Doxorubicin coupled to lactosaminated human albumin: a hepatocellular carcinoma targeted drug

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. There is a substantial need for new chemotherapeutic drugs effective against this tumor. Doxorubicin (DOXO), used for chemoembolization of HCCs, is poorly efficacious when administered systemically at conventional doses; dose escalation is hindered by unacceptable toxicity. Here, we review preclinical experiments showing that the efficacy of DOXO against HCCs and its safety increased following conjugation to lactosaminated human albumin (L-HSA). L-HSA–DOXO was initially prepared to improve the anticancer activity of the drug on well-differentiated HCCs, which actively internalize L-HSA by means of the asialoglycoprotein receptor. Unexpectedly, it was found that the conjugate enhanced DOXO concentrations in all forms of HCCs, independently of their differentiation grade.

Introduction

Hepatocellular carcinoma (HCC) is a major worldwide health problem and there is an active search for new, systemic chemotherapeutic drugs for this tumor [1]. In the treatment of HCCs that retain the asialoglycoprotein receptor (ASGP-R), the chemotherapeutic index of drugs that display side effects on extrahepatic tissues could be increased by coupling them to macromolecules that bind this receptor [2–4]. ASGP-R is a glycoprotein that is present only on the surface of hepatocytes. It mediates the uptake and lysosomal degradation of galactosyl-terminating peptides [5,6], which can be used as carriers for the selective delivery of drugs to parenchymal liver cells [7,8]. In a study using needle biopsies of 60 human HCCs, ASGP-R was histochemically detected on all cells in 28 out of 35 (80%) well differentiated (WD) and in 5 out of 25 (20%) poorly differentiated (PD) forms of the tumor [9]. This result justified the attempts to develop HCC chemotherapy via the ASGP-R. In line with this approach, doxorubicin (DOXO) was coupled to lactosaminated human albumin (L-HSA), a galactosyl-terminating neoglycoprotein [10] the safety of which has been established through its administration over 28 consecutive days as a carrier of adenine arabinoside to patients with chronic hepatitis B infection [11,12]. In rats with HCCs induced by diethylnitrosamine (DENA), it was unexpectedly found [13] that L-HSA–DOXO accumulated not only in WD HCCs, but also in the poorly differentiated forms of the rat tumor, the large majority of which did not express the ASGP-R [14]. This result indicated that the conjugate could be possibly administered for the treatment of all forms of HCCs, independent of their differentiation grade; moreover, its use would not require preliminary search for the presence of ASGP-R, thus avoiding the need of a tumor biopsy with the related risks.

Here we report experiments that clearly demonstrated that in rats with HCCs, L-HSA–DOXO accomplished an efficient targeting of the drug with an increased anticancer efficacy and tolerability.

Coupling of DOXO to L-HSA

Lactose was coupled to lysine ε-amino groups of HSA by reductive amination [10,15]. By this procedure the galactose moiety of lactose remains unchanged and can bind to ASGP-R. When lactose is coupled to HSA at a molar ratio of 20–25:1, the resultant conjugate, L-HSA, is capable of selectively entering into hepatocytes [10]. For

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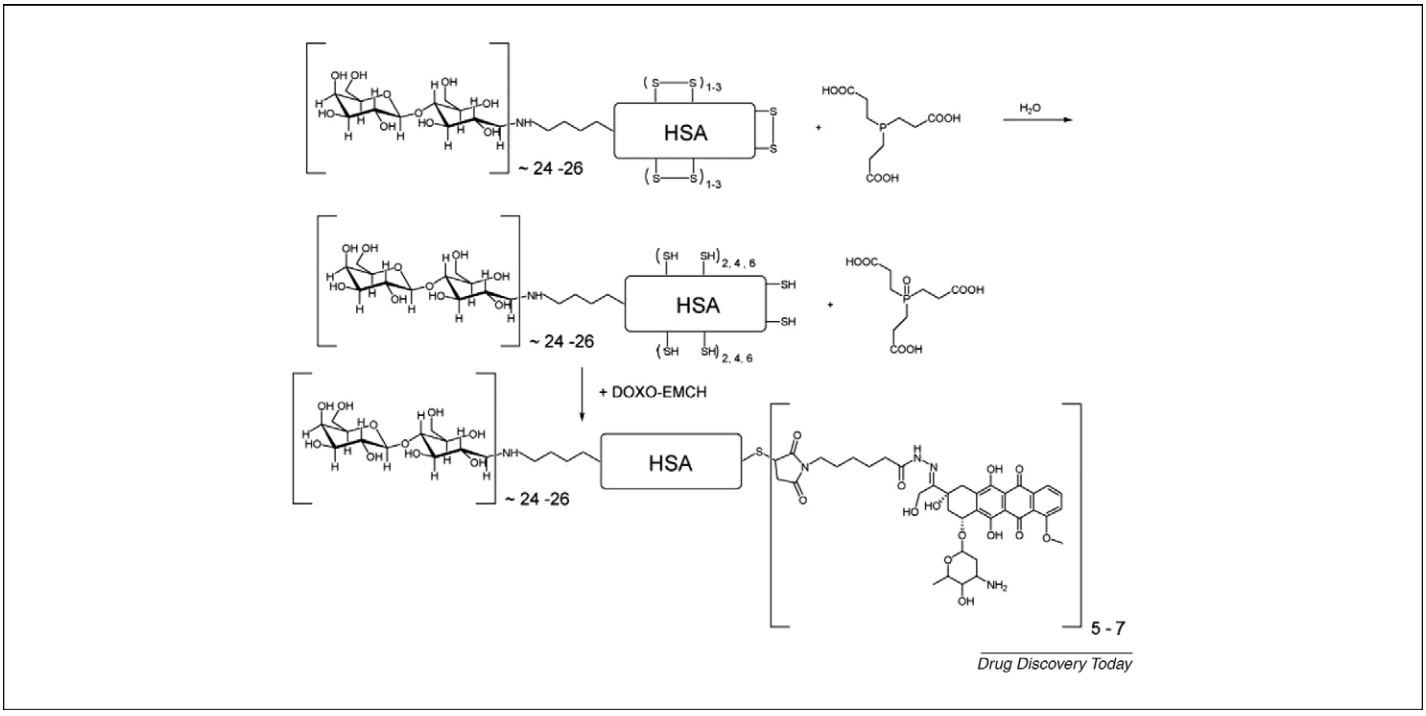

