Controlled masking and targeted release of redox-cycling ortho-quinones via a C–C bond-cleaving 1,6-elimination

Lavinia Dunsmore1, Claudio D. Navo2,1, Julie Becher1,1,1, Enrique Gil de Montes1,1, Ana Guerreiro3,1, Emily Hoyt5, Libby Brown1,4, Viviane Zelenay4, Sigitas Mikutis1, Jonathan Cooper5, Isaia Barbieri6, Stefanie Lawrinowitz7, Elise Siouve4,8, Esther Martin4, Pedro R. Ruivo5, Tiago Rodrigues3, Filipa P. da Cruz1, Oliver Werz7, George Vassiliou5, Peter Ravn4,10, Gonzalo Jiménez-Osés2,9✉ and Gonçalo J. L. Bernardes1,3✉

Natural products that contain ortho-quinones show great potential as anticancer agents but have been largely discarded from clinical development because their redox-cycling behaviour results in general systemic toxicity. Here we report conjuction of ortho-quinones to a carrier, which simultaneously masks their underlying redox activity. C-benzylation at a quinone carbonyl forms a redox-inactive benzyl ketol. Upon a specific enzymatic trigger, an acid-promoted, self-immolative C–C bond-cleaving 1,6-elimination mechanism releases the redox-active hydroquinone inside cells. By using a 5-lipoxygenase modulator, β-lapachone, we created cathepsin-B-cleavable quinone prodrugs. We applied the strategy for intracellular release of β-lapachone upon antibody-mediated delivery. Conjugation of protected β-lapachone to Gem-IgG1 antibodies, which contain the variable region of gemtuzumab, results in homogeneous, systemically non-toxic and conditionally stable CD33+–specific antibody-drug conjugates with in vivo efficacy against a xenograft murine model of acute myeloid leukaemia. This protection strategy could allow the use of previously overlooked natural products as anticancer agents, thus extending the range of drugs available for next-generation targeted therapeutics.

The application of new chemical entities with innovative mechanisms of action is necessary for the development of next-generation therapeutics. Natural products are often a source of anticancer agents or act as an inspiration for rational molecular design. However, many natural products identified with medicinal value in vitro remain underexploited due to dose-limiting toxicity in vivo. Toxicity can stem from low cancer-cell specificity, off-target reactivity or, sometimes, built-in metabolic reactivity of functional groups. Such liabilities can be addressed with prodrug strategies that can mask pharmacophores to prevent secondary pharmacology or accelerated metabolism and can widen the therapeutic window. Drug targeting strategies can also be useful. For example, antibody-mediated delivery for cancer-cell discrimination has facilitated the use of auristatins, maytansinoids and calicheamicins. However, some medicinal compounds contain a reactive moiety that cannot be effectively protected by existing chemistry or prodrug strategies. This cannot be overcome, only highly functionalized molecules that contain amine or hydroxyl groups tend to be suitable for conjugation to carriers. Toxic molecules that do not contain such amenable functional groups are often discarded if a carrier cannot be attached without laborious synthetic derivatization. Therefore, improvements in protection of functionalities and attachment of drug carriers to alternative functional groups will extend treatment options with already-discovered compounds.

One underused family of cytotoxic natural products is the ortho-quinones. Several low-molecular-weight compounds that contain an ortho-quinone group, such as β-lapachone, tanshinones (I, IIA, IIB and crypto), mansonones A–G, dunnione, miltirone, salvicine and caryopteron A, display wide antiproliferative effects in vitro. For example, β-lapachone (1, Fig. 1a), a natural product from Brazilian lapacho tree bark, exhibits good efficacy against leukemias and NQO1+ cancers, such as breast, non-small-cell lung and pancreatic. However, untargeted ortho-quinones have dose-limiting toxicity and metabolic liabilities because of their NQO1-dependent redox-cycling behaviour that results in the formation of reactive oxygen species (ROS; Fig. 1a). ROS disrupt the function of proteins and can lead to irredeemable DNA oxidation, PARP1 hyperactivation and cell death. Although this mechanism is of use against cancer cells, systemic propagation of ROS is undesirable and may lead to anaemia and methaemoglobinemia, as observed in clinical trials of 1 (refs. 24,25). Additional ortho-quinone toxicity can result from their ability to react as electrophiles with critical cellular proteins, peptides, nucleic acids or glutathione, which interferes with redox homeostasis.

1 Yusuf Hamied Department of Chemistry, University of Cambridge, Cambridge, UK. 2 Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Derio-Bizkaia, Spain. 3 Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal. 4 Biologics Engineering, R&D, AstraZeneca, Cambridge, UK. 5 Wellcome-MRC Cambridge Stem Cell Institute, Department of Haematology, University of Cambridge, Cambridge, UK. 6 Division of Cellular and Molecular Pathology, Department of Pathology, University of Cambridge, Cambridge, UK. 7 Department of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich Schiller University Jena, Jena, Germany. 8 Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK. 9 Ikerbasque, Basque Foundation for Science, Bilbao, Spain. 10 Present address: Department of Biotherapeutic Discovery, H. Lundbeck A/S, Valby, Denmark. 11 These authors contributed equally: Claudio D. Navo, Julie Becher, Enrique Gil de Montes, Ana Guerreiro. ✉ e-mail: gjoses@cicbiogune.es; gb453@cam.ac.uk

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We hypothesized that if delivered to target cells without systemic side effects, ortho-quinones would have substantial potential as a monotherapy or in combination with mainstream anticancer agents\textsuperscript{12,13}. Their metabolic interference may be a potential avenue to tackle malignancies\textsuperscript{14,15}, and a strategy to overcome cancer drug resistance\textsuperscript{16}. Some tumour selectivity of 1 has been reported, as overexpression of oxidoreductase enzymes (for example, NQO1) can increase redox-cycling rates selectively in the tumour\textsuperscript{17}. However, no therapeutic ortho-quinone has yet reached the clinic due to generalized ROS-mediated systemic toxicity. In contrast, para-quinones, such as doxorubicin (Adriamycin), geldanamycin, mitoxantrone and mitomycin C, have lower redox-cycling rates and are successfully used in the clinic\textsuperscript{18}. If the side effects of ortho-quinones could be prevented through selective protection and targeting strategies, this may lead to new cancer treatment options.

Prodrug approaches to mask redox activity and limit side effects of ortho-quinones, such as 1, have been described. Boothman and co-workers developed a number of approaches, including formation of esterase-cleavable hydroquinone alkyl esters\textsuperscript{19,20}, and pH-sensitive aminobenzyl imine, acyl hydrazone, ketal\textsuperscript{21,22}, aminoalkyl alcohol and amino aromatic phenol prodrugs\textsuperscript{23}. However, the disadvantages of these strategies have prevented their widespread adoption. For example, esters and hydrazones are often too labile under physiological conditions for effective targeting, whereas ketals are not sufficiently labile in tumours\textsuperscript{24}. Modern prodrug strategies and linkers, including those that connect targeting antibodies and their payloads, use enzymatically activatable trigger groups and release drug functionality by means of self-immolative spacers. For example, para-aminobenzyl carbamate linkers release amines and para-aminobenzyl ether linkers release alcohols upon specific protease-triggered hydrolysis\textsuperscript{25}. We hypothesize that a similar strategy is possible for ortho-quinones.

