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Pharmacokinetics and Toxicity in Rats and Monkeys of coDbait: A Therapeutic Double-stranded DNA Oligonucleotide Conjugated to Cholesterol

Anne Schlegel[1], Cyril Buhler[1], Flavien Devun[1,2], Céline Agrario[1], Saïk Urien[3], François Lokiec[4], Jian-Sheng Sun[1,5] and Marie Dutreix[2]

Increased DNA repair activity in cancer cells is one of their primary mechanisms of resistance to current radio- and chemotherapies. The molecule coDbait is the first candidate in a new class of drugs that target the double-strand DNA break repair pathways with the aim of overcoming these resistances. coDbait is a 32-base pair (bp) double-stranded DNA molecule with a cholesterol moiety covalently attached to its 5′-end to facilitate its cellular uptake. We report here the preclinical pharmacokinetic and toxicology studies of subcutaneous coDbait administration in rodents and monkeys. Maximum plasma concentration occurred between 2 to 4 hours in rats and at 4 hours in monkeys. Increase in mean AUC0–24h was linear with dose reaching 0.5 mg·h/ml for the highest dose injected (32 mg) for both rats and monkeys. No sex-related differences in maximum concentration (Cmax) nor AUC0–24h were observed. We extrapolated these pharmacokinetic results to humans as the subcutaneous route has been selected for evaluation in clinical trials. Tri-weekly administration of coDbait (from 8 to 32 mg per dose) for 4 weeks was overall well tolerated in rats and monkeys as no morbidity/mortality nor changes in clinical chemistry and histopathology parameters considered to be adverse effects have been observed.

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INTRODUCTION

Nucleic acid-based molecular therapies used alone or in association with currently available chemotherapies and/or radiotherapies are promising cancer treatments. This family of therapeutic molecules encompasses various structures and compositions, such as synthetic small interfering RNA (siRNA), plasmid DNA-based vectors, aptamers, ribozymes, and antisense oligonucleotides, all of which target specific genes, mRNAs or proteins. The latest member of this family is signal interfering DNA (siDNA), which consists of short, stabilized DNA molecules that target and interfere with DNA repair pathways (see ref. 1, for a recent review).

Despite their promising bioactivity, several challenges must be resolved before nucleic acid molecules can be fully developed into clinically effective therapies. First, with the exception of aptamers targeting cell surface proteins or receptors, nucleic acid molecules must cross cell membranes to be effective. Attempts have been made to overcome this barrier by formulating the molecules with nanoparticles or conjugating them to lipophilic moieties or receptor-mediated endocytosis ligands. Second, nucleic acids are subjected to nucleophilic degradation in sera or human fluids resulting in short-lived molecules. Strategies using phosphorothioate (PS) substitutions and other modifications have been shown to increase oligonucleotide stability.[2] However, such substitutions may result in increased toxicity and decreased activity, which limit their therapeutic potential.

The aim of siDNA technology is to manipulate the DNA repair pathway in cancer cells by mimicking DNA damage, which then leads to pathway activation and disorganization. The first siDNA molecule under clinical development, coDbait, is a double-strand break (DSB)-mimicking molecule designed to target the DSB sensing, signaling and repair pathways. siDNA represents the beginning of a new era of supramolecular therapy as it targets an entire pathway: the nonhomologous end-joining DNA repair pathway. We recently demonstrated that a prototype siDNA (called Dbait) disorganizes the DNA repair pathway in cells.[3,4] Dbait association with DNA-damaging agent, such as radiotherapy, increases both tumor growth control and survival in various xenografted tumor models in mice, including human melanoma[3] and colon cancer carcinoma[5] providing initial proof of efficacy in animals.

Dbait molecules are 32-base pair (bp) long double-stranded hairpin DNA molecules protected from exonucleases and helicases by tethering the two strands with a linker on one extremity and by using PS substitutions at the 3′- and 5′-terminal nucleotide residues on the other end (**Figure 1**). As naked DNA does not efficiently enter cells, we covalently linked a cholesterol group to the 5′-end of Dbait in order to facilitate its delivery into cells.[6] This molecule is called coDbait (clinical name DT01). Cholesterol-conjugated siRNAs or antisense oligonucleotides have demonstrated markedly improved pharmacological properties *in vitro* and *in vivo*.[7–9] Cholesterol-conjugation has the added benefit of functioning

[1]DNA Therapeutics, Evry, France; [2]Institut Curie, Centre de Recherche, Centre National de Recherche Scientifique (CNRS) UMR3347, Institut National de la Santé et de Recherche Médicale (INSERM) U1021, Université Paris-Sud 11, Centre Universitaire, Orsay, France; [3]Université Paris Descartes, Sorbonne Paris Cité, Paris, France; [4]Institut Curie, Hôpital René Huguenin, Saint-Cloud, France; [5]Muséum National d'Histoire Naturelle, USM503, Paris, France. Correspondence: Cyril Buhler, DNA Therapeutics, Pépinière Genopole Entreprises, 4 rue Pierre Fontaine, F-91058 Evry, France. E-mail: c.buhler@dna-therapeutics.com
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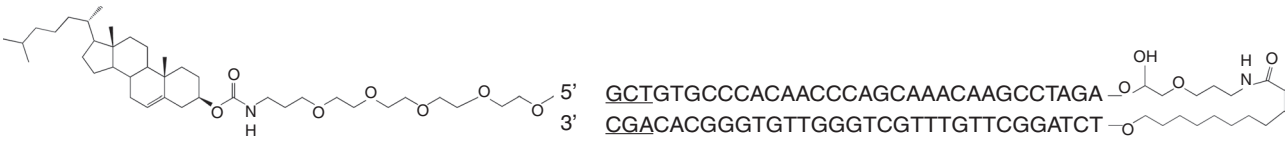


Figure 1 Structure of coDbait. coDbait is a double-stranded oligonucleotide of 32 bp in length. The two strands are connected by a linker. The last three nucleotides on the free ends are substituted with phosphorothioates nucleotides (underlined nucleotides) to increase resistance against nucleases. The hairpin 32-bp double-stranded DNA (dsDNA) is tethered at the 5′-end to a cholesterol through a triethyleneglycol (TEG) linker.

as a carrier for oligonucleotides in the blood through its binding to plasma proteins, thereby improving oligonucleotide pharmacokinetic properties. Furthermore, cholesterol improves the uptake of oligonucleotides into cells, by a mechanism that is not yet clearly understood.[10]

We have previously observed that treatment of xenografted human melanoma with coDbait in association with radiotherapy results in an increase in survival.[6] Comparison of different administration procedures showed that a combination of intratumoral and subcutaneous injections resulted in efficient diffusion of the molecule throughout the tumors.[6] Concomitant administration of chloroquine enhanced cellular uptake and coDbait-induced radiosensitization, most likely by facilitating the release of coDbait from endosomes into the cytosol,[11] whereas chloroquine or coDbait treatment alone did not result in radiosensitization.[6] All these observations served as the basis for the design of a clinical protocol to evaluate coDbait radiosensitization activity on melanoma by subcutaneous administration.