FIGURE 1

The coupling reaction and the structure of L-HSA–DOXO conjugate.

the preparation of DOXO conjugates, L-HSA preparations with molar ratios of sugar/protein ranging from 24 to 26 were used. DOXO was coupled to L-HSA using its (6-maleimidocaproyl) hydrazone derivative (DOXO-EMCH) (Fig. 1), which was first used to conjugate DOXO to a monoclonal antibody recognizing a tumor-associated antigen [16]. For a conjugate to be pharmacologically active, the bond linking the drug to the carrier must be stable in the bloodstream, but rapidly cleaved upon entering the target cells. The hydrazone bond of DOXO-EMCH fulfils this property, because it is stable at the neutral pH of plasma [17], yet is rapidly cleaved at the acidic pH of the endosomal and lysosomal compartments of the cells [18]. A demonstration of the rapid splitting of this bond *in vivo* was provided by the finding that in rats with chemically induced HCCs that received the conjugate i.v., the drug that entered the liver and tumors in the coupled form was completely set free from the carrier in two to three hours [19]. DOXO was also bound to proteins via the hydrazone bond using different maleimide spacers [20]. DOXO-EMCH has an advantage over these other drug derivatives, because it was found in a clinical phase I study to be well tolerated by patients [21]. The hydrazone bond is clearly superior to DOXO (or daunorubicin) linked through a peptide bond to galactosylated copolymers of *N*-(2-hydroxypropyl)metacrylamide (pHPMA) in another cytotoxic complex prepared to target DOXO to HCCs expressing the ASGP-R [3,22]. In fact, the peptide bond releases only small amounts of the drug within the cells [23] (see the Conclusion section). In DOXO-EMCH conjugation the maleimido moiety forms a stable thioether bond with the SH– groups of the protein, made available by reducing agents (Fig. 1). The coupling reaction with L-HSA was simplified by substituting the thiolic reducing agents [16] with tris(2-carboxyethyl) phosphine that eliminated the need for an inert atmosphere and allowed a one-step coupling reaction, without the purification of the reduced protein before the addition of DOXO-EMCH [24]. The DOXO/L-HSA molar ratio of conjugate preparations used in the experiments reported here ranged from 5 to 7 (1 mg conjugate contained 36–50 μg DOXO).

Concentrations of DOXO in HCCs and in organs of rats injected with L-HSA–DOXO

Rats with HCCs induced by DENA were used as an animal model of patients with HCCs [14]. DENA was given in the drinking water (100 mg/l) for two months [25]. One month after the last day of DENA administration, multiple, roughly spherical nodules can be observed on the liver surface, with a size ranging from 2 to 10 mm in diameter. All histologically examined nodules were classified as HCCs with either a trabecular (WD), solid (PD) or intermediate (MD) pattern. All WD HCCs studied accumulated L-[^{14}C]HSA, in contrast to PD HCCs, the majority of which internalized only L-[^{14}C]HSA to the same extent as the extra-hepatic tissues [14]. To estimate the potential increase in the chemotherapeutic index of DOXO administered in the conjugated form, the same dose of free DOXO (F-DOXO) or L-HSA coupled DOXO was injected in rats with HCCs and the drug concentrations were measured in tumors, surrounding liver and target organs of drug toxicity (heart and intestine) [13]. F- and L-HSA coupled DOXO were administered intravenously at 1 mg/kg, which was two times higher than the DOXO dose administered weekly to humans (20 mg/m^2, corresponding to approximately 0.5 mg/kg [26]). In HCC-bearing rats, 1 μg/g of L-HSA coupled DOXO (corresponding to 24 μg conjugate) was completely lost from the bloodstream within two to three hours [19]. Also, in L-HSA–DOXO injected animals, the drug form determined in both tumors and organs was free DOXO (i.e. the drug liberated from the carrier after intracellular penetration of the conjugate).