Here we present a modular strategy that uses self-immolative benzyl linkers for protection and controlled release of ortho-quinones. The self-immolative 1,6-elimination of a para-aminobenzyl linker attached at the quinone carbon as a benzyl ketol enables release of the hydroquinone. The released hydroquinone then oxidizes spontaneously to give the desired quinone by using the redox-cycling ability of the payload (Fig. 1b). The strategy is compatible with peptide linkers, such as those activated by the cysteine protease cathespin B\textsuperscript{26}. In this work, we explore the mechanism and properties of this C–C bond-cleaving elimination and prospectively apply it to protect and target 1 as an antibody–drug conjugate (ADC) for treatment of acute myeloid leukaemia (AML).

**Synthesis of self-immolative quinone models**

Initially, the synthesis of model derivatives of quinones that fragment upon removal of a protecting group was investigated using 9,10-phenanthrenequinone (PhQ), 4, as a model compound (Fig. 2a). Phenolic alcohol-containing drugs are typically protected as para-aminobenzyl ethers\textsuperscript{27}. However, when we investigated reductive alkylations of 4 with Boc-protected para-aminobenzyl bromide linker 5 (Fig. 2a), with a view to forming O-benzyl derivatives, we instead obtained the C-benzyl derivative Boc-para-aminobenzyl phenanthrene-ketol (Boc-PAB-PhQ), 6, in good yield (60–70%). Indeed, O-benzyl derivatives of hydroquinones have been reported to be unstable and to rearrange to their corresponding C-benzyl isomers\textsuperscript{28–30}. Intrigued by 6, we tested its stability following N-Boc deprotection to give para-aminobenzyl phenanthrene-ketol (PAB-PhQ), 7, which to our surprise appeared to be unstable, and as it was consumed, formation of quinone 4 was observed (Fig. 2b and Supplementary Figs. 1–6).

Complete consumption of 7 and formation of 4 was confirmed by \(^1\)H NMR spectroscopy. Appearance of 4 proceeded with a half-life \((t_{1/2})\) of 2.94 ± 0.08 h at 25 °C in a moderately acidic methanolic solution with complete release within 20 h. Data fitting suggested that release followed first-order kinetics and the rate of consumption of 7 \((k_{obs} = 6.18 ± 0.12 \times 10^{-3} \text{ s}^{-1})\) was consistent with the rate of formation of product 4 \((k_{obs} = 6.55 ± 0.22 \times 10^{-3} \text{ s}^{-1}; \; \text{Fig. 2c and Supplementary Figs. 4–6})\). Through \(^1\)H NMR spectroscopy, we identified the presence of an aminobenzyl side product from the spontaneous reaction of methanolic solvent with an aza-quinone methide generated by self-immolative elimination (Supplementary Figs. 2 and 18). This, and the necessity for aniline deprotection for quinone release, suggests a self-immolative elimination mechanism. Under basic conditions, appearance of 4 was slower \((k_{obs} = 1.16 ± 0.18 \times 10^{-4} \text{ s}^{-1})\) and not complete after 40 h (Supplementary Figs. 4–6), which suggested a pH dependence for the elimination reaction.

We anticipated that self-immolative release might generate an unstable hydroquinone intermediate (8) that could auto-oxidize to the corresponding quinone. 9,10-Phenanthrene dihydroquinone 8 was not observed when elimination was performed under standard, oxygenated conditions as a result of its instability to auto-oxidation (Supplementary Fig. 2). However, in degassed solvent, intermediate 8 was observed (Supplementary Figs. 16 and 17), which confirms...
that the transient hydroquinone intermediate exists. Elimination from \(7\) was also followed by ultraviolet–visible spectroscopic analysis (Supplementary Figs. 20–22). Elimination from benzyl ketols, such as \(7\), in this manner has not been previously documented and thus prompted further investigations.

**Generality of C–C bond elimination reaction to ortho-quinones**

We next investigated whether the observations described above also applied to additional ortho-quinones. From \(\beta\)-lapachone \(1\) (Figs. 1a and 2d), N-Boc-protected \(para\)-aminobenzyl \(\beta\)-lapa-ketol (Boc-PAB-BL) \(14\), 3-hydroxy-\(\beta\)-lapachone (HBL) \(11\), dunnione (DN) \(12\) and cryptotanshinone (CTN) \(13\), protection of ortho-quinones \(1\), \(11\), \(12\) and \(13\) as benzyl ketol derivatives. Upon deprotection the derivatives eliminate to reform the quinones.
(Boc-PAB-BL) 9 could be synthesized under identical conditions in similar yield. N-Boc deprotection of 9 gave (PAB-BL) 10, which was observed to be unstable under acidic conditions and eliminated to reform 1 via hydroquinone intermediate 2 (Fig. 2e and Supplementary Figs. 8, 9 and 20).

Three additional medicinal orthoquinones, (±)-3-hydroxy-β-lapachone (HBL) 11, (±)-dunnione (DN) 12 and cryptotanshinone (CTN) 13 (Fig. 3a), were also successfully converted into their corresponding PAB-ketol analogues as diastereoisomeric mixtures (14–16; Fig. 2e). In the case of HBL, an intermediate hydroxyl protection and the use of an indium(0)-mediated Barbier reaction were required to achieve Boc-PAB-HBL 11. In all cases, the removal of the Boc protecting group in acidic media led to reformation of their respective ortho-quinone precursors (17–19 to 11–13; Fig. 2e and Supplementary Figs. 10–15). These investigations validated the generality of the methodology for a number of medicinally relevant ortho-quinones with structural similarity to 4.

**pH-dependent elimination profiles of benzyl ketol derivatives**

The mechanism and kinetics of the elimination of model compound PAB-PhQ 7 were investigated. Initially, deprotected (R = H) derivative 7 and a theoretical protected (R = Ac) derivative 20 were compared (Fig. 3a). The energy profile along the breaking C=C bond (green) was calculated quantum mechanically at the PCM(H2O)/M06-2X/6-31+G(d,p) level of theory with the purpose of locating the transition state (TS) structures for the elimination. However, no maximum was detected, indicating that neutral species 7 remained unreactive and did not eliminate, contrasting with the observed release of 4. Comparisons were made with different heteroatom-based leaving groups for the elimination of similar neutral para-amino benzyl species (Supplementary Fig. 106). This showed that reagents with chloride or activated alcohol (mesyl or triflate) leaving groups had low enough activation barriers (7.8–16.9 kcal mol⁻¹) to eliminate. A carbamate leaving group was calculated to have a higher activation barrier (28.1 kcal mol⁻¹). However, carbon-based leaving groups needed three electron-withdrawing groups (CN or NO₂) or protonated carbonyl groups to have acceptable activation barriers (15.3–28.1 kcal mol⁻¹) for elimination.

