Defined lipid analogues induce transient channels to facilitate drug-membrane traversal and circumvent cancer therapy resistance

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Design and efficacy of bioactive drugs is restricted by their (in)ability to traverse cellular membranes. Therapy resistance, a major cause of ineffective cancer treatment, is frequently due to suboptimal intracellular accumulation of the drug. We report a molecular mechanism that promotes trans-membrane movement of a stereotypical, widely used anti-cancer agent to counteract resistance. Well-defined lipid analogues adapt to the amphiphilic drug doxorubicin, when co-inserted into the cell membrane, and assemble a transient channel that rapidly facilitates the translocation of the drug onto the intracellular membrane leaflet. Molecular dynamic simulations unveiled the structure and dynamics of membrane channel assembly. We demonstrate that this principle successfully addresses multi-drug resistance of genetically engineered mouse breast cancer models. Our results illuminate the role of the plasma membrane in restricting the efficacy of established therapies and drug resistance - and provide a mechanism to overcome ineffectiveness of existing and candidate drugs.

The main barrier for a drug to diffuse over the body and enter its target cell is the plasma membrane. This barrier, enriched in sphingolipids and sterols, forms a dense hydrophobic sheet, almost impermeable for hydrophilic compounds. In drug design, therefore, amphiphility is an essential molecular requirement (Lipinski’s rules). Consequently, the number of potential drug candidates from the vast chemical space is greatly restricted, and the efficacy of existing drugs is limited. Indeed, poor accumulation of cytotoxic drugs in tumour cells is a major limitation in cancer therapy, contributing to chemotherapy resistance.

Therefore, we questioned whether the membrane barrier function can be modulated in order to specifically enhance drug traversal and increase its therapeutic window. As this approach should be systemically applicable, we rationalized that the underlying mechanism requires concerted action with the drug molecule (instead of permeabilization for any compound). Doxorubicin is a stereotypical amphiphilic compound, whose membrane traversal is among the best characterized: the molecule’s hydrophobic anthraquinone inserts into lipid bilayers spontaneously. Membrane translocation (flip-flop) of its hydrophilic daunosamine sugar, however, is slow and energetically unfavorable. In the clinic, doxorubicin is widely used as an anti-cancer agent, but in various situations optimal efficacy is lacking. A long-circulating formulation, 100-nanometer pegylated liposomes (Doxil/Caelyx®), was developed to counteract the rapid plasma clearance of the free drug. The liposomal entrapment significantly reduced toxicity (e.g. life-threatening cardiac failure), however failed to improve efficacy.

The short-chain sphingolipid analogues N-hexanoyl-sphingomyelin and N-octanoyl-glucosylceramide (GC) strongly enhance the intracellular accumulation of doxorubicin, which we found not to be due to trivial phenomena, such as non-specific detergent-like membrane fluidization; however, any underlying mechanism remained obscure.
Here, we develop a paradigm of facilitated drug-membrane translocation based on defined lipid analogues; the mechanism is elucidated at molecular detail. Moreover, plasma membrane targeting, by systemic co-administration of GC, widens the therapeutic window of doxorubicin and overcomes multi-drug resistance.

**Results**

Membrane traversal of doxorubicin is facilitated by defined truncated phospho- and glycolipids. We previously demonstrated that GC acts at the level of the plasma membrane to enhance doxorubicin accumulation in the cell, but not by inhibition of ATP-dependent drug efflux pumps. We first questioned whether GC acts entirely independent on membrane proteins. To that end, we developed a doxorubicin translocation assay using model membranes of well-defined lipid compositions in absence of proteins (Fig. S1A–D). In liquid-disordered palmitoyl oleoyl phosphatidylcholine (POPC)/cholesterol (CH) membranes, incorporation of GC significantly decreased doxorubicin translocation half-time ($p < 0.05$) (Fig. 1A). The effect of GC was even more pronounced in entirely liquid-ordered di-palmitoyl phosphatidylcholine (DPPC)/sphingomyelin (SM)/CH membranes, which by themselves are most impermeable for doxorubicin (Fig. 1A). In a POPC/CH/SM composition, where the liquid-disordered phase co-exists with liquid-ordered (rigid) domains, like in plasma membranes, the overall effect of GC was intermediate (Fig. S1E). These data show that lipid organization of the membrane, independent of proteins, determines the degree of GC-enhanced doxorubicin translocation.

