The DNA repair inhibitor DT01 as a novel therapeutic strategy for chemosensitization of colorectal liver metastasis

Nirmitha I. Herath\textsuperscript{1, 2}, Flavien Devun\textsuperscript{1, 2}, Marie-Christine Lienafa\textsuperscript{1, 2}, Aurélie Herbette\textsuperscript{1, 2}, Alban Denys\textsuperscript{3}, Jian-Sheng Sun\textsuperscript{2}, Marie Dutreix\textsuperscript{1, 4, 5}

\textsuperscript{1}\textit{Recombination, Repair and Cancer Laboratory, Institut Curie, Centre de Recherche, 91405 Orsay, France.}

\textsuperscript{2}\textit{DNA Therapeutics, Genopole, 4 rue Pierre Fontaine, 91058 Evry cedex, France.}

\textsuperscript{3}\textit{Department of Radiology and Interventional Radiology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.}

\textsuperscript{4}\textit{CNRS UMR3347, Centre universitaire, 91405 Orsay, France.}

\textsuperscript{5}\textit{INSERM U120, Centre universitaire, 91405 Orsay, France.}

\textbf{Running title:} DT01 in pre-clinical models of CRC liver metastasis

\textbf{Keywords:} Colorectal metastases, Liver, DT01, conventional chemotherapy, mouse models

\textbf{Financial support:} This study was funded by the Institut Curie and DNA Therapeutics.

\textbf{Corresponding author:} Dr. Nirmitha Herath, Recombination, Repair & Cancer Laboratory, Institut Curie – Section Recherche, 15 rue Georges Clemenceau, Centre universitaire Bât.112 91405 ORSAY Cedex, France. Phone: +33(0)1 69 86 31 43, nirmitha.herath@curie.fr

\textbf{Conflict of interests:}

N.I. Herath, MC Lienafa, A. Herbette and F. Devun are employees of DNA Therapeutics. M. Dutreix and J-S. Sun are cofounders of DNA Therapeutics. The other authors have no conflicts of interests or financial ties to declare.
Title: 117/165 characters

Text: 4342 (including figure legends)

References: 22/50

Figures and Tables: 4/6 Figures

Abstract: 249/250 words

Abbreviations:
CT (chemotherapy) CRC (colorectal cancer), DNA-PK (DNA dependent protein kinase), DOX (doxorubicin), 5′-FU (5′-fluorouracil), FOLFOX (folinic acid, 5-fluorouracil and oxaliplatin), IP (intraperitoneal), LDL (low density lipoproteins), OXA (oxaliplatin).
ABSTRACT

Metastatic liver disease from colorectal cancer (CRC) is a significant clinical problem. This is mainly attributed to non-resectable 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 CRC 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 activate damage signaling, consequently inhibiting single- and double-stranded DNA repair enzyme recruitment. In vitro, Dbait treatment increases sensitivity of HT29 and HCT116 CRC 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 CRC liver metastasis. The high uptake of DT01 indicates that the liver is a specific target. We demonstrate significant anti-tumor 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 histological 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.
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 ablative and transarterial therapies demonstrate a limited effect with low survival benefit (3, 4).

Chemotherapy is widely used in the treatment of metastatic colorectal carcinoma (CRC). However, patients respond poorly to chemotherapy regimens combining folinic acid, 5-fluorouracil and either oxaliplatin (FOLFOX) or irinotecan mainly due to multi-drug resistance, preventing the eradication of metastatic disease (5, 6). Therefore, improvements in overcoming CRC chemo-resistance is 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.

Chemo-resistance presents a major obstacle to the efficacy of cancer treatment. DNA repair plays a key role in chemo-resistance 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) (9) and Poly-ADP-Ribo-Polymerase (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 \textit{in vitro} and \textit{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 however to low systemic exposure (14). In order to increase the efficiency of cellular uptake, the Dbait molecule was modified by covalently linking a cholesterol moiety to the 5'-end (DT01) (11). We demonstrated that local administration of DT01 by intra-tumoral 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 \textit{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 CRC metastatic liver tumor model.
MATERIALS AND METHODS

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

CRC 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% fetal bovine serum, 1% sodium pyruvate, 100mg/ml streptomycin and 100mg/ml penicillin (Invitrogen, Carlsbad, CA), 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 200µg/ml hygromycin B. All cell lines were additionally subjected to mycoplasma testing in-house and were free of mycoplasma contamination (Biovalley, France).

