($P < 0.001$) compared to 2 hours ($P = 0.02$). Despite similarities in the antitumor effect on tumor growth at both the 2 and 4-hour treatment schedules, histologically the efficacy was significantly more pronounced at the 4-hour timepoint, in terms of necrosis, apoptosis, proliferation, and angiogenesis.

Unexpectedly, tumors treated with a delay of 4 hours between DT01 and chemotherapy and sampled 22 days after treatment presented with a proportion of lysed hepatocytes within the tumor and slight edema in the adjacent nonmalignant liver (Fig. 4A), in the absence of further clinical signs of toxicity such as loss of weight (Supplementary Fig. S3). Histologic analyses did not reveal morphologic signs of toxicity in the other groups (Fig. 3). In addition, liver enzyme tests did not reveal significant differences between the control and the combination treated groups (Supplementary Fig. S4).

Interestingly, no further edema was observed when animals receiving the same treatment were sacrificed between 30 and 65 days (Fig. 4A). This suggests that the edema observed at day 22 is reversible over time. Despite the significant tumor efficacy observed 22 days post treatment, tumors monitored after this time point resumed progression (Fig. 4B). Histologic
analysis revealed that the proliferative component reached approximately 50% at 30 to 45 days after treatment, only slightly below the level observed in nontreated tumors (Fig. 4C).

To confirm that combination treatment did not induce additional toxicity to the liver, we analyzed the tolerability of DT01 in association with OXA or 5-FU for extended treatment cycles. We determined the toxicity of escalating doses of DT01 (total doses of 30, 50, or 80 mg) following systemic administration for two cycles (5 × DT01 administrations per treatment cycle) associated to OXA or 5-FU in a cohort of 50 mice. No loss of weight was observed in animals during or after treatment (Supplementary Fig. S5A). Similarly, other clinical signs of toxicity such as diarrhea or behavioral changes were not noted in these mice. At autopsy 6 weeks after the second cycle of treatment, all abdominal organs, the thoracic cavity, and contents appeared normal. No major variations in liver weights or histology were observed between the vehicle and combination treated groups (Supplementary Fig. S5B).

These results suggest that the reversible edema detected after combined treatment (Fig. 4) in animals bearing hepatic tumor is likely an acute reaction to the tumor response to efficient combination treatment.

Peritoneal metastasis treatment

Colorectal cancer often metastasizes to the liver and the peritoneum. Interestingly, 90% of the mice intrahepatically xenografted with colorectal cancers developed peritoneal metastasis. This property allowed us to monitor the effect of DT01 not only on liver tumors but also on peritoneal metastasis. Animals receiving a combination of DT01 and chemotherapy displayed significantly decreased peritoneal tumor volumes when compared with chemotherapy alone at both the 2 (mean volume: 300.31 vs. 867.20 mm², respectively, P < 0.01) and 4-hour time intervals (mean volume: 259.51 vs. 867.20 mm², respectively, P < 0.01; Fig. 5A and B). Although a slight decrease in tumor volume was observed in the group treated with DT01 alone, this did not reach statistical significance.

Discussion

Approximately 50% of patients with colorectal cancer will present either with liver and/or peritoneal metastases or develop them throughout the course of their disease (18). A majority of patients with colorectal cancer hepatic metastases present with nonresectable disease and systemic chemotherapy represents the main if not the only form of therapy. However, the therapeutic window of chemotherapy is limited due to tumor resistance and high toxicity to nontargeted tissue. In such clinical situations, an aggressive chemotherapy regimen alone may not only fail to improve survival, but may also adversely affect the quality of life. Consequently, the mortality of these patients remains high. Therefore development of new agents specifically targeting DNA repair to circumvent chemoresistance and sparing healthy tissues is imperative in the treatment of these cancers. DT01 is an attractive drug candidate based on its central role in DNA repair.