We next determined the molecular requirements of the lipid analogues to facilitate membrane traversal of doxorubicin. Using BAEC cells, we first compared the effect of GC with that of other short-chain (phospho) lipids. While di-myristoyl-phosphatidylcholine (PC) only minimally affected the intracellular accumulation of doxorubicin over 1 hour, di-nonanoyl-PC substantially elevated this accumulation in a dose-dependent manner, similar to GC (Fig. 1B).

We next varied in a series of sphingo- and glycerophospholipids the total aliphatic chain volume (i.e. the two acyl chains in PC, or acyl plus sphingosine hydrocarbon chain in sphingolipids) and the headgroup size, thus ranging from a strongly conical to an entirely cylindrical molecular geometry (see for definition of these geometries ref. 12). Fig. 1C shows that 25–27 carbons were optimal for

Figure 1 | Short-chain glycero- and sphingolipids facilitate doxorubicin-membrane traversal, depending on (A) membrane biophysical environment, (B, C) total acyl chain length, and (C) lipid headgroup size. (A). Doxorubicin translocation over liquid-ordered (DPPC:SM:Chol, 2:2:1 molar ratio) or liquid-disordered (POPC:Chol, 7:3 molar ratio) DNA-enclosed large unilamellar vesicles was determined in a fluorescence quenching assay (see Supplemental). GC significantly reduced translocation half-time ($K_{\text{translocation}}$ (in seconds; SEM, $n = 3$; $p < 0.05$). (B). Confluent BAEC cells were pre-incubated for 15 min with short-chain sphingolipid (GC) or phosphatidylcholines (PC 9:0/9:0 and PC 14:0/14:0), and doxorubicin was applied for 60 min. Intracellular doxorubicin accumulation was quantified (% of control, no lipid added) (SD, $n = 5$). (C). Doxorubicin accumulation in BAEC cells plotted against the total hydrocarbon chain length (solid lines) at constant headgroup size (180 Da, phosphocholine or glucose); or against headgroup size at constant total chain length of 24–26 carbons (dashed line), using C6-Cer (17 Da; OH-group), C8-Cer-1-P (95 Da), C6-SM (184 Da), C8-LacCer (342 Da) (headgroup size in brackets); (mean, SD, $n = 5$).
sphingolipids, whereas around 22 carbons appeared most effective in case of glycerophospholipids. The shift of the optimum peak is well explained by the 3 carbons in the glycerol backbone of the glycerophospholipids.

With respect to the hydrophilic head group, at a constant total sphingolipid chain length of 24–26 C atoms, a minimum size of 180 Da was required for optimal doxorubicin accumulation (Fig. 1C, Table S1). Together, our findings indicate that analogues of natural lipids as defined by molecular geometry, are capable of enhancing doxorubicin traversal over preferentially the liquid-ordered lipid (raft) constituent of the plasma membrane.

**Short-chain lipids assemble a transient membrane channel, facilitating doxorubicin traversal.** The mechanism behind facilitated doxorubicin traversal by short-chain lipids, and the contribution of the drug molecule in silico using molecular dynamics (MD) simulations. This technique provides detailed insight in the behavior and interaction of individual molecules in lipid bilayers. Lipid bilayers were modeled at atomic detail in the presence or absence of 20% short-chain di-octanoyl-phosphatidylcholine (PC 8:0/8:0; DOctPC). In line with experimental data, doxorubicin insertion and translocation over the membrane caused disordering of the local lipid and acyl chain organization (Fig. 2A, Movie S1). The presence of DOctPC caused a visible broadening and stabilization of such membrane perturbation, assembling a transient lipid channel for doxorubicin traversal. This DOctPC-mediated gateway afforded substantial solvation of doxorubicin (its daunosamine sugar in particular) within the lipid bilayer core, which involved lipid headgroups lining the pore’s lumen and water molecules remaining associated with the sugar moiety (Fig. 2B, Movie S1).

Free energy landscapes were obtained for the translocation of doxorubicin across the lipid bilayers (Fig. 2C). Binding from the bulk phase (>3.0 nm distance to the center of the lipid bilayer) to the membrane surface (1.5–2.5 nm) is energy-favorable, by more than −25 kJ/mol, and indifferent for the presence of the short-chain lipids. Translocation of doxorubicin across the bilayer center (0.0 nm), on the other hand, surmounts an energy barrier of 45 kJ/mol for pure DPPC bilayers. This net energy barrier of 20 kJ/mol is in good agreement with reported data. The presence of the short-chain lipids significantly reduced the net energetic barrier almost two-fold, by 7.8 kJ/mol (Fig. 2C).