The objectives of this study were to determine the pharmacokinetic properties and the toxicity of coDbait in rats and monkeys. The designed treatment protocol consisted of subcutaneous injections of coDbait, three times per week for 2 weeks in association with an oral chloroquine treatment. We first validated the efficacy of this protocol by administrating 4 mg per dose of coDbait in association with chloroquine and radiotherapy in a xenografted human melanoma model in mice. Pharmacokinetics was studied in rats and monkeys using increasing doses from 8 to 32 mg. As the clinical intent is to administer coDbait locally, the dose regimens were not adjusted to the bodyweights (BWs) of the animals but kept constant locally. coDbait toxicity studies were focused on identifying potential adverse effects on the central nervous system and on cardiovascular function, as previous toxicity studies have reported that these are the main sites of adverse events associated with oligonucleotide-based treatments.[12]

RESULTS

Antitumoral activity of coDbait in association with radiotherapy in xenografted tumors

A preliminary study of coDbait administration in association with radiotherapy has shown a statistically significant increase in survival.[6] We have extended this initial observation by increasing the dose of coDbait using a protocol of administration relevant for an association with a radiotherapy treatment of melanoma (**Figure 2**). This protocol consisted of one subcutaneous (2 mg) and one intratumoral (2 mg) injection of coDbait, three times per week for 2 weeks in association with a daily oral chloroquine treatment. Chloroquine was daily administrated a week before and during the 2-week

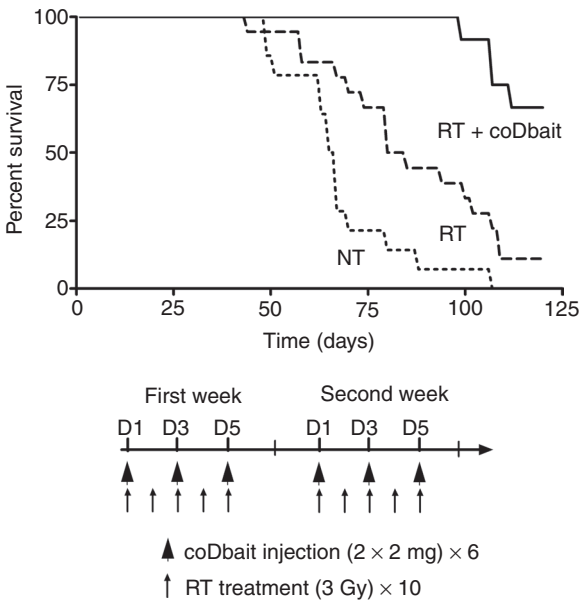


Figure 2 coDbait treatment increases survival time in radiation-treated tumor-bearing mice. Kaplan–Meier survival representation of mice with flank-xenografted SK28 melanoma tumors treated with six subcutaneous injections of coDbait (two injections of 2 mg each) and ten irradiation doses of 3 Gy (RT + coDbait, n = 12), or irradiation alone (RT, n = 18) or no treatment (NT, n = 14). All animals received a concomitant chloroquine treatment (1 mg/animal, per os). For combined radiotherapy and coDbait treatment, irradiation was administered 5 hours after coDbait injection.

treatment to reach a steady state level in the animals. In order to assess the radiosensitizing activity of coDbait but not a chloroquine-associated radiosensitization, all mice were treated with a same chloroquine regimen. Radiotherapy was administered 5 hours after coDbait injection and given in doses of 3 Gy delivered five times per week for 2 weeks. As shown in **Figure 2**, this protocol of coDbait administration in the presence of chloroquine provided a significant increase in survival over radiotherapy and chloroquine alone (relative risk = 0.4125; P value <0.011).

Pharmacokinetics of coDbait in rats

Rats were treated with subcutaneous doses of coDbait at 8, 16, and 32 mg per animal, which were combined with simultaneous oral chloroquine treatment. We determined coDbait concentrations in plasma samples using a hybridization-based immunoassay. Pharmacokinetic parameters are presented in **Table 1**. The mean plasma concentrations versus time and the dose-proportionality graphs are presented in **Figure 3**.

Table 1 Pharmacokinetic parameters of coDbait in rats

Dose (mg/animal)	Sex (number)	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (hour)	AUC_{0-24h} (ng·hour /ml)	AUC_{0-inf} (ng·hour/ml)	$t_{1/2}$ (hour)	Cl/F (l/hour)/animal	Vd/F (l/animal)
	Male (6)	40.5	17,526	2	99,635	99,642	1.64	0.0803	0.190
8	Female (6)	53.8	20,575	2	127,322	127,332	1.63	0.0628	0.148
	Ratio M/F		1.13	NA	1.04	1.04	NA	NA	NA
	Male (6)	81.0	27,597	4	229,760	229,782	1.62	0.0696	0.163
16	Female (6)	107	29,975	2	241,022	241,049	1.65	0.0664	0.158
	Ratio M/F		1.22	NA	1.26	1.26	NA	NA	NA
	Male (6)	161	47,970	2	456,093	456,141	1.62	0.0702	0.164
32	Female (6)	216	47,991	4	527,799	528,023	1.97	0.0606	0.172
	Ratio M/F		1.34	NA	1.15	1.15	NA	NA	NA

Abbreviations: AUC, area under the curve; Cl/F, clearance; C_{max}, maximum concentration; NA, non applicable; $t_{1/2}$, elimination half-life; T_{max}, time to reach maximum concentration; Vd/F, volume of distribution.

coDbait plasma concentrations were measured in all treated animals. No significant signal was detected in plasma samples from the control group. For more than 87% of the time points, the coefficients of variation (CV) for the plasma concentrations were <30% for the three animals in the same time point group. This minimal variation indicated that our plasma concentration estimates were sound. Pharmacokinetic parameters were determined from the mean plasma concentrations for each sex and group using a noncompartmental method. The maximum plasma concentrations occurred between 2 and 4 hours after coDbait administration. Neither dose nor sex impacted the half-life of coDbait in plasma, its clearance, or its volume of distribution. Both systemic exposure values (maximum concentration (C_{max}) and AUC_{0-24h}) correlated linearly with dose (R^2 = 0.9987 and 0.997, respectively) and were not significantly different for males and females.

Pharmacokinetics in monkeys

Since coDbait administration was local, the doses were not adjusted to be proportional to body weight when the assays were performed in monkeys. Thus, we used identical dose regimens of 8, 16, and 32 mg/animal combined with simultaneous oral chloroquine treatment to analyze the pharmacokinetic properties of coDbait in monkeys. The mean pharmacokinetic parameters are presented in **Table 2**. The mean plasma concentrations versus time and the dose-proportionality graphs for C_{max} and AUC_{0-24h} are presented in **Figure 4**. coDbait was not detected in plasma from the control group and coDbait was detected in the plasma of all treated animals. The pharmacokinetic parameters of the monkeys were more variable than those of the rats, as 59% of the time points were associated with CV values >30%.