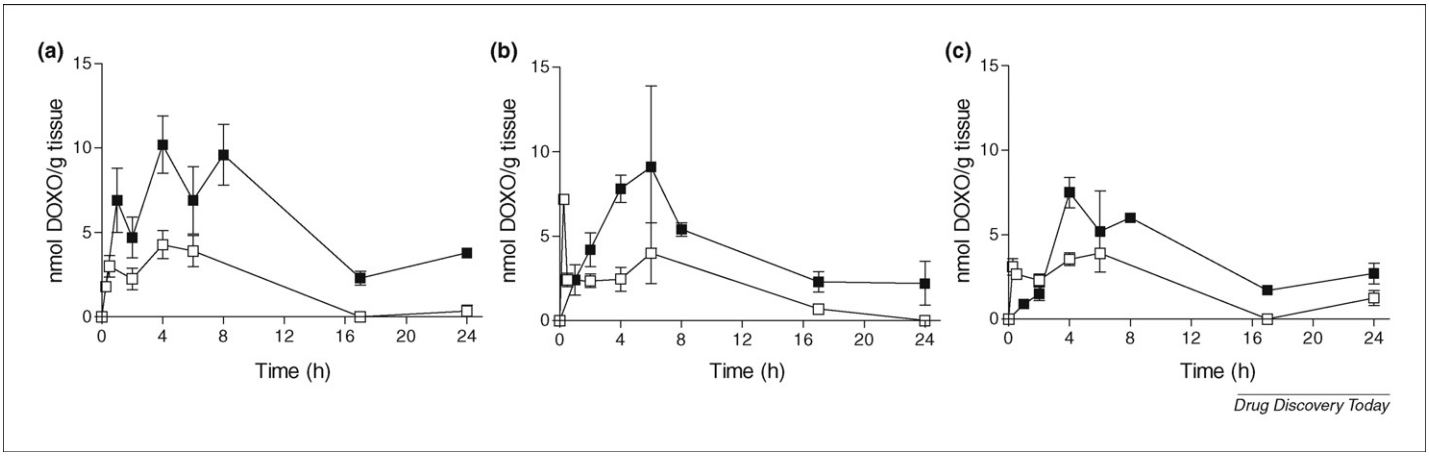

FIGURE 2

DOXO concentrations in well **(a)**, moderately **(b)** and poorly **(c)** differentiated hepatocellular carcinomas after i.v. injection of 1 μg/g of F-DOXO (□) or L-HSA coupled DOXO (■). For each compound, the time interval and form of HCCs (WD, MD and PD) 3–15 neoplastic nodules were studied. They were obtained from three to six animals. The study was performed using a total of 310 HCC nodules. In both F-DOXO and coupled DOXO-treated rats, some DOXO determinations were performed at the same time intervals (2, 4, 6, 17 and 24 hours). For each differentiation form of the tumor, the differences between the values measured at these times in the two groups of animals were statistically evaluated by two-way ANOVA, using the time and the drug form (free or coupled) as factors. A statistically significant difference was found between the two drug forms ($P < 0.0001$ for WD HCCs; $P < 0.05$ for MD and PD HCCs). From Di Stefano *et al.* [13] with permission from Blackwell, Munksgard.

The levels of DOXO in tumors are reported in Fig. 2 and Table 1. Unexpectedly, conjugate administration produced drug concentrations two times higher than those achieved by F-DOXO, not only in WD HCCs, but also in PD tumors, which, in spite of the lack of ASGP-R [14], actively internalized the conjugate. This was demonstrated by measuring the levels of radioactivity in PD tumors from rats injected with a L-[^{14}C]HSA–DOXO conjugate (labeled in the protein moiety); the levels were shown to be four times higher than those determined in animals given the same amount of the carrier L-[^{14}C]HSA [13]. The enhanced penetration of L-HSA–DOXO in PD HCCs results from nonspecific adsorption to neoplastic hepatocytes mediated by the coupled DOXO molecules, as demonstrated by experiments *in vitro*, which showed that DOXO coupling greatly increased the quantities of L-[^{14}C]HSA adsorbed by cell lines derived from HCCs, as well as from other tumors [27]. DOXO molecules interact with the cell surface mainly because at physiological pH they are positively charged and bind to anionic phospholipids of the cell membrane [28]. The lactose residues of the conjugate further increased the concentrations of DOXO in WD HCCs, as shown by the data reported in Table 1. In MD and PD forms of the tumors of rats injected with L-HSA–DOXO the drug levels were two times higher than those of animals given F-DOXO; the difference was three times higher in WD HCCs, which express ASGP-R and can internalize the conjugate by both adsorptive and receptor-mediated endocytosis. The different pathways by which L-HSA–DOXO and F-DOXO enter into cells can, in part, account for the higher levels of drug achieved by the former compound in HCCs. In fact, after the internalization of the conjugate, the DOXO released from the carrier in the endocytic compartment can, in part, escape drug efflux caused by the pumps located at the external membrane, as suggested by other experiments with macromolecular drugs [29]. In accordance with the rationale of DOXO conjugation with L-HSA, drug concentrations in the heart and intestine were lower in rats injected with L-HSA coupled DOXO compared to animals given the same dose of F-DOXO (Fig. 3 and Table 2). As expected, the concentrations of DOXO in liver were higher in rats injected with L-HSA–DOXO. In rats injected i.v. with a conjugate prepared with rat albumin (L-RSA–DOXO), the levels of DOXO in tumors and organs were similar to those measured in animals administered with L-HSA–DOXO [13], indicating that, with respect to cell penetration, the conjugate prepared with a homologous albumin did not compete with rat plasma albumin.