We next determined the distribution of the DOctPC in the membrane in relation to the position of doxorubicin. When doxorubicin localized at the water-bilayer interface (membrane surface), no...
preferred association with the short-chain lipids was observed (Fig. 2D, lower panel). In contrast, when doxorubicin positioned in the bilayer hydrophobic core, the short-chain lipids self-assemble in its vicinity, adapting to doxorubicin (Fig. 2D, upper panel). The notion that doxorubicin triggered channel formation was supported experimentally, as short-chain lipids without doxorubicin did not affect membrane permeability and GC instead significantly stabilized the membrane in an erythrocyte lysis assay (Fig. S2).

To further elucidate the self-assembling behavior of the short-chain lipids, the space occupancy of the different molecules was averaged over a time period of 120 ns, as shown in Fig. 2E (see also Movie S2). Short-chain lipids preferentially twisted around the doxorubicin molecule and localized at the curvature of the channel. The data show that self-assembly of short-chain lipids, triggered by doxorubicin’s infringement of normal lipid order, forms a transient channel for the doxorubicin to traverse the membrane.

**GC improves doxorubicin therapy and overcomes multi-drug resistance in a heterogeneous GEM mammary tumour population.** As the above-described mechanism of membrane channel formation is unrelated to drug resistance mechanisms involving transporter proteins, we predicted that the lipid channels would thus be able to counteract unresolved conditions of multi-drug resistance. The WAPCre:Ecad<sup>−/−</sup>:p53<sup>−/−</sup> (WEP) mammary tumour model is a genetically engineered mouse (GEM) model that closely mimics human invasive lobular breast carcinoma (ILC)<sup>15,16</sup>. To characterize the drug sensitivity of the Ecad<sup>−/−</sup>:p53<sup>−/−</sup> mammary tumours, we set up a mouse clinical trial by transplanting series of individual WEP tumours into syngeneic recipient female mice. Thus, a heterogeneous population of mammary tumour-bearing mice was established, albeit within the limits of a defined, clinically relevant breast cancer subtype (ILC). Mice with tumours of 200 mm<sup>3</sup> were selected for treatment at maximum tolerated dose and randomized against untreated controls. Conventional chemotherapeutics (doxorubicin, docetaxel, topotecan, cisplatin) were administered as well as the poly(ADP-Ribose) Polymerase (PARP) inhibitor olaparib.

The large variations (SEM), already evident from the control group, reflect the inter-individual heterogeneity characteristic for the set-up (Fig. 3). Although some of the mice responded to cisplatin therapy, overall these tumours appeared resistant towards chemotherapy, and to (free) doxorubicin in particular (Fig. 3).

We next investigated whether the induction of transient membrane channels by GC would sensitize to doxorubicin therapy. As GC in its free form is not water soluble, GC was co-formulated with liposomal doxorubicin (LDox GC), without affecting the main pharmacological parameters of the carrier<sup>17</sup>. Due to the outer PEG shielding, the liposomes do not directly interact or fuse with cells. However, GC transfers from the liposomes via the aqueous phase into the cell membrane (refs. 18, Fig. S3), thus remaining available to the tumour cell membrane. Cell lines were derived from untreated WEP Ecad<sup>−/−</sup>:p53<sup>−/−</sup> mammary tumours. Pre-incubation of these WEP cell cultures with free GC elevated intracellular doxorubicin accumulation in a GC dose-dependent fashion (Fig. 4A). We subsequently tested liposomal doxorubicin with and without 10 mol% GC incorporated into its liposome bilayer on the WEP cells. LDox was of the same composition as commercial Caelyx<sup>®</sup> (Doxil<sup>®</sup>) used in the clinic. In line with its clinical features, LDox poorly delivered its contents into the WEP cells, even after 24 hours incubation. In contrast, LDox GC established substantial intracellular doxorubicin levels, an 8-fold increase over conventional LDox (Fig. 4B, C), and the increased intracellular doxorubicin accumulation correlated with improved cytotoxicity (Fig. 4D). As a control, empty liposomes of the same lipid composition but without doxorubicin showed no cytotoxicity at all in the same concentration range for either formulation (data not shown). Also in other WEP cell lines a similar gain of cytotoxicity by LDox GC was observed, up to EC50 values obtained by free doxorubicin (Table S2).