The maximum coDbait plasma concentrations occurred between 4 and 6 hours after injection and coDbait remained detectable up to 24 hours after administration. The mean half-life values were between 2.74 and 7.14 hours. No clear sex-related differences were observed for C_{max} and AUC_{0-24h}, but interindividual variabilities for both of these values were high (CVs up to 75%). The increase in mean AUC_{0-24h} values was linear with dose (R^2 = 0.9993 and 0.997 for males

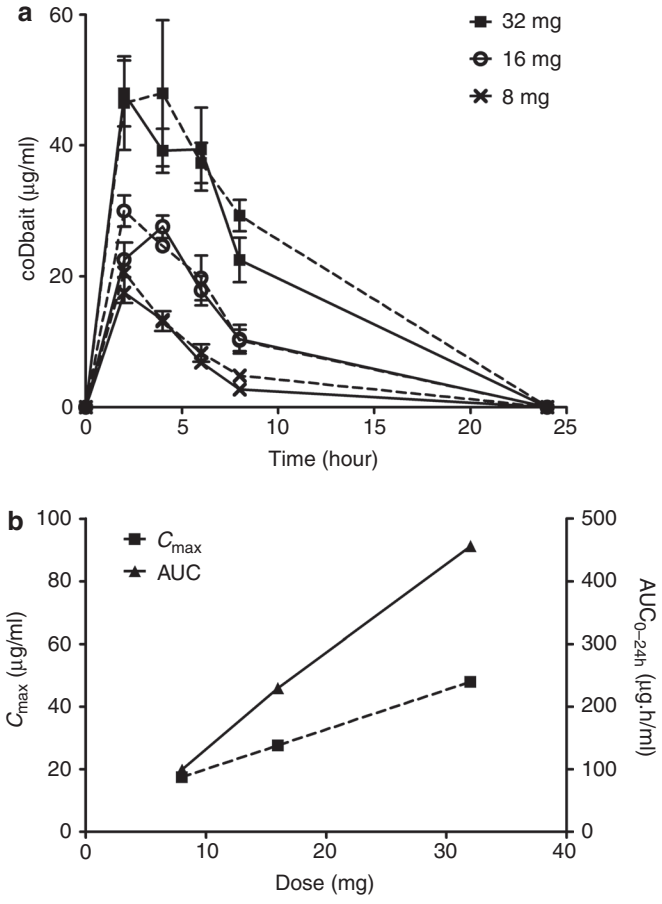


Figure 3 coDbait pharmacokinetics in rats. (**a**) Wistar rats were subcutaneously injected with 8, 16, or 32 mg coDbait at two sites after 1 week of orally administrated chloroquine (9 mg/kg/day) treatment. Blood was withdrawn from the retro-orbital sinus under isoflurane anesthesia in tubes containing K2-EDTA at different time points. coDbait concentration was measured in the plasma using a hybridization-ELISA assay. Mean plasma concentrations per time-point with SD were calculated from three animals. Results from male rats are represented with solid lines and those from females are shown with dashed lines. (**b**) Analyses of the dose-proportionalities of C_{max} and AUC for male rats.

Table 2 Pharmacokinetic parameters of coDbait in monkeys

Dose (mg/animal)	Sex (number)	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (hour)	AUC_{0-24h} (ng·hour /ml)	AUC_{0-inf} (ng·hour /ml)	$T_{1/2}$ (hour)	Cl/F (l/hour)/animal	Vd/F (l/animal)
8	Male (3)	2.41–3.69	9,467	4	106,109	102,059	2.74	0.0813	0.328
	Female (3)	2.81–3.57	9,172	4	106,236	104,990	2.74	0.0769	0.305
	Ratio M/F		1.07	NA	1.04	1.01	NA	NA	NA
16	Male (3)	4.79–6.56	20,017	4	233,328	240,280	4.75	0.0753	0.561
	Female (3)	5.03–7.44	17,703	4	222,311	228,103	4.08	0.0733	0.425
	Ratio M/F		1.24	NA	1.15	1.15	NA	NA	NA
32	Male (3)	10.74–13.73	41,544	4	517,941	611,937	7.14	0.0620	0.506
	Female (3)	12.03–16.16	44,444	4	515,578	578,606	4.19	0.0633	0.363
	Ratio M/F		0.977	NA	1.05	1.11	NA	NA	NA

Abbreviations: AUC, area under the curve; Cl/F, clearance; C_{max}, maximum concentration; NA, non applicable; $t_{1/2}$, elimination half-life; T_{max},Time to reach maximum concentration; Vd/F, volume of distribution

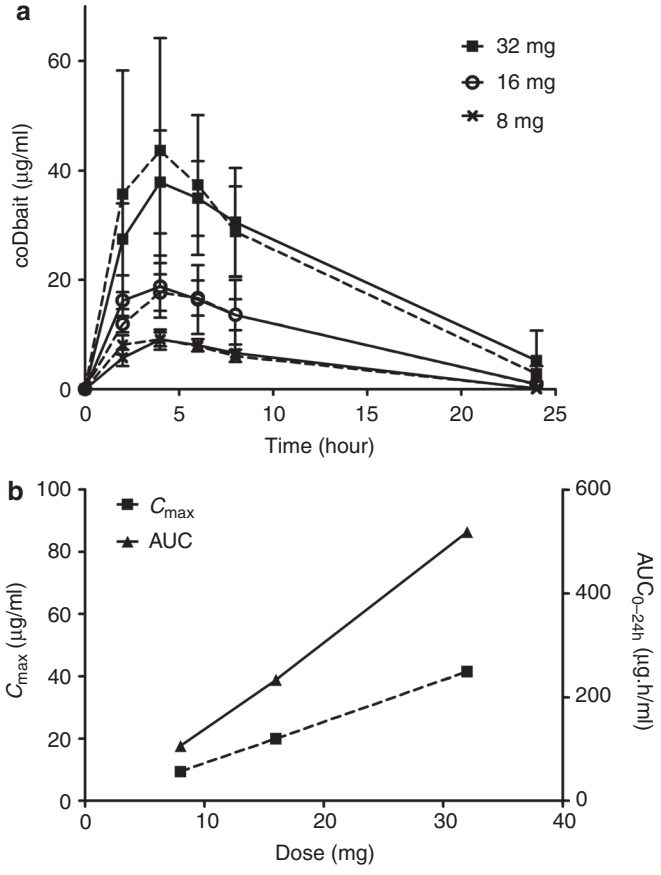


Figure 4 coDbait pharmacokinetics in monkeys. (**a**) Cynomolgus monkeys were subcutaneously injected with 8, 16, or 32 mg coDbait at two sites after 1 week of orally adminstrated chloroquine (5 mg/kg/day) treatment. Blood samples were withdrawn from the femoral vessels of unanesthetized manually restrained animals and collected in tubes containing K2-EDTA at different time points. coDbait concentration was measured in the plasma using the hybridization-ELISA assay. Mean plasma concentrations per time-point with SD were calculated from three animals. Results from male monkeys are represented with solid lines and those from females are shown with dashed lines. (**b**) Analyses of the dose-proportionalities of C_{max} and AUC for male monkeys.

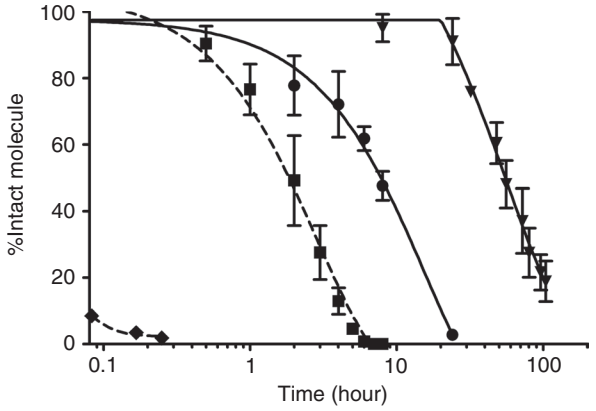
and females, respectively) but more than dose-proportional between 8 and 32 mg/animal/day, for both sexes. Whereas the high/low dose ratios were equal to 4 (32 mg/8 mg), the ratios of the high/low AUC_{0-24h} values for male and female monkeys were 4.88 and 4.85, respectively.