TABLE 1

$AUC_{(0–24\ hours)}$ of DOXO concentrations in WD, MD and PD HCCs in rats injected with the same dose (1 μg/g) of F-DOXO or L-HSA coupled DOXO

Compound	nmol h/g tissue		
	WD HCCs	MD HCCs	PD HCCs
F-DOXO	42.2	52.9	43.9
L-HSA–DOXO	132.6	98.5	84.7

AUCs were calculated from the data of Fig. 2, using the software GraphPad Prism 3.02. From Di Stefano *et al.* [13] with permission from Blackwell, Munksgard.

L-RSA–DOXO was also used to verify whether a conjugate prepared with homologous albumin induced antibodies in rats. Ten animals received four weekly i.v. injections of the conjugate (1 μg/g coupled DOXO per single injection; the schedule successfully used in the study of anticancer efficacy [30]). They were bled one week after the last administration. No antibodies binding the conjugate (IgG class) were detected by ELISA (unpublished). This result is in agreement with the finding that in mice [31] and in humans [11], the conjugate of adenine arabinoside with lactosaminated homologous albumin, injected i.v., did not induce the production of antibodies.

Effect of L-HSA–DOXO on hepatic function in rats with liver fibrosis/cirrhosis

The high drug concentrations of DOXO in liver of rats injected with the conjugate raises the possibility that hepatic damage could occur during clinical use, particularly because the majority of