Cells were transfected with 2.5µgs of Dbait (5’-GCTGTGCCCAACCCCAGCAAAAAGCTAGA-(H)TCTAGGCTTGTTGCTGGTGTGGGCACAGC-3’) (Eurogentec, Belgium) where H is a hexaethyleneglycol linker and underlined nucleotides are phosphorothioates. The cells were sham transfected with an 8bp 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-phospho-Histone H2A.X (Ser139) Antibody, clone JBW301 (1:500 dilution; 05-636, Millipore, USA) (9).

In vitro proliferation assay
Cells were seeded at a density of $3 \times 10^4$ cells/60mm dishes and transfected with Dbait. Following treatment, cells were washed and left untreated or treated with a combination of 5μM of oxaliplatin (OXA, Sigma) and 2.5μM of 5-fluouracil (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 5μM of OXA and 2.5μM of 5-FU for 1 hr. 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 NMRI\textsuperscript{NU/NU} mice (Janvier, France) weighing 20-22g were housed in specific pathogen free environment on a 12h light and 12h 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 (1x10^6/10μL 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, Boulder, CO) (11).

Pharmacokinetics of DT01

HT29L grafted mice were treated with a single intraperitoneal (IP, n=4) or intravenous (IV, n=3) injection of 5 mg of DT01. Blood samples were harvested prior to treatment and 1, 5, 10, 30mins, 1hr, 2hrs, 4hrs and 6hrs post 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). NMR1/NU mice were injected with 1mg of the DT01 fluorescent molecule (DT01-Cy5) through IP (n=3) or IV (n=3) administration. The fluorescent DT01 (DT01-Cy5) incorporates a cyanine 5 at the thymidine located immediately after the linker. Six hours after injection, fluorescence imaging was performed using a Typhoon scanner (GE Healthcare).

DT01 and chemotherapy treatment

HT29L grafted animals (n=49) were allocated into treatment groups and administered one cycle of treatment (Table S1). DT01 was systemically administered through IP injection at a dose of 5mgs/day for 5 consecutive days starting on day 0 (D0). OXA (6mg/kg, 1x per cycle, Day 1) and 5-FU (25mg/kg, 3x per cycle, Days 1-3) were administered 2 or 4 hours after DT01 treatment. These mice were sacrificed 22 days post 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 post treatment. Plasma alanine transaminase (ALT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), amylase (AMYL) and total bilirubin (TBIL) were measured using an MS-Scan II (Melet Schloesing Laboratories, France).

Toxicity Assays

NMRI\textsuperscript{NU/NU} mice (n=50) were treated with two cycles of DT01 at escalating doses of 3mg/day (30mg total), 5mg/day (50mg total) or 8mg/day (80mg total) through IP injection in combination with OXA or 5\textsuperscript{'-}FU. OXA and 5\textsuperscript{'-}FU were administered through systemic IP injection at doses of 1x6mg/kg or 3x25mg/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) in a blinded fashion. Viable and necrotic components (indicated by increased cell size, indistinct cell border, eosinophilic cytoplasm, loss or condensation of the nucleus, or associated inflammation) were expressed as a proportion (%) of the total tumor surface. Apoptosis was estimated (weak-<5%, moderate 5-10%, significant 10-20% and very significant 20-50%) from representative non-necrotic fields at high power.

Digitization and image capture was performed using a whole-slide scanning system (Philips digital pathology solutions).

\textbf{Ki67 and CD31 Immunohistochemistry}

Immunohistochemistry was performed using rabbit anti-Ki67 (ab28364, 1/500; Abcam, UK) and rabbit anti-CD31 (ab15580, 1/500; Abcam, UK) antibodies. This was
followed by a secondary biotinylated goat anti-rabbit IgG antibody (BA-1000; Vector, USA) 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 +ve and -ve cells, in five randomly selected microscopic fields per section. Average micro-vessel density was determined by CD31 staining. CD31 positive 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 histological data. Error bars indicate standard error of the mean (SEM), except when specifically indicated. All statistical analyses were performed using StatEL software (adScience, France) 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 CRC cell lines (HCT116 and HT29) by monitoring the pan-nuclear phosphorylation of H2AX (Figure 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 oxaliplatin (OXA) and 5-fluorouracil (5-FU) or a combination of Dbait with chemotherapy (CT) (Figure 1B). As already observed in fibroblasts (9) Dbait alone appears to have no effect on cell proliferation in both cell lines (Figure 1B). Treatment with OXA and 5-FU resulted in a decrease in cell proliferation. However, 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 to chemotherapy alone (p<0.001 and p<0.02, respectively). These differences become apparent particularly at later time points (>5 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 (Figure 1C) revealing their
dependency in repair activity for survival whereas no significant effect was noted in HT29. Since the sensitivity of HCT116 to Dbait was not detected during the first 8 days of proliferation (Figure 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 CT with Dbait.