Population pharmacokinetic analysis

The pharmacokinetics of coDbait fit well with a one-compartment model with linear resorption and elimination. The effects of BW on clearance (Cl) and the volume of distribution (Vd) were significant with P values of 10^{-7} and 10^{-10}, respectively (Wald's test). The corresponding power exponent (PWR) estimates for the BW effect were 0.467 and 0.155. Because these estimates were clearly different from the theoretical values of 1 and 0.75, the extrapolation of Vd and Cl values for a 70 kg individual was performed using the estimated PWR values, which provided Vd and Cl estimates of 3.12 l/70 kg and 0.23 l/hour/70 kg, respectively. From these values, the corresponding coDbait half-life was calculated to be 9.4 hours.

coDbait degradation in animal and human sera

Unlike small chemical molecules, oligonucleotides are not metabolized by cytochrome P450 but are mainly hydrolyzed by nucleases present in the serum, extracellular fluids and in cells.[13] We evaluated the resistance of coDbait to nucleolytic degradation in mouse, rat, monkey and human sera. coDbait degradation was assessed on polyacrylamide gels by quantifying the band corresponding to the intact molecule. As the coDbait molecule was strongly associated with serum proteins through its cholesterol moiety, we developed a simple extraction protocol using methyl-β-cyclodextrine, a reagent known to solubilize cholesterol. For each time point, we added methyl-β-cyclodextrine and the sample was heated for 10 minutes at 95 °C before loading onto the acrylamide gel. We found that coDbait was rapidly degraded in rodent sera, with a half-life of <5 minutes in rat serum and a half-life of ~2 hours in mouse serum (**Figure 5**). The half-lives in primate sera were much longer than the half-lives in rodent sera, and were 11 and 57 hours in monkey and human sera, respectively. This higher stability of oligonucleotides in primate



Species	$t_{1/2}$ (hour)
◆ Rat	0.02
■ Mouse	2.00
● Monkey	10.75
▼ Human	57.13

Figure 5 coDbait stability in human and animal sera. coDbait (20 µg/ml) was incubated in mouse, rat, monkey and human sera at 37 °C for various lengths of time. Samples were treated with cyclodextrine and analyzed by acrylamide gel electrophoresis. Bands corresponding to intact coDbait were quantified using ImageJ software and the data were fitted to an exponential decay curve.

serum versus rodent serum has been reported previously by Bouchard and colleagues.[14,15]

Toxicity of coDbait after subcutaneous injection in rats and monkeys

The second main objective of this study was to evaluate the toxicity of coDbait following a 4 weeks subcutaneous administration and to determine whether toxic signs dissipated during the 2 weeks following treatment. As for pharmacokinetics studies, coDbait was administered in association with oral chloroquine at 5 or 9 mg/kg/day for monkeys and rats respectively in order to enhance coDbait potency. Although chloroquine is a widely used antimalarial and no toxicity was expected at the prophylactic doses used in this study, it was necessary to evaluate the toxicity of the combined treatment. coDbait was administered three times per week during 4 weeks at dose levels of 8, 16, or 32 mg per animal per day in chloroquine-treated rats and monkeys. Overall, the 4-week administrations of coDbait were well tolerated in rats and monkeys, and we detected no changes that could be considered adverse events in any of the measured parameters (see **Supplementary Table S1**). No drug-related deaths occurred in any of the treated animals. No toxicologically relevant changes were observed in food consumption, BW, ophthalmological parameters or urine composition.

Blood analysis performed using samples obtained during either the treatment or the recovery phases, revealed no relevant treatment-related effects on platelets, on activated partial thromboplastin time, on prothrombin time or on fibrinogen concentration, compared to the values of the control group. In monkeys, no meaningful variations were noted in Bb and C3a complement factor values. Hematological parameters were slightly affected, and characterized by lower numbers of lymphocytes and monocytes and higher numbers of neutrophils. Due to the dose-dependency of these changes in white blood cell populations, they were considered related to treatment. These effects could be related either to an unknown systemic effect or more likely to a cutaneous local reaction at the injection sites. As these were only slight to moderate changes and were reversible, they were not considered to be toxicologically relevant.

Toxicity studies revealed no gross abnormalities in the major organs of any animal during the observation periods. In rats, the only deleterious effect was a slight to moderate, dose-dependent and reversible inflammatory response at the injection sites. We observed dose-dependent skin thickening at the injection sites, with induration/erythema and the presence of dark foci, and we considered these events to be treatment-related. Histopathological evaluation indicated a dermal/subcutaneous subacute infiltration of inflammatory cells. At the end of the recovery period, these effects were present with low frequency and minimal severity. Their persistence in the recovery period indicated that they were partially reversible in all dose groups. In monkeys, local inflammation reactions, such as induration and edema, were also observed at the injection sites. This subacute inflammation in the subcutis at the injection sites was associated with the presence of granular macrophages. The inflammatory cell infiltrates in the subcutis and deep dermis were also associated with hemorrhage and/or oedema, necrotic foci, occasional fibrinous microthrombi and diffuse epidermal acanthosis/hyperkeratosis. The incidence and the severity of these reactions were dose-related as they were observed in all animals in the 32 mg group but occurred sporadically in the groups receiving 8 and 16 mg per animal per day. These effects were partially reversible since those at the injection sites were judged to be minimal to slight in recovered animals whereas they were minimal to moderate in animals that were examined at the completion of the dosing. In addition, the secondary coDbait-related effects found in the animals examined at the completion of the dosing (such as hemorrhage, edema, and necrosis) were no longer found in animals examined after the recovery period.

Another coDbait-related finding in rats was a reversible increase in mean spleen weight, which reached statistical significance in females in the 32-mg dose group. This size increase correlated with the minimal to marked red pulp vacuolation (coarse droplet) found on histological analysis. However, these findings were not observed in a group that received the 32-mg dose per animal per day without chloroquine, which suggested that they were secondary effects due to chloroquine treatment.

Other coDbait-related microscopic findings in monkeys were diffuse infiltration of the digestive mucosa by granulocytes (only for some animals in the 32-mg dose group); increased development of lymphoid follicles and/or paracortex in lymphoid organs (i.e., mesenteric and mandibular lymph nodes and spleen); and an increased granulopoiesis and/or erythropoiesis in the sternal bone marrow. After the

2-week recovery period, the coDbait-related microscopic findings in the bone marrow had completely reversed, those at the injection sites and in the lymphoid organs had partially reversed, and those in the digestive tract persisted.

We also assessed central nervous system function in rats and found no relevant behavioral alterations were observed after a single dose (day 0) or after repeated doses (day 26). In monkeys, we found no treatment-related effects on systolic and diastolic blood pressures, heart rate, cardiac conduction and cardiac rhythm six hours after the last coDbait administration on day 26.

Under the experimental conditions of the study, the no-observed-adverse-effect-level in rats and monkeys was considered to be the highest tested dose of 32 mg/animal/day (dose range of 161–216 mg/kg/day for rats and 10.7–16.2 mg/kg/day for monkeys), corresponding to 16 mg of coDbait administered per injection site.

DISCUSSION

DNA repair pathways play vital roles in normal metabolic activities and in cellular protection against environmental mutagens. Many tumors exhibit intrinsic resistance to DNA-damaging radiotherapy and chemotherapy due to the hyperactivation of their DNA repair functions. Therefore, inhibitors of specific DNA repair pathways may provide novel opportunities for targeting genetic differences between tumor and normal cells.[16] siDNA molecules have been developed to enhance DNA-damaging treatment by interfering with DSB damage sensing and signaling. These molecules mimic DSBs and "jam" DSB repair pathway signals. This leads to DNA repair system disorganization and ultimately inhibits non-homologous end-joining and homologous recombination.[3,4] Importantly, the selectivity of siDNA for tumor cells that spontaneously suffer DNA damage and the absence of side effects on normal cells confer a strong advantage of this approach over other chemotherapies.