In the current study, we show for the first time that systemic DT01 treatment sensitizes colorectal cancer cells to conventional chemotherapies by in vitro and in vivo assays. In a colorectal cancer metastatic model, we demonstrate significant antitumor efficacy in the liver and the peritoneum (regarded as a terminal condition) with DT01 treatment in combination with OXA and 5-FU. It is of interest to note, that the significant antitumor effect was limited to DT01 association with both OXA and 5-FU and not with single-agent chemotherapy (Supplementary Fig. S1). This demonstrates that in agreement with the clinical conventional setting, combination with DT01 must be associated to double chemotherapy rather than single-agent chemotherapy in the treatment of colorectal cancer metastases. This study further highlights that tumors receiving double chemotherapy combined with DT01 restart proliferation and regrowth at later time points (post 22 days). Therefore repeated cycles of treatment would be necessary to achieve long-term disease control similar to current conventional chemotherapy protocols. This would be possible as no added toxicity was observed with DT01 alone or in combination with OXA or 5-FU.

DT01 preferentially accumulate in the liver and intestines after systemic injection. Although the entire liver appeared to be uniformly fluorescent after Cy5-DT01 injection, the activation of DNA-PK revealed by the phosphorylation of H2AX was observed exclusively in tumor cells and not in the healthy tissue surrounding the tumor. This observation indicates that either DT01 does not enter nontumor cells and/or that DT01 is not active in healthy liver tissue. DT01 was specifically designed by cholesterol conjugation, first, to increase the bioavailability and second, to play on the difference in the substrate uptake between cancer and normal cells. Low-density lipoproteins (LDL) are a major component of...
the cholesterol pathway (19). High requirement for LDL by malignant cells and thus the consequent overexpression of LDL receptors has been shown in many types of cancer cells making tumor cells specific targets of DT01 (20, 21). In addition, an extensive analysis of normal and cancerous human tissues by immunohistochemistry revealed that either DNA-PKcs or Ku80 were consistently absent in the liver and the mammary epithelium, a specific post-transcriptional regulation that was not found in the other tissues and most of the tumors (22). Taken together, these data highlight that DT01 is likely to be an efficient drug for the treatment of liver cancers (Supplementary Table S2).

In conclusion, there is an urgent need for new treatment options targeting secondary hepatic malignancies, a rapidly progressive disease with a poor prognosis and an alarming rate of mortality. Our study strongly suggests that combining systemic administration of DT01 with conventional chemotherapy may prove to be a safe and effective therapeutic strategy in the treatment of colorectal cancer metastasis of the liver and the peritoneum.

**Disclosure of Potential Conflicts of Interest**

J.-S. Sun has ownership interest in DNA Therapeutics. M. Dutreix has ownership interest in a DT01 patent and is a consultant/advisory board member for DNA Therapeutics. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: N. I. Herath, M. Dutreux

Development of methodology: N. I. Herath, F. Devun, M.-C. Lienafa

References


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.). N. I. Herath, F. Devun, A. Herbette, A. Denys

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis). N. I. Herath, F. Devun, A. Denys, M. Dutreux

Writing, review, and/or revision of the manuscript: N. I. Herath, F. Devun, A. Denys, J.-S. Sun, M. Dutreux

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. I. Herath, M.-C. Lienafa, M. Dutreux

Study supervision: N. I. Herath, J.-S. Sun, M. Dutreux

**Acknowledgments**

The authors thank Sophie Dodier (Histology platform of Institut Curie) for histologic services, Professor Michel Huere for analyses of tumor sections, and André Nicolas for scanning of histologic sections (Pathology service, Hôpital Curie). This study was also supported by the technical staff of the Institut Curie animal facility.

**Grant Support**

This study was supported by the interdisciplinary translational program of the Institut Curie, the National de la Recherche, the Institut National de la Santé et de la Recherche Medicale, Institut National du Cancer (TRANS13-081) and the conseil général de l’Essonne (A.S.T.R.E). This study was also funded by the Institut Curie and DNA Therapeutics.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 18, 2015; revised September 28, 2015; accepted October 14, 2015; published OnlineFirst December 4, 2015.
The DNA Repair Inhibitor DT01 as a Novel Therapeutic Strategy for Chemosensitization of Colorectal Liver Metastasis

Nirmitha I. Herath, Flavien Devun, Marie-Christine Lienafa, et al.