These results suggested that charged species might be necessary to promote the C=C bond elimination reaction. In aqueous solution, several charged species are in equilibrium albeit in various proportions. We considered nine possible charged states for 7 (Fig. 3b). Three of the nine species (7NH–, 7Ac and 7OH) were reactive enough to allow an effective flow of electronic density from the para-amino group to the ketone to promote elimination of the hydroquinone, by either making the former more nucleophilic (NH⁻) or the latter more electrophilic (C=O⁻¹). The activation barriers calculated for these species (ΔG*+1 = 11.8 kcal mol⁻¹; ΔG*+1H2O = 6.5 kcal mol⁻¹; ΔG*+1Zw1 ≤ 0.1 kcal mol⁻¹) suggest fast to extremely fast intrinsic elimination rates (Fig. 3c; k = 3.13 × 10⁸ s⁻¹; k = 1.70 × 10⁸ s⁻¹; k∞ = 5.49 × 10⁷ s⁻¹). However, the global rate depends on the concentration of these reactive species in solution, which are exceedingly low due to the high pKₐ values for aniline deprotonation (≥21) and low pKₐ for carbonyl protonation (≤2), values well beyond the practical range in aqueous solution. Hence, the theoretical global kinetic constant (k∞) can be modelled as a function of the intrinsic kinetic constants for each reactive species (k), the equilibrium constants for all species in solution both reactive and non-reactive (K), and pH; this expression for the theoretical rate constant can be related to the experimentally observed rate constant (kobs) (Fig. 3b (equation) and Supplementary Information).

It became apparent that despite many attempted approximations to cancel out some of the terms in the equation, kobs calculated at different pH values was exceedingly dependent on the equilibrium constants involving the extremely low populated, but very reactive species (K₁H₂O, K₁HL and K₁OH), which resulted in erratic pH dependency profiles depending on the numbers used. With no experimental values available for some equilibrium constants, and because tabulated¹⁰ and calculated values by using different programs such as Marvin 19.19.0 (2019), ChemAxon (https://chemaxon.org) or Epik (Schrodinger Suite)¹³,¹⁴ were not reliable (Supplementary Fig. 109), we decided to examine the elimination reaction kinetics for 7 at different pH values to derive more realistic equilibrium constants by using experimental data.

For N-protected derivative 20, Ac-PAB-PhQ, we also considered nine different species. We found three of them to be reactive, but with higher elimination activation bar- riers (ΔG*+1Ac = 24.8 kcal mol⁻¹; ΔG*+1H2O = 10.7 kcal mol⁻¹; ΔG*+1Zw1 = 4.5 kcal mol⁻¹; Fig. 3c, Supplementary Fig. 111 and Supplementary Table 5) relative to those calculated for the unprotected (R = H) derivative 7. The activation barrier for the negatively charged acetanilide (20,−1Ac−) was substantially increased with respect to the deacetylated analogue due to the large decrease in nucleophilicity of the nitrogen lone pair delocalized along the acetyl group. The dramatic obstruction of this reaction channel causes the global elimination rate to slow down by 2–5 orders of magnitude at pH 3–10 (Supplementary Fig. 110). Therefore, as for the widely used para-aminobenzylalcohol linkers⁶, elimination is self-immolative and faster with free aniline.

For comparison, we also modelled the elimination of the amibenzyl ketol species derived from the medicinal natural product of most notable interest, β-lapachone, PAB-BL 10 and dunnione, PAB-DN 18 (Supplementary Fig. 111 and Supplementary Table 5). Similarly to PAB-PhQ 7, the neutral species of PAB-BL 10 and PAB-DN 18 were unreactive to elimination. We considered the possible reactive species in a similar manner. An additional species—arising from protonation of the cyclic vinyl ether group—was considered but was calculated to be unreactive. In comparison to unprotected phenanthrenequinone derivative 7, slightly higher activation barriers were obtained for the three charged reactive species of PAB-BL 10 (ΔG*+1H2O = 13.5 kcal mol⁻¹; ΔG*+1H1O = 13.5 kcal mol⁻¹; ΔG*+1Zw1 = 1.4 kcal mol⁻¹) and PAB-DN 18 (ΔG*+1H2O = 13.0 kcal mol⁻¹; ΔG*+1H1O = 14.1 kcal mol⁻¹; ΔG*+1Zw1 = 1.6 kcal mol⁻¹) and were noticeably higher for cationic 16OH⁻ and 18OH⁻. This increase is due to the reduced aromaticity around the carbonyl groups in 10 and 18, which in turn affects their protonation ability (K*). Given the calculated differences in the intrinsic kinetic accessibility to the different reaction channels, and the expected thermodynamic differences in the relative populations of each reactive charged species, it was likely that both PAB-BL 10 and PAB-DN 18 had a slightly different pH-rate dependence relative to 7.

To experimentally validate predictions and determine the pH-rate dependence of the elimination and the critical reaction parameters in aqueous solution, we synthesized models protected with a penicillin-G amidase-cleavable phenylacetamide group⁴⁶. Addition of penicillin G amidase to the protected models triggered amide hydrolysis and formation of the desired para-aminobenzyl ketol in neutral aqueous solution (Fig. 4a).

With this strategy, we showed that at physiological pH and temperature (pH 7.4, 37 °C), formation of PhQ 4 occurred at rate kobs = 1.15 ± 0.66 × 10⁸ s⁻¹ following addition of the enzyme to 21. Intermediate 7, PAB-PhQ, could be identified (Fig. 4b and Supplementary Figs. 24–26) and elimination of 7 occurred at rate kobs = 9.72 ± 2.88 × 10⁷ s⁻¹. Without added enzyme, 21 was stable and formation of 4 negligible. As mentioned above, hydroquinone 8 was not observed by NMR spectroscopic analysis of non-degassed solutions, suggesting that in oxygenated aqueous solution the intermediate has a very short t₁/₂, relative to that of species 7.

As enzyme-mediated amide hydrolysis was fast (<5 min), immediate kinetic analysis of the subsequent elimination rate was possible without use of acidic or basic deprotection reagents, thus allowing control of pH. Focusing on the phenanthrenequinone, β-lapachone
Fig. 3 | Modelling of the mechanism of elimination of para-aminobenzyl ketols. a, Theoretical potential energy surface calculated with PCM(H2O)/MO6-2X/6-31+G(d,p) for the elimination of species 7, 10 and 20 along the breaking C–C bond (green). b, General chemical scheme of nine different species denoted as Q (where subscript indicates neutral, cationic, anionic and zwitterionic) that are in equilibrium for the protected and unprotected para-aminobenzyl ketol derivatives in an aqueous solution. Species inside a dashed box (QNH−, QOH+ and QZw1) are those for which an elimination TS structure was found. The equation describes the effect of pH on the observed kinetic rate (kobs) and its derivation is described in the Supplementary Information. c, TS structures for the elimination of reactive species calculated at the same level of theory. Green dashed lines show interatomic distances (in angstroms) for the breaking C–C bond. Colour blue denotes nitrogen, orange denotes oxygen, grey denotes carbon and white denotes hydrogen. Intrinsic first-order kinetic constants (k−, k+, and kzw) were calculated from the theoretical activation energies (ΔG‡ in kcal mol−1) at 310 K using the Eyring equation.
and dunnione derivatives, elimination from 7, 10 and 18 generated enzymatically from 21, 22 and the (R,R/S,S) pair of enantiomers of 23, respectively, was followed across a range of pH values at 37 °C (Fig. 4c and Supplementary Figs. 27 and 38). Reactivity of the quinone products under basic conditions precluded analysis of product formation at pH >10. As predicted computationally, the reaction rate was dependent on pH. Compound 7 tends to show acid-promoted elimination, which peaks at pH 5–6 ($k_{obs}$ pH 6 = 1.7 ± 0.3 × 10⁻⁵ s⁻¹).
C-benzylaition prevents quinone redox activity