Many therapeutic oligonucleotides harboring diverse structures (antisense oligonucleotides, immunostimulatory oligonucleotides, aptamers, siRNAs, miRNAs, decoys, etc.) and different chemical modifications (PS, modified 2′-ribose sugar, locked nucleic acid, etc.) are in early stage clinical trials. coDbait molecules differ from other oligonucleotides in terms of size, structure and chemical modification. Therefore the pharmacokinetics properties of other double-stranded oligonucleotides[14,17] cannot be compared to those of coDbait. The pharmacokinetics of unmodified, naked nucleic acid-based drugs make them especially unsuitable as systemic therapeutics because these molecules bind plasma proteins only weakly, resulting in rapid kidney filtration and very short half-lives of ~5 minutes.[18,19]

The most widely used modifications to increase the stability of oligonucleotides in biological fluids are PS group substitutions. PS oligonucleotides have higher stability in serum and bind tightly to plasma proteins.[20] However, extensive PS modifications are known to be toxic.[13] The PS modifications in coDbait, which are confined to the last three oligonucleotides on each strand, and the tethering of both strands on the opposite end of the double-stranded region by a linker confer substantial protection from nuclease-mediated degradation.[21] Consequently, the coDbait half-life in human serum was 57 hours.

Despite many favorable characteristics and signs of possible clinical victories, nucleic acid-based drug development for humans is still in its infancy, and success has been rare. The slow development of these drugs can be attributed, in part, to their poor cellular uptake profile *in vivo*. In a previous work, we demonstrated that cholesterol conjugation enhanced Dbait delivery.[6] Similar observations have been reported using cholesterol-conjugated siRNAs to silence specific genes *in vivo*.[7,22] Cholesterol conjugation prolongs the half-life of circulating siRNAs by promoting their association with circulating lipoprotein particles and albumin, which renders it resistant to filtration by the kidney.[7,23] Cholesterol modification may also provide additional protection from plasma nucleases.[24] Moreover, side effects induced by cholesterol conjugates have been shown to be qualitatively similar and only moderately more pronounced than those induced by the unconjugated oligonucleotides.[10,25] This is a great advantage over other forms of oligonucleotide delivery approaches which are often found to be toxic.

In the present study, we validated the efficacy of a protocol of coDbait administration in association with radiotherapy using human melanoma xenografted mice. Concomitant chloroquine treatment was added to the protocol to increase coDbait uptake and efficacy[6] resulting in an increase of the radiosensitivity of xenografted tumors by coDbait. Although chloroquine has been suggested to enhance radiotherapy through the inhibition of autophagy and the release of ROS in tumor cells,[26,27] the radiosensitization observed in our study can be attributed to coDbait as all groups received the same chloroquine regimen.

We studied the pharmacokinetics of subcutaneously injected coDbait (8, 16, and 32 mg) in rats and monkeys. Because coDbait administration was local (subcutaneous injection), the doses were not adjusted to the BWs of two different animal species but were kept constant. No major sex-related differences were observed with respect to C_{max} and AUC_{0-24h}. The relationship between dose and systemic exposure (AUC_{0-24h}) was linear and dose-proportional in rats but in monkeys, the AUC_{0-24h}/dose ratios were higher for the different doses. The clearance (Cl/F) parameters are very close for rats and monkeys. Surprisingly, the Vd/F parameters in monkeys were only two- to threefold higher than those of the rats, despite the monkeys' 15–18 times higher blood volumes. This could be partially explained by the large differences of coDbait degradation rates in rat and monkey sera. The rapid degradation of coDbait is probably the main force driving its elimination from the bloodstream in rats.[14] In the monkey, coDbait stability in serum is much longer than its persistence in the bloodstream, so its predominant pathways of elimination likely involve renal clearance and/or tissue absorption. C_{max} was reached ~4 hours after dosing in both species. The model-based analysis using a population approach showed a clear relationship between BW and Vd. The relationship between Cl and BW was also highly significant ($P = 10^{-7}$), but the change in Cl as a function of BW is somewhat flat, as the PWR exponent was only 0.15. This allowed us to estimate the expected mean Vd and Cl in a 70 kg human.

We sought to determine the toxicity of coDbait following twelve subcutaneous doses (three administrations per week for 4 weeks) of 8, 16, or 32 mg in chloroquine-treated rats and monkeys. We also evaluated the possible regression of any toxic signs during a 2-week treatment-free recovery period. Overall, the treatments were well tolerated in rats and monkeys, and no drug-related deaths occurred in any of the treated animals. We detected dose-dependent but reversible inflammatory responses at injection sites in rats and monkeys. These inflammatory responses were characterized by skin thickening, induration, erythema, edema, and the presence of dark foci. Histopathological evaluation indicated dermal/subcutaneous subacute inflammatory cell infiltration associated with the presence of granular macrophages. The incidence and the severity of the reactions were dose-related, sporadically observed in the 8 and 16 mg/animal/day dose groups and observed in all animals receiving 32 mg. At the end of the recovery period, these effects were present at low frequency and were of minimal severity. These results suggest that it would be preferable to use multiple injection sites for doses higher than 32 mg rather than giving a high dose of coDbait at one injection site, within the limit of systemic tolerance of the treatment in patients. We also found that 6 and 12 subcutaneous injections of 32 mg in rats and monkeys respectively did not trigger any adverse effect (data not shown). The maximal tolerated doses (MTD) of 192 mg in rats and 384 mg in monkeys were above the highest dose we administered in this study.

To stabilize the coDbait against nuclease-mediated degradation, the molecule possesses three PS linkages at one 3′-end and three at one 5′-end. Several non sequence-related toxic effects have been attributed to PSs. In rodents, splenomegaly, lymphoid hyperplasia, and mononuclear cell infiltrates in several organs have been reported.[28] In monkeys, PS oligonucleotide toxicity has been associated with prolongation of aPPT, complement activation and even cardiovascular collapse and death.[29,30] These effects are most frequently associated with fully PS-linked oligonucleotides of ~20 bp in length. In contrast, coDbait possesses only six PS linkages and we detected no cardiac abnormalities or increases in Bb, C3a or activated partial thromboplastin time associated with its administration. The C_{max} of coDbait was also below the threshold concentration associated with complement activation in cynomolgus monkeys treated with the PS oligonucleotide ISIS2302 (50 µg/ml).[30]

In rodents, for various classes of therapeutic oligonucleotides, the liver and kidney are the major sites of accumulation, followed by the spleen, bone marrow and lymph nodes.[13,31] coDbait in mice accumulated in these same target organs (unpublished data), which can therefore be considered the primary target organs for toxicity. No apparent or histologically observable signs of toxicity were found in the livers or kidneys of rats or monkeys, even at the highest dose. Moreover, we observed no increases in aspartate and alanine aminotransferases; urea or creatinine were observed, confirming the lack of liver and kidney toxicities. After a 2-week treatment-free period, the coDbait-related microscopic findings in the bone marrow reversed completely whereas those found in the digestive tract persisted. It is possible that the coDbait-sensitivity of these two tissues with high renewal rates is due to the sporadic occurrence of replication errors. Indeed, the single layer of epithelial cells in the mammalian intestine is renewed every 4–6 days.[32]

Another source of toxicity in nucleic acid-based therapeutics is the presence of CpG motifs in the oligonucleotide sequence. Oligonucleotides or double-stranded DNAs (dsDNA) containing CpG sequences trigger immune responses via Toll-like receptors. These responses induce proinflammatory effects characterized by cytokine production, splenomegaly, infiltration of monocytes/macrophages in several organs,[33] lymphopenia, thrombocytopenia, and elevated levels of liver transaminases.[13] coDbait is an optimized dsDNA fragment without any CpG sequences and induces few proinflammatory effects. Immune competent mice challenged with the coDbait DNA sequence (seven injections of 0.12 mg/animal, 5 mg/kg, within 24 hours) produced no significant cytokine responses (interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12P70, interferon-γ, and tumor necrosis factor-α), whereas challenge with a CpG-containing dsDNA fragment of the same length strongly stimulated IL-6 and IL-12P70 production.[3] Similarly, coDbait induced few hematological changes in rats and monkeys and no liver damage, both of which are consistent with the absence of cytokine release.