**Pharmacokinetic and biodistribution analyses of intraperitoneal vs intravenous administration of DT01**

To avoid transfectant adjuvant toxicity, all in vivo 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 intraperitoneal (IP) or an intravenous (IV) dose of 5 mg of DT01. IP administration resulted in a C_{max} of 578μg/ml, a T_{max} of 1 hour and an AUC_{0-6} of 799 whereas IV led to a C_{max} of 1,917μg/ml, a T_{max} of 0.08 hours and an AUC_{0-6} of 799 (Figure 2A). Pharmacokinetic analyses revealed that following IP injection, the plasmatic exposure of DT01 was longer than that of IV bolus injection with an AUC corresponding to approximately 70 % of the AUC with IV administration (Figure 2A).

We used a fluorescent labelled cy5-DT01 molecule to monitor the biodistribution in excised whole-organs (Figure 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 IP 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 (Figure 2A), significant amounts of DT01 were still detectable in the liver indicating a specific retention in this organ (Figure 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 γ-H2AX was specifically observed in the tumor and not in the surrounding healthy tissues (Figure 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 frontline treatment for metastatic CRC, we used a HT29 xenografted liver tumor model, since previous reports and our in vitro data demonstrate this line to be highly chemo-resistant 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 (Figure 3A). The two schedules consisted of either two or four-hour intervals between the two treatments, since the maximum DT01 levels in the liver were observed at one and three hours after treatment (Figure 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 CT alone and DT01 had only a moderate effect when administered alone (Figures 3B, 3C). Interestingly, the
association of DT01 to OXA and 5-FU significantly decreased the liver tumor size in both combination treated groups compared to CT alone when administered at two (mean volume: 525.80 vs 872.01 mm$^2$, p=0.03) and four hours (mean volume: 501.05 vs 872.01 mm$^2$, p=0.02) before CT (Figures 3B, 3C). This effect was not observed when DT01 was associated with a single chemotherapy agent, either OXA or 5-FU (Figure S1). Detailed blinded histological analyses including measures of the viable tumor area, necrosis and apoptosis were assessed in haematoxylin-eosin-saffron (HES) stained sections, by an experienced pathologist. Both groups with DT01 and CT combined treatment showed higher treatment efficacy than the groups receiving single treatment, with a marked increase in necrosis in the group treated with a four-hour interval between CT and DT01 (p<0.0001) than two hours (p<0.01), compared to CT alone (Figure 3D). Furthermore, a high apoptotic index was apparent in both groups treated with DT01 and CT (Figure S2). Similar to other histological parameters, the extent of apoptosis was elevated in animals treated with a four-hour delay (p<0.0001). These histological 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 (Figures 3E, 3F). Ki67 immunoreactivity indicated that tumors treated with either DT01 or chemotherapy alone were densely packed with a high degree of proliferation. Treatment with a two-hour interval between DT01 and CT resulted in a moderate decrease in proliferating cells (p=0.02) (Figure 3E). Strikingly, immunoreactivity of Ki67 was 10-fold reduced in the group treated with a four-hour interval between DT01 and CT (p<0.001) (Figure 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 CT, compared to
CT alone (Figure 3F). However, the mean microvessel density was even more notably
reduced in the group treated with a four-hour interval (p<0.001) compared to two hours
(p=0.02). Despite similarities in the anti-tumor effect on tumor growth at both the two and
four-hour treatment schedules, histologically the efficacy was significantly more pronounced
at the four-hour time point, in terms of necrosis, apoptosis, proliferation and angiogenesis.

Unexpectedly, tumors treated with a delay of four hours between DT01 and CT and
sampled 22 days post treatment presented with a proportion of lysed hepatocytes within the
tumor and slight edema in the adjacent non-malignant liver (Figure 4A), in the absence of
further clinical signs of toxicity such as loss of weight (Figure S3). Histological analyses did
not reveal morphological signs of toxicity in the other groups (Figure 3). In addition, liver
enzyme tests did not reveal significant differences between the control and the combination
treated groups (Figure S4).