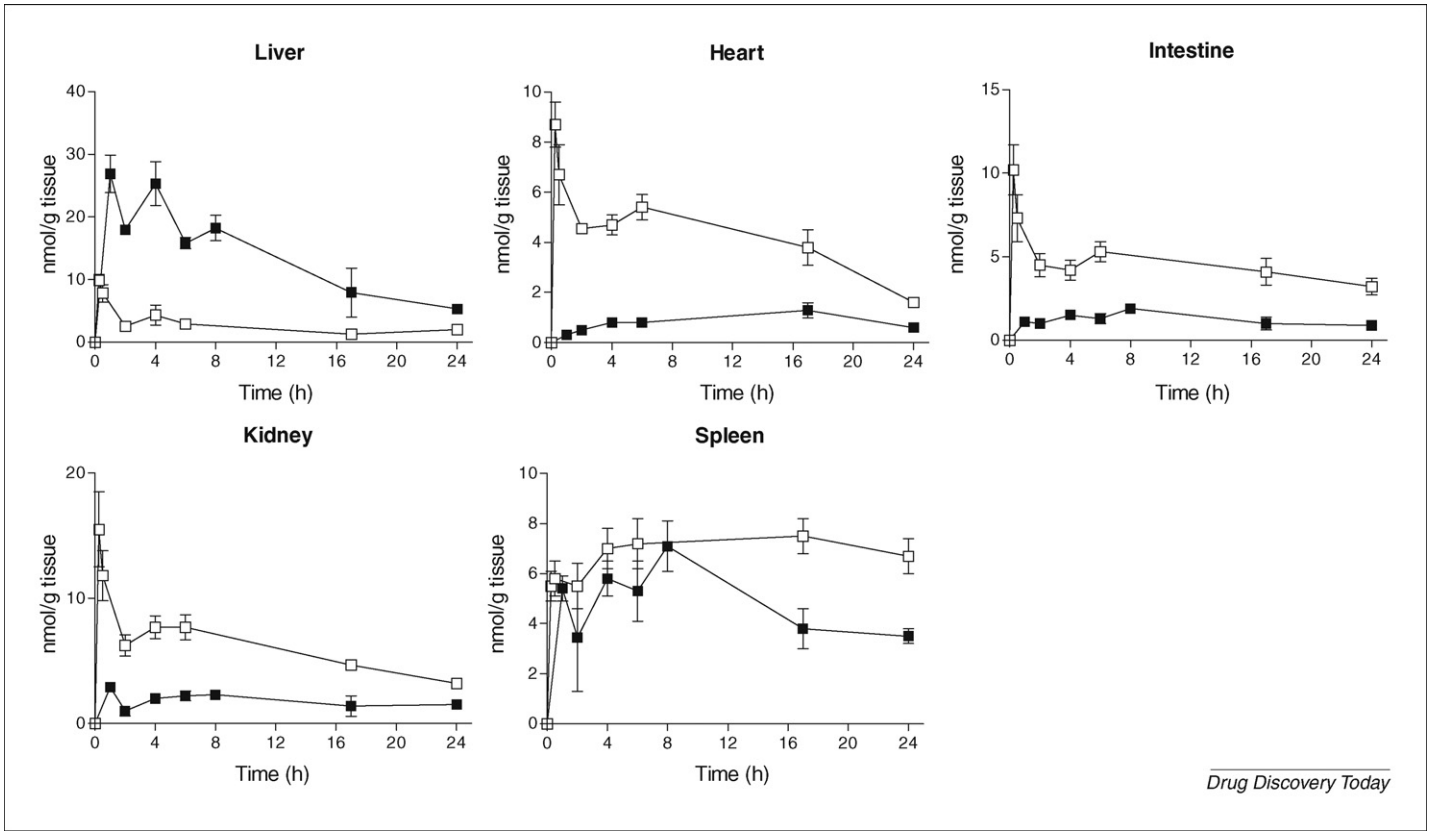

FIGURE 3

DOXO concentrations in organs of rats after i.v. injection of 1 μg/g of F-DOXO (□) or L-HSA coupled DOXO (■). Data were obtained from the same animals used in the experiments outlined in Fig. 2. The differences between the values of DOXO concentrations measured in both F-DOXO and coupled DOXO-treated animals at the same time intervals (2, 4, 6, 17 and 24 hours) were evaluated by two-way ANOVA (see legend of Fig. 2). In liver, heart, intestine and kidney the differences were statistically significant ($P < 0.0001$). No statistical difference was found in spleen. From Di Stefano *et al.* [13] with permission from Blackwell, Munksgard.

HCCs in Western Countries arise in cirrhotic livers. This issue was addressed by studying the effect of administration of L-HSA coupled DOXO given by four weekly i.v. injections at 1 μg/g on serum parameters of liver function and viability in normal rats [32], in rats whose livers were regenerating following partial hepatectomy [33] and in rats with fibrosis/cirrhosis induced by carbon tetrachloride (CCl_4) [32] or by DENA [34]. In normal rats L-HSA–DOXO did not modify any serum liver parameter. L-HSA coupled DOXO given (2 μg/g) to partially hepatectomized rats neither impaired the viability of regenerating hepatocytes nor produced changes in their ultra-structure and caused only moderate delay of hepatic DNA recovery. Administration of L-HSA–DOXO to rats with fibrosis/cirrhosis induced by CCl_4 during the last month of CCl_4 poisoning caused only moderate increases in GOT and GPT serum levels that were not statistically different from those produced by F-DOXO [32]. F-DOXO produced a decrease in albumin concentration, which was probably owing to kidney damage and proteinuria caused by DOXO in rats [35]. Its absence in animals treated with L-HSA–DOXO can be explained by the lower drug concentrations raised in kidney by the conjugate (Table 2). Most importantly, L-HSA–DOXO did not cause the decrease in the number of peripheral leucocytes which was caused by F-DOXO [32]. In the fibrotic livers of rats treated with DENA and bearing HCCs, neither L-HSA–DOXO nor F-DOXO affected the levels of bilirubin, GOT and GPT. Compared to values in saline injected rats, F-DOXO produced a decrease in albumin concentration, whereas L-HSA–DOXO caused an increase in alkaline phosphatase (ALP) levels which was not due to cholestasis, as indicated by the levels of gamma-glutamyl transferase, which were not modified [34]. It was probably caused by the carrier L-HSA because it was also observed in patients with chronic type B hepatitis receiving the L-HSA conjugate with adenine arabinoside and in Cynomolgus monkeys treated with nonconjugated L-HSA [11].

TABLE 2

$AUC_{(0–24\ hours)}$ of DOXO concentrations in rat organs after the administration of the same dose (1 μg/g) of F-DOXO or L-HSA coupled DOXO

Compound	nmol h/g tissue				
	Liver	**Heart**	**Intestine**	**Kidney**	**Spleen**
F-DOXO	60.5	100.0	109.0	145.6	169.4
L-HSA–DOXO	360.0	28.8	33.2	48.3	119.6

AUCs were calculated from the data of Fig. 3, using the software GraphPad Prism 3.02. From Di Stefano *et al.* [13] with permission from Blackwell, Munksgard.

Role of lactose residues in the safety of L-HSA–DOXO

A DOXO conjugate with nonlactosaminated albumin (HSA–DOXO) was injected in rats bearing HCCs (1 μg DOXO/g) and the concentrations of DOXO in tumors and in organs were measured [13]. The conjugate produced drug concentrations in HCCs similar to those raised by the conjugate prepared with L-HSA;