The protocol of coDbait administration described in this study revealed an absence of severe adverse effects in animals and provide useful informations on the pharmacokinetics of the molecule. It also procured a method of administrating high doses of coDbait in order to avoid local toxicity. Based on the results of a pharmacokinetic simulation in humans and on the lack of severe adverse effects, coDbait has entered a phase I clinical trial for the treatment of in-transit melanoma (DRIIM: NCT01469455). To study the potential cumulative toxicity of coDbait and radiotherapy on healthy tissues, patients receiving radiotherapy will be given escalating doses of coDbait—between 16 and 64 mg—administered at two to four injection sites for three times a week for 2 weeks with daily oral administration of chloroquine.

MATERIALS AND METHODS

coDbait molecules. coDbait is 32-bp double-stranded oligonucleotide protected from nucleolytic degradation by a 1,19-bis(phospho)-8-hydraza-2-hydroxy-4-oxa-9-oxo-nonadecane linker loop on one end and by PS nucleotides on the other end (**Figure 1**). The sequence is: 5′-L-GCTGTGCCCACAACCCAGCAAACAAGCCTAGA-(X)-TCTAGGCTTGTTTGCTGGGTTGTGGGCACAGC-3′, where X is a carboxylic acid loop, L is a cholesterol triethylene glycol and underlined bases mark the position of the PS nucleotides. coDbait was synthesized using automated solid-phase oligonucleotide synthesis and purified by denaturing reversed-phase high-performance liquid chromatography and/or high-performance liquid chromatography–ion exchange, in compliance with GMP regulations (Agilent Technologies, Boulder, CO).

Efficacy study in mice. SK28 xenograft tumors were obtained by injecting 10^6 tumor cells into the flank of adult female nude mice (Charles River Laboratory; L'arbresle, France). The animals were housed in the laboratory for at least 1 week before commencing experiments. There were five to

six animals per cage, under controlled conditions of light and dark cycles (12:12 hours), relative humidity (55%), and temperature (21 °C). Food and tap water were available *ad libitum*. After ~12 days, when the subcutaneous tumors measured 150–200 mm^3, the mice were separated into homogeneous groups of at most 12 each, to receive different treatments. All animals received three per OS administrations of 200 µl of a 5 mg/ml chloroquine solution (Sigma, Saint-Quentin Fallavier, France) per week for 3 weeks. The chloroquine was administered 1 week before treatment and during the 2 weeks of treatment, 1 hour before coDbait injection. Irradiation was performed in a 37 °C unit (0.5 Gy/minute) with a shield designed to protect about two-thirds of the animal's body. Doses were controlled by thermoluminescence dosimetry. A total dose of 30 Gy were delivered in 10 sessions at intervals of five sessions of 3 Gy per week for a period of 2 weeks. coDbait molecules were prepared in 100 µl of 5% glucose. The group treated with coDbait and irradiation received two subcutaneous injections of 2 mg coDbait each at the proximity of the tumor (~ 0.5 cm), three times per week for 2 weeks. Injections of coDbait were performed 5 hours before each radiotherapy session. Mock-treated animals were injected with 100 µl of 5% glucose. Tumor size was assessed by caliper measurements every 3 days, and size was calculated using the formula ($0.5 \times \text{length} \times \text{width}^2$). Mice were weighed and pictures of tumors were taken every week during 120 days. For ethical reasons, the animals were killed when their tumors reached 2,000 mm^3. The endpoint used in survival analysis was death day. Overall survival curves were assessed by Kaplan–Meier estimates and compared using the nonparametric log-rank test because the data do not follow a normal distribution. The relative risk and the *P* value were obtained using S-Plus 6.2 version software (MathSoft Inc., Seattle, WA) and statEL (ad Science, Paris, France). The Local Committee on Ethics of Animal Experimentation (Orsay, France) approved all experiments.

Pharmacokinetic study in rats. Wistar rats (*Rattus norvegicus*) were between 136 and 163 g for males and between 116 and 140 g for females and ~6-week old at the start of treatment. Each animal received daily oral doses of chloroquine (9 mg/kg/day). After 1 week of chloroquine treatment, animals were divided into four groups (12 animals per group: six males and six females) and were treated with two subcutaneous injections (bolus with constant volume of 0.4 ml each) of coDbait per animal. Two injection sites in the dorsal region were used. Each injection site was separated by ~2 cm latero-laterally and cranio-caudally. Group 1 (control) received vehicle (5% glucose solution). Group 2 received a total of 8 mg of coDbait, group 3 received 16 mg and group 4 received 32 mg. Blood was withdrawn from the retro-orbital sinus under isoflurane anesthesia. Samples were collected in tubes containing K2-EDTA at the following times: before treatment, at 2 and 6 hours for the first three animals per group, and at 4, 8, and 24 hours for the last three animals per group. The samples were centrifuged at 1,800*g* for 10 minutes at 4 °C and the plasma was recovered and frozen at –80 °C. Mean plasma concentrations at each time-point, the SD and CV were calculated from three animals. Animals were housed at Ricerca facility in Lyon, France that holds certification from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Pharmacokinetic study in monkeys. Cynomolgus monkeys (*Macaca fascicularis*) were between 2.2 to 3.3 kg for males and between 2.0 to 3.1 kg for females and ~35 months old at the start of treatment. Animals received daily oral doses of chloroquine (5 mg/kg/day). After 1 week of oral chloroquine, animals were divided into four groups (six animals per group: three males and three females) and were treated with two subcutaneous injections (bolus of 0.4 ml each) of coDbait per animal. Two injection sites in the dorsal region were used. Each injection site was separated by ~2 cm. Group 1 (control animals) received vehicle (5% glucose solution). Group 2 received a total of 8 mg of coDbait, group 3 received 16 mg and group 4 received 32 mg. Blood samples (1 ml) were withdrawn from the femoral vessels of unanesthetised manually restrained animals and collected in tubes containing potassium-EDTA at the following times: before treatment, and at 2, 4, 6, 8, and 24 hours after the coDbait injections. The samples were centrifuged at 1,800*g* at 4 °C for 10 minutes and the plasma was separated and frozen at –80 °C. Mean plasma concentrations at each time-point, the SD and CV were calculated from three animals. Animals were housed at Ricerca facility in Lyon, France that holds certification from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Bioanalysis procedures. Standards were prepared by spiking known amounts of coDbait in control rat plasma. Samples were thawed and kept on ice during the sample analysis. Homogenization RLT Buffer (Qiagen, Toronto, Canada) was added to the standards and samples at a ratio of three parts RLT to two parts plasma. The samples were then incubated at 95 °C for 12 minutes and cooled to room temperature for 20 minutes. Homogenates (50 µl) were mixed with 300 µl of the capture probe (5′- /5BioTEG/GCT GTG CCC ACA ACC -3′) at 20 nmol/l in 5× saline-sodium citrate Tween buffer (SSCT) (0.75 mol/l NaCl, 0.075 mol/l sodium citrate, 0.01% Tween-20). The samples were heat-denatured at 95 °C for 10 minutes in a heating block and cooled to room temperature for 20 minutes. Hundred microliter of this mixture was then added to duplicate wells of a 96-well streptavidin-coated plate (StreptaWell; Roche Applied Science, Melan, France). The plate was covered and incubated for 45 minutes at room temperature on an orbital shaker set at 200 rpm and was then washed three times with 2× SSCT (300 mmol/l NaCl, 30 mmol/l sodium citrate, 0.01% Tween-20). A detection probe (5′- CAG CAA ACA AGC CTA GA/3DigN/ -3′) at 20 nmol/l in 5× SSCT was added to all wells (100 µl). The plate was incubated for 45 minutes at room temperature on an orbital shaker set at 200 rpm, washed three times with 2× SSCT and excess liquid was removed by blotting on paper. An anti-digoxigenin peroxidase-conjugated antibody diluted 1:10,000 in phosphate-buffered saline containing 0.005% Tween-20 was added to all wells (100 µl). The plate was incubated for 45 minutes at room temperature on an orbital shaker set at 200 rpm, washed three times with 2× SSCT and excess liquid was removed by blotting on paper. Detection