The toxicity of the benzyl ketol pharmacophore generated upon quinone derivatization was investigated to determine the usefulness of the protection strategy for prodrug generation. C-benzylaition disrupts the quinone scaffold, so it was expected that the reducto-cycling ability of an ortho-quinone and associated undesirable systemic toxicity would be lost upon derivatization. We focused our attention on β-lapachone, the most well-studied medicinal ortho-quinone. Redox activity of a stable, non-releasable model of benzyl β-lap-lketol, 24, which lacks a para-amino-group necessary for self-immolative release, was compared to parent drug 1, and control non-redox cycling protected 1,4-dioxine derivative 25. Compound 24 was found to be non-redox active in an in vitro redox cycling assay (Fig. 5a and Supplementary Fig. 39) and, unlike 1, did not generate detectable ROS in AML cell line HL-60, a cell line in which H2O2 generation by deacidification (NH4Cl) or inhibition (E64d) that occurred as previously observed. The Cbz group was not necessary for cathepsin-B-labile dipeptides. Dipeptide units 49 Cbz-Val-Cit-, Cbz-Val-Ala- and Cbz-Phe-Cit- were added by sequential amide coupling from deprotected aniline intermediate 10 (26–28, Fig. 5f). After incubation of 26–28 with cathepsin B (Fig. 5g and Supplementary Figs. 53–55), compound 1 was observed. Aniline 10 was identified as a common intermediate and release of 1 from 10 occurred as previously observed. The Cbz group was not necessary for cathepsin B action (Supplementary Fig. 56). This demonstrated that the dipeptide-para-aminobenzyl ketols are compatible with cathepsin B, in an identical manner to dipeptide-para-aminobenzyl alcohol and carbamate linkers. Like benzyl model 24, dipeptide prodrugs 26–28 generated less methaemoglobin in blood in an in vitro model relative to 1 (Fig. 5h). Additionally, they generated less methaemoglobin than an acyl-hydroquinone β-lapachone prodrug 29, previously described by Ma et al., due to the increased stability of the dipeptide over labile ester bonds.

Cbz dipeptide prodrugs 26–28 were fully stable in human serum (Supplementary Figs. 58 and 59) and did not exhibit toxicity to AML cell line MOLM-13 at 5 μM concentration, unlike 1 and derivative 29 (Extended Data Fig. 1c and Supplementary Fig. 57) that achieved complete cell death at this concentration. Compound 26 exhibited 10-fold higher IC50 relative to 1 in AML cell lines HL-60 and MOLM-13 (Supplementary Fig. 57). After lysosomal deacetylation (NH4Cl) or inhibition (E64d), 26 was less toxic, suggesting that acidic compartments help promote toxicity of the prodrug (Extended Data Fig. 1d). It was interesting that with NH4Cl at 30 mM, an increase in toxicity of 1 was seen, a result previously described for menadione (Supplementary Fig. 60). Prodrugs 26–28 may have suitability for treatment of NQO1+ solid tumours with concurrent overexpression of proteases, for example, cathepsins and acidic pH in the extracellular malignant environment.

β-Lapachone is a promising treatment for AML

In our work, we were particularly interested in testing quinone 1 for the treatment of AML, a cancer for which 1 displays potent toxicity, as described in ref. and confirmed by us on a panel of AML cell lines (Supplementary Fig. 43). AML is the most common form of acute leukaemia among adults. It is characterized by immature myeloid cell proliferation and bone marrow failure and is a cancer for which new treatments are urgently needed. AML has a poor 5 yr survival rate of ~20% and a large proportion of patients relapse. Interference in redox homeostasis is an appealing treatment angle for AML. Recent observations note that despite differences between the mechanisms of action of clinically used AML therapeutics, most share oxidative stress as a mediator of the cytotoxic effect. For example, anthracyclines and arsenic trioxide induce rapid ROS accumulation.

Quinone 1 also has relevant protein targets in AML. As mentioned above, in previous work, we show that β-lapachone strongly inhibits enzyme 5-LO, an enzyme that is a candidate target for therapeutic targeting of the stem-cell-like blasts in AML. In our experiments, 5-LO gene knock-out (KO), using CRISPR-Cas9 gene editing, impaired proliferation of leukaemia cell line HEL (Supplementary Figs. 44–47). Experiments also suggested that KO or shRNA-mediated knockdown (Ko) may make AML cells more resistant to 1 (Supplementary Figs. 45–52). Both redox sensitivity and 5-LO target relevance made β-lapachone a valuable experimental drug for AML treatment. For AML, the data strongly supports that the benzyl protection unit, while intact, has the capacity to mitigate the blood-borne redox-mediated side effects of quinones.
the development of an intracellular targeting strategy to enable application of the quinone prodrugs.

**ADCs release β-lapachone in CD33 + AML cells in vivo**

Antibody conjugation can enable targeting and assisted delivery of payloads inside AML target cells. We have shown that an ortho-quinone protection strategy can eliminate redox toxicity from 1, and indeed, protection should therefore prevent any potential redox damage to the antibody carrier.

We reasoned that cell killing should depend on effective cellular trafficking of a conjugate to a low-pH cellular compartment inside target cells because pH 4–5 is required for the efficient release of 1 in vitro. Furthermore, in the system described, the slow release of 1 from the quinone protection unit PAB-BL 10 at physiological pH should limit systemic drug release, that is, any linker deconjugated from the antibody while in circulation should not release drug, even if a protecting dipeptide unit is broken. This can limit the toxicity profile of the ortho-quinone to that produced by non-specific cell uptake.

To test this strategy, we designed IgG1 antibodies for payload conjugation with a binding region based on the well-characterized CD33 targeting antibody gemtuzumab [64] and synthesized payload linker 30. Linker 30 is equipped with a Val-Cit cleavable moiety, as used in brentuximab vedotin [65], and a 3-benzylocrylic acid conjugation moiety, as previously developed by our group [66,67]. for
site-specific cysteine conjugation (Fig. 6a). By using variants with engineered cysteine mutations, we generated homogeneous conjugates with a drug-antibody ratio (DAR) of 2. Three single-cysteine mutation sites were compared, one in the light chain and two in the heavy chain (Fig. 6b). Complete conversion was achieved for all conjugates. The positioning of the engineered cysteines on the antibody influenced the ease of conjugation. A Gem-HC-239iC mutant with a cysteine introduced in the hinge region of the heavy chain at position 239 offered favourable reactivity over Gem-LC-V205C and Gem-HC-S442C mutants with cysteine residues in more exposed positions on the light chain (Val to Cys mutant at position 205) and heavy chain (Ser to Cys mutant at position 442), respectively. With Gem-HC-239iC, complete conversion was achieved using 20 equivalents of linker per cysteine for 6 h to generate conjugate Gem-HC-239iC-BL. Gem-HC-V205C and Gem-HC-S442C mutants required more equivalents of linker (40 and 30 equivalents per cysteine, respectively) to achieve complete conversion. These conjugates may also be termed antibody–prodrug conjugates (APDCs) because they contain a protected payload.