Interestingly, no further edema was observed when animals receiving the same
treatment were sacrificed between 30-65 days (Figure 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 (Figure 4B).
Histological analysis revealed that the proliferative component reached ~50% at 30-45 days
post treatment, only slightly below the level observed in non-treated tumors (Figure 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
80mg) following systemic administration for two cycles (5xDT01 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 post treatment (Figure S5.A). Similarly, other clinical signs of
toxicity such as diarrhea or behavioral changes were not noted in these mice. At autopsy 6 weeks post 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 (Figure S5.B).

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

**Peritoneal metastasis treatment**

CRC often metastasizes to the liver and the peritoneum. Interestingly 90% of the mice intrahepatically xenografted with CRC tumors 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 to chemotherapy alone at both the two (mean volume: 300.31 vs 867.20 mm$^2$, respectively, p<0.01) and four hour time intervals (mean volume: 259.51 vs 867.20 mm$^2$, respectively, p<0.01) (Figure 5A, 5B). 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 CRC will present either with liver and/or peritoneal metastases or develop them throughout the course of their disease (18). A majority of patients with CRC hepatic metastases present with non-resectable 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 non-targeted 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 present study, we show for the first time that systemic DT01 treatment sensitizes CRC cells to conventional chemotherapies by in vitro and in vivo assays. In a CRC metastatic model, we demonstrate significant anti-tumor 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 anti-tumor effect was limited to DT01 association with both OXA and 5-FU and not with single agent chemotherapy (Figure 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 CRC metastases. This study further highlights that tumors receiving double
chemotherapy combined with DT01 restart proliferation and re-growth 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 non-tumor cells and/or that DT01 is not active in healthy liver tissue. DT01 was specifically designed by cholesterol conjugation firstly, in order to increase the bioavailability and secondly, 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). Additionally, 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 (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 CRC metastasis of the liver and the peritoneum.

Acknowledgments

The authors would like to thank Sophie Dodier (Histology platform of Institut Curie) for histological services, Prof. Michel Huerre for analyses of tumor sections and Mr. André Nicolas for scanning of histological sections (Pathology service, l’hôpital Curie). This study was also supported by the technical staff of the Institut Curie animal facility. This study benefited from the support of the interdisciplinary translational program of the Institut Curie, the National de la Recherche, the Institut National de la Sante et de la Recherche Medicale, Institut National du Cancer (TRANSLA13-081) and the conseil général de l’Essonne (A.S.T.R.E)
REFERENCES


FIGURE LEGENDS

Figure 1: Dbait treatment increases sensitivity of CRC and HCC cancer cell lines to chemotherapy

CRC (HCT116, HT29) cell lines were transfected with a sham control or Dbait. (A) γHA2X Immunostaining. Cells were transfected with Dbait and immunostaining was performed with a γHA2X antibody (pink) and DAPI. (B) Cell proliferation. Cells were seeded at a density of $3 \times 10^4$ cells/60mm dish left untreated or treated with Dbait alone (grey) or in combination with CT (5µM OXA + 2.5µM 5-FU) (black) for 1 hour. Live cell counts were performed on days 1, 3, 5, 6, 7 and 9 using trypan blue post Dbait transfection. (C) Clonogenic survival of HT29. Following transfection, cell lines were either left untreated or treated with Dbait alone (grey) or treated in combination with CT (black). After 14 days, the clones were stained with crystal violet and counted manually. Results are expressed as an average % of clones. * p<0.01: The level of proliferation was significantly reduced by day 9 in cells transfected with Dbait prior to CT treatment in both HCT116 and HT29 cell lines compared to CT alone.

Figure 2: Pharmacokinetics and bio-distribution of DT01

(A) DT01 pharmacokinetics. Intrahepatic tumor bearing NMRI$^{NU/NU}$ mice were treated with a dose of 5 mg of DT01 through a single IP (black) or IV (grey) bolus administration. DT01 concentration in plasma was measured at 1, 5, 10, 30 mins and 1, 2, 4 and 6 hours post treatment using an ELISA assay. (B) DT01 biodistribution with fluorescent DT01-cy5. Mice were injected with 1mg of DT01-cy5 through IP or IV administration. Six hours post 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 1mg of DT01 and the tumors were harvested 6 hours after treatment and subjected to immunolabeling with a γH2AX antibody (green) and DAPI (in blue).