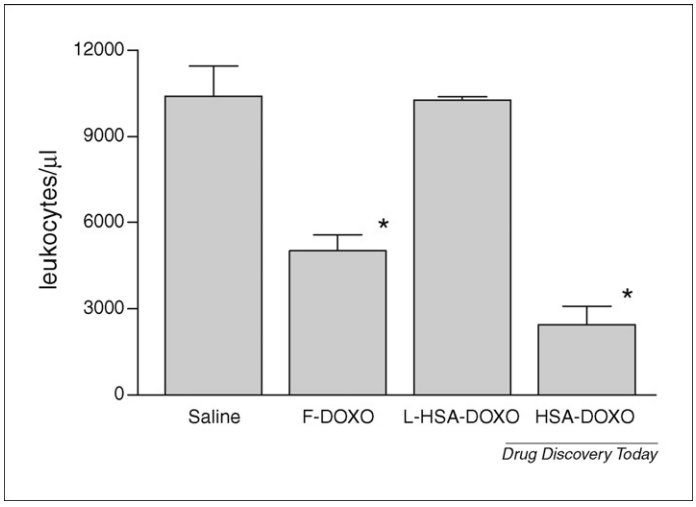

FIGURE 4

Number of peripheral leukocytes of rats, counted after i.v. administration of free DOXO, HSA–DOXO or L-HSA–DOXO. Compounds were given for three consecutive days at the single dose of 1 μg DOXO/g. One week after the last administration, rats were bled from the vena cava and sacrificed under isoflurane anesthesia. Each entry is the mean value ± SE from four animals. *Statistically significant difference compared to saline and L-HSA–DOXO groups. From Di Stefano *et al.* [13] with permission from Blackwell, Munksgard.

however, in spleen and bone marrow it caused DOXO levels six times higher than those produced by L-HSA–DOXO. The high drug concentrations were the consequence of an enhanced uptake of the conjugate by these organs as ascertained using a radioactive complex [13]. HSA–DOXO caused leucopenia, which was not produced by L-HSA–DOXO (Fig. 4). Leucopenia was also caused by F-DOXO, although its concentrations in bone marrow were not much higher than those measured in L-HSA–DOXO-treated animals [13]. An explanation might be that F-DOXO uniformly entered all bone marrow cells, including leukocyte precursors, whereas coupled DOXO may have been taken up predominantly by cells of the reticulo-endothelial system. Because L-HSA–DOXO disappears from bloodstream more quickly than the nonlactosaminated conjugate, it was postulated that the lower drug concentrations in spleen and bone marrow in rats injected with L-HSA–DOXO were a consequence of shorter exposure of these organs to this conjugate or owing to hindered uptake by spleen and bone marrow cells caused by the coupled lactose molecules. In rats and mice, the plasma concentrations of L-HSA–DOXO at one hour were maintained at higher levels than those of HSA–DOXO by injecting larger quantities of the former conjugate (unpublished) or by simultaneous administration of a tenfold higher amount of the carrier L-HSA [36], which competes with L-HSA–DOXO in the hepatic internalization through the ASGP-R. In spite of the higher plasma concentrations of L-HSA–DOXO, the DOXO levels in spleen and bone marrow were lower in animals injected with this conjugate. DOXO accumulation produced by HSA–DOXO in bone marrow, a target organ of the drug toxicity, discourages clinical studies using this conjugate. The mechanism by which lactose residues hinder the accumulation of L-HSA–DOXO in spleen and bone marrow has not yet been elucidated. Possibly, in producing this effect, lactose residues could be substituted by other molecules. However, lactose molecules also display the advantageous effect of enhancing conjugate penetration in WD HCCs through the ASGP-R (see above) and L-HSA has been shown to be a safe and efficient carrier in humans [11,12].

Anticancer efficacy of L-HSA–DOXO on rat HCCs

A first estimate of the anticancer activity of L-HSA–DOXO was obtained by studying its effect on development of HCCs from rat preneoplastic liver lesions [30]. One week after the last day of DENA administration, when established tumors had not yet formed, rats were injected with saline (NaCl 0.9%; i.v.) or F-DOXO (1 μg/g) or L-HSA coupled DOXO (1 μg/g). Animals received four weekly injections. One week after the last administration, rats were killed, livers removed and the lobes were separated. Tumor nodules on the surface of nonfixed liver lobes as well as on digital enlargements of the photos of formalin-fixed lobes were counted. In rats treated with L-HSA–DOXO the number of HCC nodules was significantly lower compared to those counted in animals administered with saline or with F-DOXO, which did not exert any anticancer activity (Fig. 5). Coupled DOXO did not decrease body weight, which, on the contrary, was markedly reduced by the free drug.

Subsequently, investigations were designed to determine whether L-HSA–DOXO could also inhibit the growth of established rat HCCs [34]. To address this, it was necessary to establish protocols for the detection and longitudinal tracking of liver tumors in living animals. On the basis of the work of Graham *et al.* [37], who studied the progression of liver metastases in mice using high frequency ultrasound, their imaging modality was adopted to detect individual rat HCCs and to evaluate changes in their size over time (Fig. 6). One month after the last day of