The ADCs were characterized under reducing conditions using liquid chromatography–mass spectrometry (LC–MS) analysis (Supplementary Figs. 70–73), with the linker mass identified as an addition to the chain with the engineered cysteine in each case. Native-MS analysis confirmed that the ADCs had retained the bonding pattern of their light and heavy chains (Fig. 6c and Supplementary Figs. 74–84). All three conjugates retained a good CD33 binding profile with similar binding to their non-conjugated parent antibodies (Supplementary Fig. 64) and offered good conjugation integrity following incubation tests at physiological pH and temperature (phosphate-buffered saline (PBS), 37 °C) and in the presence of 10% human serum, over 48 h (Supplementary Figs. 85–90). The conjugates demonstrated stability to storage, retaining payload when stored at 4 °C for ~1 month (Supplementary Figs. 91–96). Compound 1 has an IC50 against CD33+HL-60 cells of ~1 μM, and the conjugates displayed toxicity to HL-60 at 0.4 μM, a concentration at which unconjugated antibodies were non-toxic (Supplementary Figs. 97–99). Gem-HC-239iC-BL was selected as our favoured conjugate because of favourable reactivity with payload 30 and stability.

**DAR 2 ADC inhibits AML tumour growth in mice**

We evaluated the antileukaemic effect of DAR 2 ADC Gem-HC-239iC-BL in a subcutaneous mouse model of human AML. NOD-SCID immunocompromised mice were inoculated with HEL cells to induce tumour growth and were treated intravenously with either ADC Gem-HC-239iC-BL, the native antibody Gem-IgG1 or PBS. In vitro, against HEL, Gem-HC-239iC-BL had similar toxicity to 1 while non-targeted NIP228-HC-239iC-BL and Gem-IgG1 showed no apparent toxicity (Fig. 6d). In vitro toxicity results were promising although variable. Results varied depending on the cell-counting method used and potentially because of conjugate aggregation arising from the hydrophobic linker and drug. In the in vivo model, a strong antitumour activity was observed with the administration of only two doses of ADC at 7.5 mg kg−1. The ADC-treated mice had decreased tumour volumes relative to the controls (Fig. 6e–g). Moreover, the antileukaemic activity of the ADC significantly improved the survival of the ADC-treated animals (mean of 52 days versus 24 days for control; Fig. 6h). Importantly, two out of the five ADC-treated animals were alive 76d post-treatment, and no signs of toxicity or metastasis were seen in the liver, spleen, kidney, brain, bone marrow, heart and lung of control mice and mice treated with the ADC as demonstrated by histopathological analysis (Supplementary Figs. 100 and 101 and Supplementary Table 1). To verify drug release, we tested samples of fixed tumour tissue of mice treated with ADC Gem-HC-239iC-BL by LC tandem MS, and in ADC-treated mice we were able to detect and quantify 1, which was present at 0.327 ng g−1 (Fig. 6i, Supplementary Figs. 102–105 and Supplementary Tables 2 and 3). These data provide evidence for the successful in vivo release of 1 through the acid-dependent C–C bond-cleaving self-immolative 1,6-elimination mechanism reported in this work. Together, these results demonstrate that the ADC antitumour activity in vivo has great potential to be further developed as an antileukaemic treatment.

Alternative payload classes are an important strategy to identify new treatments and combat drug resistance in the fight against cancer. Here we leverage prodrug chemistry and antibody-mediated delivery to develop a method for intracellular targeting and release of ortho-quinones from a protected redox-inactive form. This work begins to solve a fundamental problem of application of ortho-quinone therapeutics: the reactivity of ortho-quinones with cellular metabolism. Through protection and targeting we aim to increase the concentration of redox-active quinone in the target tissue and decrease the concentration in healthy tissues, thereby lowering associated dose-limiting toxicities.

Unlike previous approaches, our ortho-quinone protection strategy is truly modular and could be adapted to any peptide pro-moiety. The self-immolative 1,6-elimination mechanism described is unprecedented because it cleaves a C–C bond between the benzyl linker and quinone carbon. The elimination is triggered within an acidic pH range, depending on the protected quinone. We demonstrate the generality of the methodology, which we applied to protect model 9,10-phenanthrenquinone and four additional ortho-quinones, β-Lapachone, (+)-dunnione and cryptotanshinone are all ortho-quinones of medicinal interest. 3-Hydroxy-β-lapachone adds additional possibilities for functionalization compatible with the protection strategy.

As an example of the application of the ortho-quinone protection strategy, we synthesized cathepsin-B-cleavable prodrugs of β-lapachone, followed by ADCs using the protection chemistry. For β-lapachone, 1, minimal release of the payload from the quinone-protection unit occurs at physiological pH even after peptide deprotection, which for an ADC means the chemistry offers a built-in protection against premature in-circulation drug release upon linker deconjugation. The rapid cell death triggered by the ADC relative to the untargeted prodrug suggests that released protected quinone species 10 reaches a low pH compartment (~4) necessary for its fast elimination. In many cells the FcRn receptor will recycle the antibody to the cell surface directly from the endosome (pH ~5.5–6) and prevent access to more acidic compartments10, but our results suggest that our ADC does not undergo notable endosomal recycling11,12. The activity of β-lapachone is believed to be independent of endosomal/lysosomal escape, (that is, it should give a toxic effect as soon as it is released from 10).

This work also highlights the relevance of β-lapachone for the treatment of AML and verifies the relevance of 5-LO as a respective target. ADC therapy is well established in the AML disease area, with reapproval of CD33-targeting Mylotarg (gemtuzumab ozogamycin) for relapsed or refractory AML29 and with many more ADCs in development34. Our protected β-lapachone payload is efficacious in a xenograft AML model following ADC targeting and can effectively expand future treatment options upon further development and optimization. It should also be noted that β-lapachone is a lower-potency payload than those commonly used in ADCs; for example, most marketed ADCs have subnanomolar-potency payloads5. However, the concept that ADCs must contain a subnanomolar toxic payload has been recently challenged and efficacious cancer-cell killing has been obtained with lower-potency payloads53. Side effects, and hence the high attrition rate of ADCs in clinical studies, often result from payload-mediated toxicity; thus a lower-toxicity drug that is masked while in circulation with built-in deconjugation toxicity
Fig. 6 | Gem-HC-239iC-BL has an effect on subcutaneous AML tumour growth and prolongs survival of mice. a, Structure of payload 30 for attachment to antibody through engineered cystine residues on the antibody. b, IgG1 antibody containing a gemtuzumab variable domain with the three different cysteine mutation sites annotated. See Supplementary Information for details. In this study we decided to use Gem-HC-239iC. c, Native MS of refolded and conjugated ADC (green) in comparison to refolded unconjugated antibody (orange), indicates intact, homogenous conjugates. d, Toxicity to HEL after 96 h, measured by counting of number of live cells relative to control using the method of trypan blue exclusion. Line represents mean cell viability (one experiment, \( n = 6 \) or \( n = 3 \)). Data suggest Gem-HC-239iC-BL shows increased toxicity compared with non-internalizing control ADC NIP228-HC-239iC-BL, also containing payload 30 in DAR 2, and to native Gem-IgG1, which showed no toxicity at 2.5 \( \mu \)M. e, Representation of the timeline of the HEL tumour cell inoculation and ADC therapy. f, Tumour growth curve. Data are represented as mean ± s.e.m. (\( n = 5 \)). A two-way ANOVA indicates a significant effect of time versus treatment (\( P = 0.0089 \)). g, Tumour volume at day 12 (\( n = 5 \)). Data are represented as mean ± s.e.m. and a Mann–Whitney test was used to test statistical significance. P value has been deleted. h, Overall survival over time (\( n = 5 \)). Log rank test indicates a statistically significant difference between DAR-2 ADC Gem-HC-239iC-BL treatment versus PBS and versus native antibody Gem-IgG1 treatment (\( P = 0.0194 \)). i, Gem-HC-239iC-BL-treated (200 mg l\(^{-1}\)) xenograft tissue homogenate sample.
protection may be advantageous. In this respect, β-lapachone or other ortho-quinone derivatives offer opportunities for the development of ADCs with a distinct mode of action.