was achieved by adding the TMB substrate (100 µl) and the plate was incubated (in the dark) for ~45 minutes at room temperature on an orbital shaker set at 100 rpm. The reaction was stopped by the addition of 0.5 mol/l H_2SO_4 (100 µl). Absorbance was measured using a Spectramax 340PC or Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA) set at 450 nm with a 570 nm background correction. The amount of coDbait in samples was calculated from calibration standards over a working range of 3–80 ng/ml using a four-parameter logistic curve fit. Plasma concentrations below the lower limit of quantification (3 ng/ml) were taken as 0. The method was subjected to the following validations: specificity; dilution linearity; working calibration range; lower limit of quantification; interassay precision and accuracy; and evaluation of the prozone effect. Using gel electrophoresis assay, we confirmed that coDbait measured was full-length double-stranded coDbait.

Pharmacokinetic parameters calculation. Mean plasma concentrations per time-point with SD and CV were calculated from three animals for rats and monkeys. The pharmacokinetic parameters were determined from the mean plasma concentrations by noncompartmental analysis, using Kinetica 4.4.1 (Thermo Fisher Scientific, Waltham, MA) or GraphPad Prism 5 (GraphPad Software, San Diego, CA) software. The area under the plasma concentration-time curve from zero to the last quantifiable concentration (AUC_{0-t}) was calculated using the trapezoidal rule. The Cl/F was calculated using the equation:

$$Cl/F = \frac{Dose}{AUC_{0\text{-inf}}}$$

where the dose is expressed as mg/animal and F is the bioavailability.

Elimination rate (k_e) was obtained by a linear regression analysis of selected time points in the terminal phase of the concentration versus time curves. The terminal half-life ($t_{1/2}$) was calculated as follows:

$$t_{1/2} = \frac{\ln(2)}{k_e}$$

The volume of distribution (Vd/F) of the terminal phase was calculated using the equation:

$$Vd/F = \frac{Cl/F}{k_e}$$

The pharmacokinetic data from all animals were also analyzed collectively via a population approach using the Monolix program[34] in order to extrapolate the pharmacokinetic parameters to a 70 kg human. According to the allometric rule, the Vd and Cl terms are related to the individual BW with PWR of 1 and 0.75, respectively. The Vd and Cl terms are then related to BW as follows:

$$Vd = Vd_{70}\left(\frac{BW}{70}\right)^{PWR.Vd}$$

$$Cl = Cl_{70}\left(\frac{BW}{70}\right)^{PWR.Cl}$$

where Vd_{70} and Cl_{70} represent the typical values for a 70 kg individual. Therefore, in the population analysis of data from individual rats and monkeys, the Vd and Cl terms were assumed to depend upon the animal's BW using either these fixed power values or estimated values.

coDbait stability assay in serum. Nucleolytic degradation of coDbait (20 µg/ml) was evaluated by polyacrylamide gel electrophoresis after incubation in mouse, rat, monkey, or human sera at 37 °C. Samples were removed at different time points between 0 and 106 hours and degradation was stopped by addition of 20 mmol/l EDTA and immediate freezing at –20 °C. Cholesterol oligonucleotides were dissociated from plasma proteins by the addition of 15 mg/ml methyl-β-cyclodextrine (Sigma). After incubation for 12 minutes at 95 °C, samples were subjected to 15% nondenaturing polyacrylamide gel electrophoresis and bands were revealed using SybrGold nucleic acid dye (Life Technologies, Grand Island, NY). Degradation of coDbait was estimated by measuring its band intensity relative to that of the band corresponding to the intact molecule using ImageJ software (National Institutes of Health, Bethesda, MD). Half-lives of coDbait in the different sera were determined by fitting the amount of full-length oligonucleotide at different time points to a first-order exponential decay function using GraphPad Prism 5 software (GraphPad Software).

Toxicity study. After 1 week of oral chloroquine (5 mg/kg/day for monkeys and 9 mg/kg/day for rats) or water pretreatment, monkeys and rats were treated three times/week by two subcutaneous injections of coDbait per animal per day for 4 weeks. Half of the animals were kept under observation for a recovery period of 2 weeks. Dose levels were 0, 8, 16, and 32 mg per animal per day. The 32 mg dose level was tested with and without chloroquine. Rat groups contained five animals of each sex and monkey groups contained three animals of each sex. Morbidity/mortality checks were performed at least twice daily. Clinical observations were performed daily. Local reactions at the injection sites were assessed before and ~24 hours after each administration, then once weekly during the recovery time. Ophthalmological examinations were performed before the study and during the fourth week. BW was recorded weekly for each animal. Uneaten food was estimated daily for each monkey and weekly for each cage of rats. Blood sampling for activated partial thromboplastin time determination was performed pretest, then on days 0, 26, and 27, at ~6 hours after dosing. Other clinical laboratory determinations of hematology, complement factors Bb and C3a evaluation (only for monkeys), clinical chemistry parameters and urine analysis, were performed pretest and on days 27 and 41. All designated animals were euthanized at the end of the treatment period (day 27) or after a 2-week

treatment-free period (day 41) and necropsied. Selected organs were weighed. Organ/tissue samples were fixed and preserved at necropsy for all animals. Selected organs/tissues from the control group and 32 mg dose group animals were examined histopathologically. In addition, microscopic examination of injection sites, stomach, duodenum, jejunum, ileum, caecum, colon, bone marrow (sternum), mandibular lymph node, mesenteric lymph node, spleen, liver, and lung was performed for all animals in the 8 mg and 16 mg dose groups and for all recovery animals.

To assess the potential neurobehavioral effects of coDbait in rats, we performed a standard observation battery, which allows the assessment of peripheral and central nervous system activities, on day 0 and day 26. Several tests were conducted including an auditory reflex test, a gripping test and a pupillary reflex test to assess the reaction of the pupils to light and an open field test to assess reactivity to a novel environment and motor activity during 3 minutes. To assess the cardiovascular effects of coDbait treatment in monkeys, we assessed the following parameters of unanesthestized monkeys in a restraining chair before coDbait treatment and 6 hours after the last vehicle or coDbait injection on day 26: systolic and diastolic arterial blood pressure (indirect method using a thigh- or arm-cuff), electrocardiography (leads I, II, III), heart rate, analysis of rhythm, duration of QRS complex and PR and QT intervals.