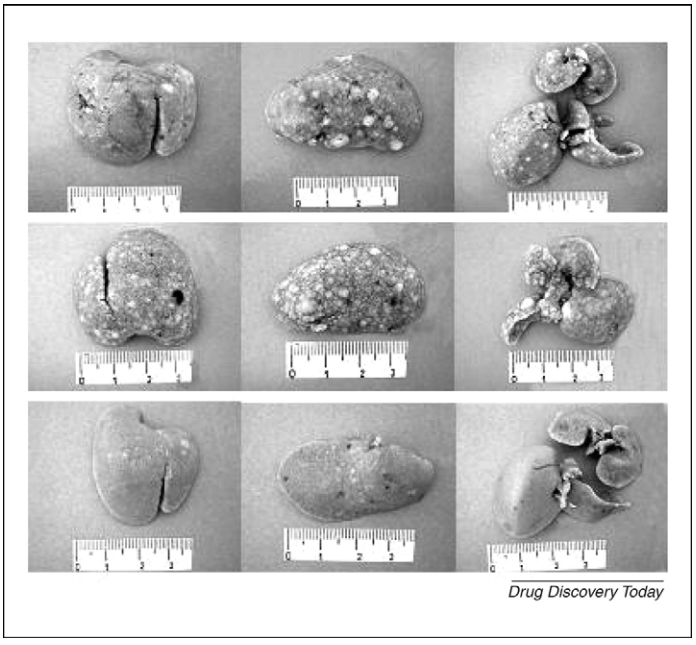

FIGURE 5

DENA-treated rats received four weekly i.v. injections of NaCl 0.9%, F-DOXO (1 μg/g) or L-HSA coupled DOXO (1 μg/g). Animals were killed one week after the last administration. The figure shows the macroscopic views of formalin-fixed liver lobes from one representative rat in the saline, F-DOXO and L-HSA–DOXO groups (upper, medium and lower set of photos, respectively). From Fiume *et al.* [30] with permission from Elsevier.

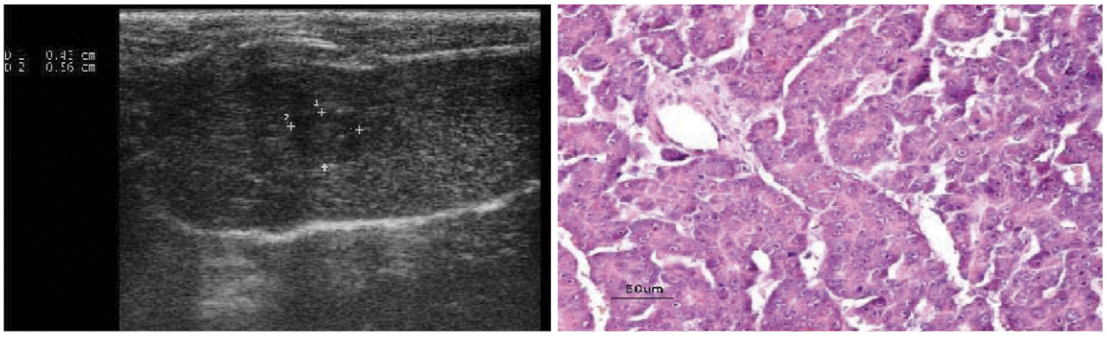

FIGURE 6

To validate ultrasound imaging as a modality to detect rat HCCs, nodules from eight rats, localized by a proper needle inserted in the liver under echographic observation, were removed, fixed and were stained. Microscopic examination confirmed that all the nodules were HCCs. The figure shows a hypoechoic nodule (left panel) that from microscopic examination (right panel) was classified as a moderately differentiated HCC (MD HCC). From Di Stefano *et al.* [34] with permission from Elsevier.