The protection strategy described here is expected to be applicable to other ortho-quinones and may encourage the development of improved synthetic routes to, or new derivatives of, these high-value natural products. Derivatives of β-lapachone with higher toxicity that retain the quinone pharmacophore have been reported. New prodrugs, drug-targeting strategies and combinations of the two will enable the application of compounds previously deemed inappropriate for medicinal use. Here, we have developed enabling chemistry that facilitates the integration of bioactive ortho-quinones into strategies.

Online content
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Penicillin-G-avidimediased kinetic analysis of elimination at physiological pH. Stocks of 2-phenylacetyl-para-aminobenzyl ketol were made up in DMSO at a concentration of 10 mM. For a reaction, 17.5 μl of ketol stock was added to 1 ml of a 10 mM stock of internal standard in DMSO and 310 μl of DMSO at pH 7.4. To initiate a reaction, 5 μl of a suspension of penicillin G amidase (PenG, Sigma-Aldrich enzyme 76427, 5−10 U mg⁻¹) was added. In control samples 5 μl of PBS was added in place of the enzyme. Test and control samples were then incubated at 37 °C with shaking. At the recorded time points, 20 μl aliquots of the reactions were analysed by HPLC (254 nm, Thermo Scientific U3000). HPLC column: Phenomenex Kinetex C18, 5 μm, 50 x 4.6 mm, 100 Å, flow, 1 ml min⁻¹. Gradient and solvent system for assay with 2-phenylacetyl-PAB-PHQ 21: A = H₂O, B = acetonitrile, t₀ = 0−10 min 0% B, t₁ = 10−11 min 0% B, t₂ = 10−11 min 0% B. Gradient and solvent system for assay with 2-phenylacetyl-PAB-Bl 22 and 2-phenylacetyl-PAB-DN 23: A = H₂O + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, t₀ = 0−10 min 0% B, t₁ = 10−11 min 0% B, t₂ = 16.1−18 min 100% B, t₃ = 18−20 min 100% B. Peaks were identified by LC−MS analysis and comparison with pure compounds. Values described represent peak area (mAU x min) divided by peak area of internal standard.

pH-rate aqueous kinetic analysis of fragmentation. pH-rate analysis was performed by adaptation of the PenG assay. To obtain deprotected para-aminobenzyl ketol species, 2-phenylacetyl protected ketol was dissolved in PBS with 33% DMSO to a concentration of 3.3 mM. Then 20 μl of a stock solution of PenG was added to 600 μl of this stock and incubated for 20 min at 37 °C, after which 20 μl of a 100 mM stock of internal standard in DMSO was added. Reactions were then set up immediately. For a reaction, 10 μl of the aminobenzyl ketol/stock solution was added to 1 ml of citrate-phosphate pH 5.5 buffer. Solutions were made to obtain an aqueous solution containing 1.8% DMSO. Test samples were incubated at 37 °C with shaking. At the recorded time points, 20 μl aliquots of the reactions were analysed by HPLC (254 nm, Thermo Fisher U3000). HPLC conditions: column Phenomenex Kinetex C18 5 μm, 50 x 4.6 mm, 100 Å; solvent system A = H₂O + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, flow, 1 ml min⁻¹. Gradient for assay with PAB-PhQ 7: t₀ = 0−10 min 0% B, t₁ = 10−11 min 0−100% B, t₂ = 10−11 min 0−100% B. Gradient for assay with PAB-Bl 10 and PAB-DN 18: t₀ = 0−1 min 0% B, t₁ = 10−11 min 0−40% B, t₂ = 10−11 min 40−50% B, t₃ = 16.1−18 min 100% B, t₄ = 18−20 min 100% B. Values described represent peak area (mAU x min) divided by peak area of internal standard. Standardised rates were determined using GraphPad Prism software. Rate and half-life measurements were calculated based on consumption of intermediate para-aminobenzyl ketol and formation of product quinone.

In vitro redox-cycling ability analysis. The in vitro redox-cycling ability of compounds was assessed by phenol red/mercaptide peroxidase (HPRP) assay by adaptation of a previously described protocol. Briefly, stocks were prepared as follows: (1) DTT to a concentration of 2.4 μM in PBS pH 7.4 buffer; (2) compound stock (500 nM) in PBS pH 7.4 buffer to the desired concentration. To a stock solution of DMSO stocks; (3) a stock of phenol red/HRP detection reagent containing 300 μg ml⁻¹ phenol red and 180 μg ml⁻¹ HRP enzyme in PBS pH 7.4 buffer: (4) a stock of 1 M NaOH in water; (5) a stock of 100 μM H₂O₂ in PBS prepared by addition of a 30% solution of H₂O₂ in water into 1,000 μl of PBS. Into each well of a 96-well clear flat-bottomed plate was then added 40 μl of compound stock in PBS followed by 40 μl of DTT stock, followed immediately by 40 μl of phenol red/HRP reagent. A positive control with 100 μM H₂O₂ added in place of compound was included. The plate was incubated in the dark at room temperature for 15 min. Following this, 20 μl of 1 M NaOH was added to each well to terminate the reaction. Absorbance was immediately measured at 610 nm (MinimMax i3x Imager, Molecular Devices). Absorbance of treated wells was normalized to control treated with PBS only (zero redox cycling).

Cellular ROS detection. To determine ROS generation by compounds in leukemic cells, HL-60 cells were washed with PBS, then incubated in serum-free media (10 ml of 1.6 x 10⁻³ mg/ml concentration) with 2,7-dichlorofluorescein diacetate at 20 μM for 30 min at 37 °C. Following this, the cells were washed with PBS and resuspended in FluorBrite–DMEM media (ThermoFisher) (10 ml). Then, 450 μl of the cell mixture was seeded into wells of a clear flat-bottomed 12-well plate, to which 50 μl of a 100 μM stock of the compounds in media had been already added, to achieve a final compound concentration of 30 μM. The 10x media stocks were made by 100-fold dilution of DMSO compound stocks, to ensure a final in plate DMSO concentration of 0.1%. DMSO 0.1% was used as a negative control. H₂O₂ was used as a positive control, for which 10 μM of a 30% aqueous solution was dissolved into 1 ml DMSO, then diluted 100-fold into media for plating, to give a final H₂O₂ concentration of 1 μM. After plating the cells were immediately incubated at 37 °C in the dark for 15 min before the fluorescence
of the wells (excitation, 485 nm; emission, 535 nm) was recorded (MiniMax i3x Imager, Molecular Devices).