After establishing efficacy in the WEP cultures in vitro, we assessed therapy response in the GEM model, using the clinical trial set-up, as described above. The clinically used doxorubicin formulations free doxorubicin (fDox) and conventional liposomal doxorubicin (LDox) were administered at MTD, LDox GC was applied at equidose to LDox (10 mg/kg), while untreated tumours served as control. As depicted in Fig. 5A, LDox (without GC) generated an initial anti-tumour effect, more so than free doxorubicin (fDox), but tumours finally progressed despite additional dosing in either group. However, when GC is present (LDox GC; 10 mg/kg) a sustained inhibition of tumour progression was observed. At day 21 of treatment in the LDox GC group, tumour size was significantly reduced over LDox (p < 0.05), by two-fold (Fig. 5A).

We subsequently analyzed the overall survival of the different treatment cohorts. The clinically used doxorubicin formulations (LDox and fDox) did not result in a significantly extended survival over the control group. Importantly, LDox GC was the only intervention that prolonged overall survival significantly over untreated controls (p = 0.001). Median survival extended from 11 days to 30 days (Fig. 5B). A separate study in a homogeneous tumour cohort, established from a single WEP donor tumour (CDB212) randomly selected from the population a priori, further underlined these results (Fig. S4).

After study exit, all major organs were collected and the samples were blinded for investigation by veterinary pathologists. The LDox GC group revealed minimal normal tissue effects; no additional toxicities were discernable as compared to the other doxorubicin formulations. In all groups, the most prevalent anomaly was depleted erythropoiesis, (Fig. 5C, right panels) in bone marrow (upper panels) and spleen (lower panels), as compared to a healthy tumour-free (FVB × 129/Ola) F1 mouse (left panels). In addition to tumour-induced anemia, enforcement of this pathology by doxorubicin is a well-known adverse effect, also in the human situation<sup>19,20</sup>. Notably, for LDox GC this normal tissue effect was less frequent, as compared to the LDox treated group (Fig. 5D). In the LDox GC cohort, the incidence of reduced erythropoiesis appeared not significantly elevated, compared to untreated controls (Chi-square test: p = 0.41), in contrast to the LDox treatment (Chi-square test: p = 0.036). Overall, we conclude that GC counteracts chemotherapy resistance in a

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**Figure 3 | Ecad<sup>−/−</sup>:p53<sup>−/−</sup> GEM mammary tumours are multi-drug resistant.** After orthotopic transplantation of a population of spontaneous tumours from WAPCre:EcadF/F:Fip53F/F mice, treatment was started with the indicated cytostatic drugs when tumour size reached 200 mm<sup>3</sup>, all at maximum tolerated dose (MTD); or mice were left untreated as controls. (Mean, SEM, n = 5).

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clinically relevant tumour model, greatly improving efficacy at reduced bone marrow and spleen toxicity.

**GC elevates intracellular doxorubicin accumulation in the GEM mammary tumour, while reducing normal tissue exposure.** In order to further elucidate the observed reduced toxicity and to shed more light on the pharmacokinetics of LDox GC, doxorubicin plasma profiles were determined (CDB212 cohort). Evidently, the sustained circulation time of LDox was preserved upon GC co-formulation (Fig. 6A), while doxorubicin plasma levels steeply declined during the first two hours. Non-compartmental kinetic analysis showed that the distribution rate in this first phase increased 4-fold as compared to LDox without GC (Table S3), which mainly accounted for the reduced systemic doxorubicin levels. Overall, plasma AUC was reduced 3-fold \((p < 0.05)\) and residual circulating doxorubicin (96 hours after administration) was decreased 8-fold \((p < 0.05)\) by GC co-administration.

We investigated whether there was a preference of GC mediated drug gateways for the tumour cell membrane. Of spleen, heart and tumour we determined the fraction doxorubicin that finally passes the membrane, ending up inside the cell, over the above mentioned time courses. The effect of GC appeared strongest on the tumour cell membrane (Fig. S6A). We confirmed this observation in vitro on various cell cultures (Fig. S6B).