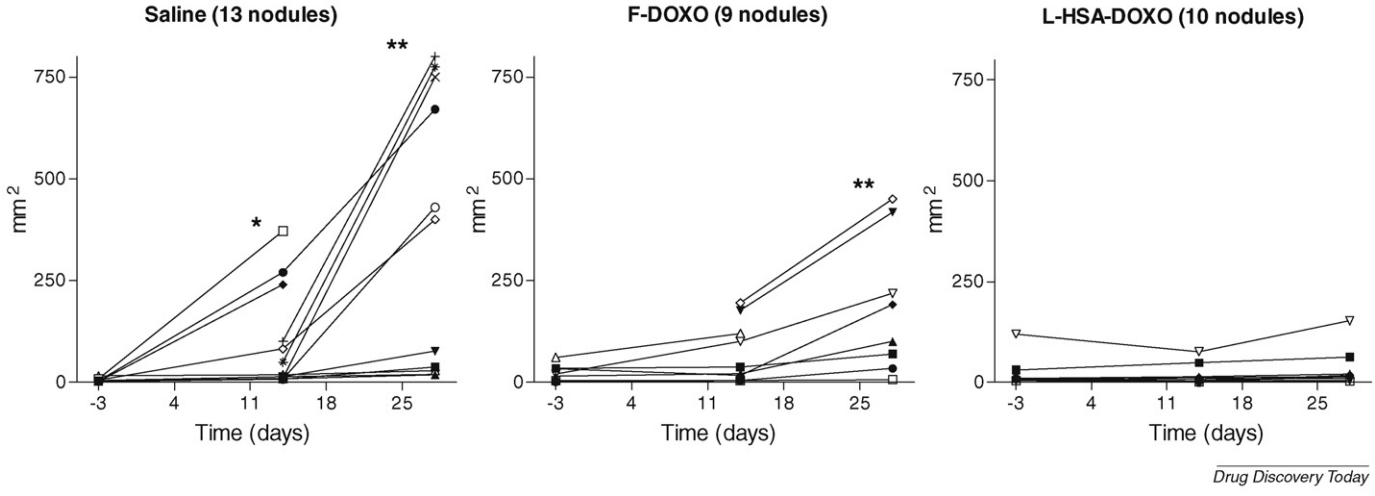

FIGURE 7

Rats with DENA induce HCCs received two weekly i.v. injections of NaCl 0.9%, F-DOXO (1 μg/g) or L-HSA coupled DOXO (1 μg/g) for four weeks. Growth of individual HCCs tracked on successive imaging sessions. Data were evaluated by paired *t*-test. *and **indicate statistically significant differences with $P < 0.05$ and 0.01, respectively, from the values of the previous imaging session. From Di Stefano *et al.* [34] with permission from Elsevier.

DENA administration, when tumors were established, rats received two weekly injections (i.v.) of saline or F- or L-HSA coupled DOXO (1 μg/g) for four consecutive weeks. Following the tumors in three imaging sessions, it was observed that L-HSA–DOXO not only hindered the development of new liver lesions, but also had the ability to inhibit the growth of already established tumor nodules (Fig. 7). By contrast, F-DOXO exerted an effect on tumor growth only in the first period of administration and did not impede the appearance of new lesions. F-DOXO caused a marked loss of body weight, which was not observed in rats treated with L-HSA–DOXO. The improved anticancer activity of L-HSA–DOXO was presumably because of the higher drug concentrations raised in HCCs. Conversely, its reduced toxicity could be explained by the lower drug levels achieved in rat extra-hepatic tissues.

Future directions

Penetration of L-HSA–DOXO in HCCs is mainly mediated by adsorptive interaction of the coupled DOXO molecules with the surface of neoplastic hepatocytes (see above). The finding that L-HSA–DOXO also binds in high quantities to the cell lines derived from tumors different from HCCs [27] suggests that studying the activity of L-HSA–DOXO on malignancies other than HCCs may well be warranted. For L-HSA–DOXO to be active requires that a tumor has sufficient vascular permeability to permit the large conjugate molecule to come in contact with the neoplastic cells. Because results have shown a particularly enhanced vascular permeability in cancers growing in liver [38], future research should study the efficacy of L-HSA–DOXO on experimental hepatic metastases.

Conclusion

L-HSA–DOXO appears to have definitive advantages over other vectors of DOXO, prepared to improve the systemic chemotherapy of HCCs. One vector, addressing liver tumors expressing ASGP-R, was obtained by coupling the drug to galactosylated pHPMA by a peptide bond with a tetrapeptidyl spacer [3]. The copolymers forming the backbone of this conjugate are largely nonbiodegradable [39]. Moreover, DOXO is scantly released from the tetrapeptidyl spacer of the pHPMA carriers inside the cells [23] (see above). Therefore, the cytotoxicity of these complexes is probably due to damage of the endosomal and lysosomal membranes caused by the drug in its coupled form [23] and, in the case of galactosylated pHPMA conjugate, is expected to be indiscriminately exerted on both normal and neoplastic hepatocytes. A second vector was prepared by encapsulating DOXO in polyethylene glycol-coated liposomes (PLD). The rationale for the use of PLD in the treatment of solid tumors is based on their ability to extravasate through 'leaky' tumor vasculature, resulting in a preferential localization of DOXO in tumor tissue [40]. However, experiments in rats demonstrated that PLD remain entrapped in the sinusoidal space of liver and only small quantities of DOXO can gain access to hepatocytes [41]. Accordingly, in a phase II clinical study, the response rate of patients with HCCs to PLD was not superior to that obtained with free DOXO [42]. In another approach [43], DOXO-loaded poly-isohexylcyanoacrylate (PIHCA) nanoparticles were administered to X/myc transgenic mice that develop liver dysplasia and subsequent lesions of WD HCCs. PIHCA–DOXO exerted an antitumor activity higher than F-DOXO; however, this nanoparticle formulation has an important drawback, because it increases the myelotoxicity of DOXO in mice [44].

L-HSA–DOXO increased both the anticancer efficacy and the safety of DOXO in rats bearing HCCs. Assuming that the results obtained in rats can be applied to patients, L-HSA–DOXO could enhance drug concentrations in human HCCs by two additive mechanisms: (1) by producing DOXO levels in tumors higher than those caused by an equal dose of the unconjugated drug and (2) by decreasing the DOXO concentrations in the target organs of its toxicity, thus allowing administration of higher doses of drug. The higher levels of drug achieved in the tumors should make L-HSA–DOXO an effective anticancer agent for systemic chemotherapy of HCCs that cannot be cured by surgery. The conjugate might also be given to prevent recurrent HCC after surgery and to hinder tumor progression in patients waiting for liver transplantation. Finally, for its better efficacy and tolerability, L-HSA–DOXO could successfully substitute uncoupled DOXO in combination with other drugs active in the systemic treatment of HCCs [45].

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