**Metaemoglobin analysis.** Deteriorated Oxiid sheep’s blood (ThermoScientific) was diluted to a 5% v/v suspension in PBS pH 7.4. In a 96-well microtitre plate, 150 µl of blood suspension was added to 50 µl of serially diluted compound in PBS containing a 4× stock of each compound of interest. Serial dilution of compounds from DMSO stocks into PBS was performed to achieve an in-place DMSO concentration of 0.1%. PBS buffer alone was used as a negative control. The plate was incubated for the specified time at 37 °C. The plate was then centrifuged at 3,500 r.p.m. for 5 min. The supernatant was removed and cells lysed by resuspension in 100 µl of a solution of Triton-X 100 (1%) in PBS. Following this, 80 µl of lysed cell contents were transferred to a second microtitre plate for ultraviolet measurement. Ultraviolet absorbance was measured immediately at 630 nm (thromboxane synthetase, Molecular Diagnostics). The variable heavy-chain and light-chain sequences for gemtuzumab were identified as previously described.81–83. Strings of cathepsin B (Abcam ab151914) was preactivated by dilution of enzyme stock (1 , 37 kDa, 12 µg) and internal standard acetophenone (0.75 µM) into activation buffer (45 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, soybean trypsin inhibitor (60 µM) (Gibco) with 10.5% DMSO, 500 µM arachidonic acid (5 equiv. per cysteine + 1 µl of a stock in DMSO) for 30 min at 37 °C. Following preactivation, activated cathepsin stock (20 µg) was diluted to a 5% v/v suspension in PBS pH 7.4. In a 96-well microtitre plate, 190 µl of blood suspension was added to 10 µl of serially diluted compound in PBS containing a 20× stock of each compound of interest. Serial dilution of compounds from DMSO stocks into PBS were performed to achieve an in-place DMSO concentration of 0.2%. PBS buffer with 0.2% DMSO was used as a negative control. Triton-X (1% v/v) was used as a positive haemolysis control. The plate was incubated for 6 h at 37 °C. Following incubation, the plate was centrifuged at 3,500 r.p.m. for 5 min at room temperature. Cell pellets were lysed by resuspending 100 µl of a solution of Triton-X 100 (1%) in PBS. Then, 80 µl of lysed cell contents were transferred to a second microtitre plate for ultraviolet measurement. Ultraviolet absorbance was measured immediately at 540 nm. The percentage of haemolysis was determined by (A_{540} − A_{630}) / (A_{630}) × 100, where A_{540} is the absorbance of the test well at 540 nm, and A_{630} is the absorbance of the negative control at 630 nm.

**Haemolysis analysis.** To analyse haemolysis, deteriorated Oxiid sheep’s blood (ThermoScientific) was diluted to a 5% v/v suspension in PBS pH 7.4. In a 96-well microtitre plate, 190 µl of blood suspension was added to 10 µl of serially diluted compound in PBS containing a 20× stock of each compound of interest. Serial dilution of compounds from DMSO stocks into PBS were performed to achieve an in-place DMSO concentration of 0.2%. PBS buffer with 0.2% DMSO was used as a negative control. Triton-X (1% v/v) was used as a positive haemolysis control. The plate was incubated for 6 h at 37 °C. Following incubation, the plate was centrifuged at 3,500 r.p.m. for 5 min at room temperature. Cell pellets were lysed by resuspending 100 µl of a solution of Triton-X 100 (1%) in PBS. Then, 80 µl of lysed cell contents were transferred to a second microtitre plate for ultraviolet measurement. Ultraviolet absorbance was measured immediately at 540 nm. The percentage of haemolysis was determined by (A_{540} − A_{630}) / (A_{630}) × 100, where A_{540} is the absorbance of the test well at 540 nm, and A_{630} is the absorbance of the negative control at 540 nm and A_{630} is the absorbance of the positive haemolysis control (100% haemolysis at 540 nm).

**Expression, purification and cell-free activity assay for recombinant human 5-LO.** Escherichia coli (BL21) was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 30 °C as described. Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, soybean trypsin inhibitor (60 µM) (Gibco) with 10.5% DMSO, 500 µM arachidonic acid (5 equiv. per cysteine + 1 µl of a stock in DMSO) for 30 min at 37 °C. Following reaction, a sample of conjugate was subjected to reduced LC–MS analysis to assess conversion. The concentration of the conjugates was determined by ultraviolet absorbance at 280 nm (A_{280}), as measured using a SpectraDrop reader (MiniMax i3x Imager, Molecular Devices). Ultraviolet absorbances were corrected by the expression A_{280} (1.929 × A_{405}) to account for light scattering.

**Antibody–drug linker conjugations were performed by incubation (with shaking) of refolded antibody stock with the specified equivalent of linker for the specified time period in a buffer of NaPi 20 mM pH 8 and a final concentration of DMSO of 10%. After completion of the reaction, antibodies were desalted with Zeba spin desalting columns (ThermoScientific) into PBS pH 7.4. Following reaction, a sample of conjugate was subjected to reduced LC–MS analysis to assess conversion. The stability of the antibody conjugates to deconjugation of payload at physiological temperature was measured by incubation of conjugate (10 µl of 20 µM stock) in PBS pH 7.4 buffer at 37 °C with shaking. After 48 h, 10 µl aliquots were taken and analysed for percentage conjugated to unconjugated antibody by reduced LC–MS analysis. Conjugate stability to storage at 4 °C in PBS pH 7.4 buffer over an extended period (3–4 weeks) was assessed. Stability of conjugate to deconjugation of payload in human serum was measured by incubation of conjugate (10 µl of 20 µM stock) with 1 µl of human serum in PBS pH 7.4 buffer at 37 °C. After 48 h, 10 µl aliquots were taken and analysed for percentage conjugated to unconjugated antibody by reduced LC–MS analysis.

**General cell culture conditions.** Cells were incubated in a humidified 10% CO₂/90% air atmosphere at 37 °C. HL-60 cells were cultured in RPMI medium (Gibco) with 10% heat-inactivated foetal bovine serum. HEL cells were grown in RPMI media with 20% heat-inactivated foetal bovine serum. Leukaemia cells were maintained at a density of 1 × 10⁶ cells ml⁻¹. Cells were split every second day to keep cell culture in exponential growth phase. SKBR-3 cells were grown in McCoy’s 5A Modified Medium (Gibco) with 10% heat-inactivated foetal bovine serum. MCF-7 cells were grown in Dulbecco’s Modified Eagle Medium (Gibco) with 10% heat-inactivated foetal bovine serum. Adherent cell lines were passaged by addition of trypsin–EDTA (0.25%) (Gibco). HL-60 was kindly donated from the group of Prof. Bruno Silva-Santos, IMM Liem. HEL was kindly donated from Dr. Mateo Soto from IMM Liem, Barcelona, Spain, and Dr. John Urbanski and Dr. Isabella Barbieri, Department of Pathology, University of Cambridge (in vitro ADC studies). HeLa, SKBr3 and HCT-116 were purchased from ATCC. MCF-7 was kindly donated from the group of Dr. Sérgio Almeida, IMM Liem.