**Discussion**

Membrane translocation strongly delineates the compounds that, from the vast chemical space, can potentially act as a bioactive drug.
Moreover, it hampers efficacy of existing drugs. Poor intracellular accumulation accounts for situations of suboptimal or ineffective therapy, including therapy resistance. We here describe a well-defined paradigm for enhancing drug-membrane translocation and subsequent elevated intracellular accumulation. Amphiphilic drug molecules by themselves impose strong distortion upon the natural lipid assembly, when traversing the membrane. We demonstrate that during this process, the assembly of short-chain lipids is triggered around doxorubicin in a short time frame, much quicker than that of doxorubicin-membrane translocation. Such spatiotemporal enrichment of the truncated lipids in vicinity of the drug favors local membrane curvature and hence allows transient channel formation of a size just fitting for doxorubicin to translocate. GC thus on its own does not permeabilize the membrane, and instead acts in rapid concert with the drug. After acceleration of the translocation process, channel disassembly can occur as soon the drug has left the membrane to bind its intracellular target or further diffuse through the body.

Liquid-disordered and liquid-ordered phases coexist in plasma membranes dependent on cell type and conditions. The anti-cancer drug doxorubicin binds strongest to the liquid-ordered lipid phase of membranes. Strikingly, the doxorubicin uptake-enhancing effect of GC is most prominent in the liquid-ordered (lipid raft) environment, where spontaneous doxorubicin traversal is slowest. In all tumour cells tested thus far, but not in most normal cells, lipid analogues (such as GC) effectively facilitated doxorubicin traversal across the plasma membrane. Characteristically, the outer leaflet of the plasma membrane is indeed enriched in sphingolipids and sterols, which form the liquid-ordered phase. One could therefore hypothesize that tumour cells are particularly rich in liquid-ordered plasma membrane outer leaflets, when compared to cells of e.g. heart, spleen and liver, as lipid compositions vary significantly between tissues and cell types. However, systematic lipidomics information is lacking, and in particular at such subcellular detail. Advanced lipidomics analyses (e.g. by imaging mass spectrometry) are needed to further elucidate (subtle) differences among cell types, or those associated with malignant transformation.

Overall, rational design of lipid analogues, medicinal chemical lipid-analogue structure optimization, and systematic high-throughput screening are approaches that are suitable for identifying selected action on cell type and the drug combination of interest. For tumour cells, we are currently employing this strategy to improve efficacy of other anti-cancer drugs, such as mitoxantrone, which is clinically hampered by inadequate delivery and bioavailability.

Conventional therapy with free or liposomal doxorubicin has its limitations: Free doxorubicin shows life-threatening cardiotoxicity and is rapidly eliminated from the body. Conventional liposomal doxorubicin (Caelyx®) stays sufficiently longer in circulation but is hampered by poor intracellular delivery of the drug. A strategy of concomitant GC membrane targeting combines the advantages of...
these two clinical formulations (Fig. 7). While long-circulation is preserved by liposomal entrapment, GC establishes intracellular delivery into the tumour cells. Moreover, towards normal tissues doxorubicin exposure is unaffected or reduced, and plasma levels as well as erythropoeitic toxicity attenuate; resulting in an increase of the therapeutic ratio.

Liposomal encapsulation protects from life-threatening cardiotoxicity. As was evident from phase III clinical trials, this beneficial effect is rather independent of pharmacokinetics of the particular liposomal formulation9,33. In contrast, palmar-plantar erythrodysesthesia (PPE), the dose-limiting toxicity of current liposomal doxorubicin (Caelyx) therapy, strongly correlates to pharmacokinetics, in particular to long-term doxorubicin plasma level34, which was 8-fold reduced by the co-formulation of GC.

We used EcadΔ/Δ; p53Δ/Δ mouse mammary tumours that, like many other spontaneous GEM tumours, are therapy resistant and closely resemble the human counterpart15,16. Uptake of free doxorubicin by these tumours was extremely low. LDox GC may challenge therapy resistance of these tumours in three ways. First, the liposomes accumulate in the tumour over time, a phenomenon known as Enhanced Permeability and Retention23, thereby reaching substantially higher doxorubicin levels in tumour tissue than with free doxorubicin. Secondly, the short-chain lipid enhances the doxorubicin flux into the tumour cell from the interstitial fluid where the liposomes leak their content. Thirdly, the efficacy of GC may be enforced by the fact that multidrug-resistance (MDR) proteins (such as the ABCB1 and ABCC1 drug efflux transporters) are lipid exporters that expel short-chain GC (but not long-chain GC) efficiently to the outer bilayer leaflet35, once it has reached the intracellular side after co-flipping with doxorubicin. The short-chain lipid is thus continuously recycled back to the cell surface, ready to aid new doxorubicin partners in traversing the membrane. This study highlights a critical role of the cellular plasma membrane in cases of drug resistance.