**Figure 3: DT01 significantly increases sensitivity to CT in a CRC (HT29) liver metastatic model**

Intrahepatic tumor bearing NMRI<sup>NU/NU</sup> mice were treated as described in the Material and Methods, and sacrificed 22 days post treatment. Livers were sampled for macroscopic and microscopic examination. (A) Sequence of DT01 and CT therapy. CT was administered 2 or 4 hours post DT01 treatment. (B) Mean liver tumor volume (mm$^3$) in each treatment group. (C) Representative macroscopic and HES sections of liver tumors in each group. (D) Tumor necrotic component assessed through HES staining. Necrosis is expressed as a proportion (%) of the total tumor surface of the tissue section analyzed. (E-F) Immunohistochemistry. Proliferation of the viable tumor component and the average micro-vessel density were determined by Ki67 (D) and CD31 (E) staining, respectively. Results are expressed as an average ± SEM.

**Figure 4: Tumor properties of mice receiving DT01 and chemotherapy at early, intermediate and later time points**

(A) Representative HES sections of tumor (T) and adjacent non-malignant (N) tissues harvested early (E) on day 22, intermediate (I) between days 30-45 and late (L) between days 45-65, receiving combination treatment. (B) Comparison of mean liver tumor volumes (mm$^3$) in the vehicle treated (V) and post treatment with DT01 + CT with a 4-hour interval when sacrificed at E, I or L time points. (C) Restart of proliferation and re-growth of tumor when harvested at I and L time points compared to E time point.
Figure 5: Association of DT01 with CT significantly decreases the peritoneal tumor volume

Intrahepatic tumor bearing NMRI\textsuperscript{NU/NU} mice were treated as described in the Material and Methods, and sacrificed 22 days post treatment. The presence of peritoneal metastasis was visually monitored during the duration of treatment and measured at the time of sacrifice. (A) Mean peritoneal tumor volume (mm\textsuperscript{3}) in each treatment group. (B) Representative macroscopic images of peritoneal tumors in each group. Results are expressed as an average ± SEM.
FIGURE 1

A

HCT116

HT29

Sham

Dbait

DAPI γH2AX Merge DAPI γH2AX Merge

B

HCT116

HT29

Days post transfection

Live cell number (millions)

8H Dbait CT Dbait + CT

C

HCT116

HT29

Clonogenic survival (%)

8H Dbait

p<0.01 p<0.01

NS

p<0.05

Copyright © 2015 American Association for Cancer Research. mct.aacrjournals.org Downloaded from on April 19, 2017.
FIGURE 2

A

DT01 Plasma Conc. (μg/ml)

Time (hrs)

B

L       Li      S       I       K       Bl       H       B       P       M

NT

IP

IV

C

Average fluorescence (AU)

NT 1h 3h 6h

D

Glucose

DT01

Tumor

Normal liver

Tumor

Normal liver

Downloaded from mct.aacrjournals.org on April 19, 2017. © 2015 American Association for Cancer Research.
FIGURE 3

A

OXA + 5-FU

DT01

Day 22

Sacrifice

Treatment Day 0 – Day 4

B

Mean Liver Tumor Volume (mm³)

C

Vehicle

DT01

CT

DT01+CT

DT01+CT

2h

4h

D

Necrosis (%)

p<0.01

p<0.0001

E

Mean Ki67 index (%)

P = 0.02

P < 0.001

F

Mean Microvessel Density (5 fields)

P = 0.02

P < 0.001

DT01

CT

2h

4h
FIGURE 4

A  

Vehicle only  

DT01+CT (E)  

DT01+CT (I)  

DT01+CT (L)  

B  

Mean tumor volume (mm³)  

V  (22)  E  (22)  I  (30-45)  L  (45-65)  

C  

Tumor surface components (%)  

V  (22)  E  (22)  I  (30-45)  L  (45-65)
Mean Peritoneal Tumour Volume (mm³)

A

B

Veh. DT01 CT DT01 +CT 2h DT01 +CT 4h
Molecular Cancer Therapeutics

The DNA repair inhibitor DT01 as a novel therapeutic strategy for chemo-sensitization of colorectal liver metastasis

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

Mol Cancer Ther Published OnlineFirst December 4, 2015.