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Supplementary Material

Table S1. Toxicological parameters studied in rats and monkeys.

REFERENCES

1. Dutreix, M, Cosset, JM and Sun, JS (2010). Molecular therapy in support to radiotherapy. *Mutat Res* **704**: 182–189.
2. Wójcik, M, Cieslak, M, Stec, WJ, Goding, JW and Koziolkiewicz, M (2007). Nucleotide pyrophosphatase/phosphodiesterase 1 is responsible for degradation of antisense phosphorothioate oligonucleotides. *Oligonucleotides* **17**: 134–145.
3. Quanz, M, Berthault, N, Roulin, C, Roy, M, Herbette, A, Agrario, C *et al.* (2009). Small-molecule drugs mimicking DNA damage: a new strategy for sensitizing tumors to radiotherapy. Clin Cancer Res **15**: 1308–1316.
4. Quanz, M, Chassoux, D, Berthault, N, Agrario, C, Sun, JS and Dutreix, M (2009). Hyperactivation of DNA-PK by double-strand break mimicking molecules disorganizes DNA damage response. *PLoS ONE* **4**: e6298.
5. Devun, F, Bousquet, G, Biau, J, Herbette, A, Roulin, C, Berger, F *et al.* (2012). Preclinical study of the DNA repair inhibitor Dbait in combination with chemotherapy in colorectal cancer. J Gastroenterol **47**: 266–275.
6. Berthault, N, Maury, B, Agrario, C, Herbette, A, Sun, JS, Peyrieras, N *et al.* (2011). Comparison of distribution and activity of nanoparticles with short interfering DNA (Dbait) in various living systems. Cancer Gene Ther **18**: 695–706.
7. Soutschek, J, Akinc, A, Bramlage, B, Charisse, K, Constien, R, Donoghue, M *et al.* (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature **432**: 173–178.
8. Bijsterbosch, MK, Rump, ET, De Vrueh, RL, Dorland, R, van Veghel, R, Tivel, KL *et al.* (2000). Modulation of plasma protein binding and *in vivo* liver cell uptake of phosphorothioate oligodeoxynucleotides by cholesterol conjugation. Nucleic Acids Res **28**: 2717–2725.
9. Ma, L, Reinhardt, F, Pan, E, Soutschek, J, Bhat, B, Marcusson, EG *et al.* (2010). Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. Nat Biotechnol **28**: 341–347.
10. Manoharan, M (2002). Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. *Antisense Nucleic Acid Drug Dev* **12**: 103–128.
11. Minchin, RF and Yang, S (2010). Endosomal disruptors in non-viral gene delivery. *Expert Opin Drug Deliv* **7**: 331–339.
12. Crooke, ST (1995). Progress in antisense therapeutics. *Hematol Pathol* **9**: 59–72.
13. Levin, AA (1999). A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim Biophys Acta* **1489**: 69–84.
14. Bouchard, PR, Hutabarat, RM and Thompson, KM (2010). Discovery and development of therapeutic aptamers. *Annu Rev Pharmacol Toxicol* **50**: 237–257.
15. Crooke, ST (1992). Therapeutic applications of oligonucleotides. *Annu Rev Pharmacol Toxicol* **32**: 329–376.
16. Helleday, T, Petermann, E, Lundin, C, Hodgson, B and Sharma, RA (2008). DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* **8**: 193–204.
17. Lee, PA, Blatt, LM, Blanchard, KS, Bouhana, KS, Pavco, PA, Bellon, L *et al.* (2000). Pharmacokinetics and tissue distribution of a ribozyme directed against hepatitis C virus RNA following subcutaneous or intravenous administration in mice. Hepatology **32**: 640–646.
18. Agrawal, S, Temsamani, J, Galbraith, W and Tang, J (1995). Pharmacokinetics of antisense oligonucleotides. *Clin Pharmacokinet* **28**: 7–16.
19. Braasch, DA, Paroo, Z, Constantinescu, A, Ren, G, Oz, OK, Mason, RP *et al.* (2004). Biodistribution of phosphodiester and phosphorothioate siRNA. Bioorg Med Chem Lett **14**: 1139–1143.
20. Geary, RS (2009). Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin Drug Metab Toxicol* **5**: 381–391.
21. Eder, PS, DeVine, RJ, Dagle, JM and Walder, JA (1991). Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. *Antisense Res Dev* **1**: 141–151.
22. Chen, Q, Butler, D, Querbes, W, Pandey, RK, Ge, P, Maier, MA *et al.* (2010). Lipophilic siRNAs mediate efficient gene silencing in oligodendrocytes with direct CNS delivery. J Control Release **144**: 227–232.
23. Wolfrum, C, Shi, S, Jayaprakash, KN, Jayaraman, M, Wang, G, Pandey, RK *et al.* (2007). Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol **25**: 1149–1157.
24. de Smidt, PC, Le Doan, T, de Falco, S and van Berkel, TJ (1991). Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acids Res* **19**: 4695–4700.
25. Henry, SP, Zuckerman, JE, Rojko, J, Hall, WC, Harman, RJ, Kitchen, D *et al.* (1997). Toxicological properties of several novel oligonucleotide analogs in mice. Anticancer Drug Des **12**: 1–14.
26. Rouschop, KM, Ramaekers, CH, Schaaf, MB, Keulers, TG, Savelkouls, KG, Lambin, P *et al.* (2009). Autophagy is required during cycling hypoxia to lower production of reactive oxygen species. Radiother Oncol **92**: 411–416.
27. Solomon, VR and Lee, H (2009). Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. *Eur J Pharmacol* **625**: 220–233.
28. Henry, SP, Taylor, J, Midgley, L, Levin, AA and Kornbrust, DJ (1997). Evaluation of the toxicity of ISIS 2302, a phosphorothioate oligonucleotide, in a 4-week study in CD-1 mice. *Antisense Nucleic Acid Drug Dev* **7**: 473–481.
29. Henry, SP, Novotny, W, Leeds, J, Auletta, C and Kornbrust, DJ (1997). Inhibition of coagulation by a phosphorothioate oligonucleotide. *Antisense Nucleic Acid Drug Dev* **7**: 503–510.
30. Henry, SP, Giclas, PC, Leeds, J, Pangburn, M, Auletta, C, Levin, AA *et al.* (1997). Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action. J Pharmacol Exp Ther **281**: 810–816.
31. Peng, B, Andrews, J, Nestorov, I, Brennan, B, Nicklin, P and Rowland, M (2001). Tissue distribution and physiologically based pharmacokinetics of antisense phosphorothioate oligonucleotide ISIS 1082 in rat. *Antisense Nucleic Acid Drug Dev* **11**: 15–27.
32. Barker, N, van de Wetering, M and Clevers, H (2008). The intestinal stem cell. *Genes Dev* **22**: 1856–1864.
33. Younis, HS, Vickers, T, Levin, AA and Henry, SP (2006). CpG and Non-CpG oligodeoxynucleotides induce differential proinflammatory gene expression profiles in liver and peripheral blood leukocytes in mice. *J Immunotoxicol* **3**: 57–68.
34. Kuhn, E and Lavielle, M (2005). Maximum likelihood estimation in nonlinear mixed effects models. *Comput Stat Data An* **49**: 1020–1038.