In summary, drug-triggered molecular assembly of transient channels facilitates drug traversal over the plasma membrane. GC-mediated channels preferentially target the tumour cell membrane to strongly elevate intracellular doxorubicin accumulation. While reducing systemic exposure, LDox GC increases anti-tumour efficacy and overcomes multi-drug resistance.

**Methods**

Doxorubicin uptake. Serum-starved BAEC cells were pre-incubated with short-chain lipids from ethanol for 15 minutes after which free doxorubicin was applied for 1 hour. Intracellular doxorubicin was quantified and corrected for protein content.
Assay for doxorubicin translocation across lipid vesicles. By reversed-phase evaporation large unilamellar vesicles (LUVs) were prepared to enclose DNA. The DNA-LUVs were injected into a doxorubicin solution (50 μM) and doxorubicin fluorescence was monitored in a spectrofluorometer. The quenching of fluorescence (Q) over time was fitted according to $Q(t) = Q(0) + k(Translocation)$, where $k(Translocation)$ is the translocation half-time (sec). The maximal quenching ($Q(max)$) was determined after releasing all DNA by LUV dissolution.

Molecular dynamics of doxorubicin translocation. Simulations of doxorubicin translocation across a membrane containing 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or DPPC and 1,2-dioctanoyl-sn-glycerol-3-phosphocholine (DOCTPC) were carried out using GROMOS 4.54 package and 52a6 GROMOS’ united-atom force field. The center of mass of doxorubicin was positioned at different distances to the bilayer center, as indicated, using a harmonic restraint in the direction of the bilayer normal. Free energy profiles were obtained after integrating the force of the restraining potential at each depth.

GEM mammary tumour model and therapeutic intervention. Ecad -`p53` mammary tumours, generated in WAPcre/ExDox/ExFp53E/F (WEP) mice were orthotopically transplanted in syngeneic wild-type mice. The first drug dose was administered when the tumour reached 200 mm$^3$. The tumour volume (in mm$^3$) was calculated using the formula: $0.5 \times \text{length} \times \text{width}^2$. Treatments (MTD): Olaparib: daily 50 mg/kg i.p.; docetaxel: weekly 25 mg/kg i.v.; cisplatin: once every two weeks 6 mg/kg i.v.; topotecan daily 4 mg/kg i.p.; free doxorubicin: weekly 5 mg/kg i.v.; LDox (conv): weekly 10 mg/kg i.v.; LDox GC: weekly 10 mg/kg i.v. Mice were sacrificed when tumour volume exceeded 1500 mm$^3$ or body weight declined below 80% of its initial value. Sacrificed tissues, including mammary lymph nodes, lung, liver, heart, bone marrow, thymus, spleen, kidneys and GI-tract, were blinded, fixed and H&E stained followed by pathological investigation. All mouse experiments were performed in accordance with Dutch law and regulatory guidelines. The ‘Dier Experimentele Commissie’ of the NKI was involved in experimental design and approved all experiments prior to start study.

Plasma kinetics. From mice with the CDB212 tumour (350 mm$^3$) blood samples were drawn by tail-clipping and collected in heparinized tubes. Plasma was obtained by centrifugation at 10 min, 100 mm Hg) via the left ventricle of the heart. Heart, liver, spleen and tumour tissue were homogenized in ice-cold nuclear isolation medium. From half of the tissue were isolated by sucrose density centrifugation. Doxorubicin was quantified by HPLC and expressed per gram injected doxorubicin. Data were corrected for tissue weight and doxorubicin dose, and sample nuclei were isolated by sucrose density centrifugation. Doxorubicin was measured and doxorubicin was quantified by HPLC and expressed per gram injected doxorubicin. Data were corrected for tissue weight and doxorubicin dose, and if applicable, nuclear creatine yield yield.

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Doxorubicin incorporation in tissues and cell nuclei. Mice with orthotopic CDB212 tumours were i.v. injected with doxorubicin when primary tumour was 350 mm$^3$. At $t = 2, 4, 24$ and 96 hours (LDox GC and LDox) or at $t = 0, 1$ and 4 hours (LDox GC) the animals were sacrificed. Body perfusion was immediately performed (5 min, 100 mm Hg) via the left ventricle of the heart. Heart, liver, spleen and tumour tissue were assayed and H&E stained followed by pathological investigation. All mouse experiments were performed in accordance with Dutch law and regulatory guidelines. The ‘Dier Experimentele Commissie’ of the NKI was involved in experimental design and approved all experiments prior to start study.

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Author contributions
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Additional information
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