**Viability assays with small-molecule lapachone models.** Assays assessing cytotoxicity of β-lapachone and derivatives were performed by CellTiter-Blue assay (Promega) according to the manufacturer’s instructions. Briefly, adherent cancer-cell lines were plated at 20,000 cells per well in 96-well plates or suspension cell lines were plated at a concentration of 2 × 10⁵ cells ml⁻¹ in 24-well plates. Compound stocks in media were made by dilution of DMSO stocks containing the compound of interest to obtain a final in-plate DMSO concentration of only 0.1%. Incubation was performed for 48 h. Following incubation, media was replaced with media containing CellTiter-Blue (Promega) in 1:10 dilution and the plates were incubated for 1.5–4 h. The fluorescence of the plates was recorded (excitation, 555 nm; emission, 585 nm; MiniMax i3x Reader, Molecular Devices). Cell viability was calculated by division of the fluorescence intensity of treated wells by that of the calculated average fluorescence intensity of replicates without weight. RSD values were calculated using GraphPad Prism 8 software.

**N-Acetyl cysteine assay.** For assays involving cytotoxicity of β-lapachone derivatives with and without antioxidant N-acetyl cysteine, HL-60 cells were plated into 96-well plates at a concentration of 2 × 10⁵ cells ml⁻¹ in 450 µl. To this well was then added 25 µl of drug diluted in PBS to obtain the correct in-plate drug concentration, and 25 µl of PBS or a stock of N-acetyl cysteine at 12 mM in PBS.
DMSO was maintained at an in-plate concentration of 0.1%. After 96 h incubation, toxicity was assessed by counting of live cells using the trypan blue exclusion method with a Countess II Automated Cell Counter (ThermoFisher Scientific) according to the manufacturer’s instructions. Cell viability was determined by dividing the average live cell number of treated wells by the average live cell number for control wells treated with 0.1% DMSO. Viability for wells treated with N-acetyl cysteine and 0.1% DMSO. IC50 values were calculated using GraphPad Prism 8 software.

Viability assays with small-molecule prodrugs and ADCs using the trypan blue exclusion method. For assays assessing cytotoxicity of β-lapachone versus small-molecule prodrugs, antibodies and ADCs, suspension cells were plated into 24-well plates at a density of 2.5 × 10⁴ cells/ml in 240 µl. Stocks of ADC or antibody in PBS were normalized to 12.5 µM. Serial dilutions of compound were made up in PBS. Then, 60 µl of each stock of either PBS or compound in PBS was added to the cells to achieve the desired final concentration. After the specified incubation time, toxicity was assessed by counting of live cells using the trypan blue exclusion method with a Countess II Automated Cell Counter (ThermoFisher Scientific) according to the manufacturer’s instructions. Cell viability was determined by dividing the average live cell number of treated wells by the average live cell number for control wells treated with PBS only. IC50 values were calculated using GraphPad Prism software. Note: for the assay performed over 96 h, 5% human serum was added to media instead of 10% fetal bovine serum, to limit non-specific antibody internalisation. This had little effect on ADC toxicity.

Lysosomal inhibition assay with small molecule prodrugs. Assays to assess the effect of lysosomal activity on prodrugs were performed as follows: HL-60 cells were plated into 96-well plates at a concentration of 2.5 × 10⁴ cells/ml in 160 µl media. Cells were treated with 10 µl of a stock of NH4Cl in PBS (to obtain an in-plate concentration of 3 or 30 mM), 10 µl of a dilution of a DMSO stock of 166 µl in PBS (to obtain an in-plate concentration of 30 µM), PBS only or a dilution of DMSO in PBS. Cells were incubated for 30 min. Following this, 10 µl of a stock of test compound made by dilution of a DMSO stock into media was added. DMSO was present in plate at a maximum of 0.2%. Cells were incubated overnight for 16 h. Following incubation, media was replaced with media containing CellTiter-Blue (Promega) in 1:4 dilution and the plates were incubated for 6 h. The fluorescence of the plates was recorded (excitation, 555 nm; emission, 585 nm; MiniMax i3x Reader, Molecular Devices). Cell viability was calculated by division of the fluorescence intensity of treated wells by that of the average fluorescence intensity of the relevant negative control wells.

Protein mass spectrometry. LC–MS to analyse protein samples was performed on a SQ Detector 2 connected to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 µm, 2.1 mm × 50 mm). Flow rate was set at 0.2 ml min⁻¹ with eluents of solvent A (water with 0.1% formic acid) and solvent B (71% acetonitrile, 29% water with 0.075% formic acid). The gradient was from solvent A/B (72:28) to 100% B over 25 min, followed by solvent B for 2 min and then up to solvent A/B (72:28) over 18 min. A capillary voltage of 2.0 kV and a cone voltage of 40 V were used for the electrospray source. The desolvation gas was nitrogen at a total flow of 800 l/h. Mass spectra were reconstructed using the MaxExtract algorithm preinstalled on MassLynx software (Waters) from the ion series. MS/MS spectra of the ions selected for further analysis were collected for ESI+ and ESI−/ESI−, ions were fragmented with collision energy of 35 and 25 eV, respectively. The extracted samples were injected onto a Waters Acquity UPLC HSS T3 1.8 µm OD 2.1 mm × 100 mm column and a detection was performed at 210 nm. The extracted samples were collected for histopathology. Samples were immersed in 10% neutral buffered formalin, routinely processed for paraffin embedding, sectioned at 4 µm, and stained with haematoxylin and eosin. Lesions were classified according to previous published criteria (International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice) and scored according to a six-tier severity scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. Distant metastasis were scored according to a five-tier severity scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked. Representative haematoxylin and eosin pictures were obtained using NDPview2 software (Hamamatsu) in slides digitally scanned in the Hamamatsu NanoZoomerSQ (20X for heart, lung, liver, spleen, kidney and primary tumour; and 10X for the central nervous system).

Quantitative LC–MS/MS of β-lapachone in formalin-fixed xenograft mouse tumour and formalin-superfused tumor. Concentrations of β-lapachone in formalin-fixed tumour and the respective formalin superfused samples were quantified using reversed-phase ultra-high-performance liquid chromatography tandem mass spectrometry as previously described. Care was taken during sample handling to minimize exposure to light, and amber glass/plastic and foil wrapping were used where possible. Tumour tissue was homogenized to a concentration of 200 mg ml⁻¹ with PBS. An aliquot of homogenate or formal superfused sample was transferred to a fresh tube with cryptotanshinone (internal standard) in ethyl acetate. Samples—liquid–liquid extraction samples were mixed with an equal volume of ethyl acetate layer transferred to a 96-well plate, evaporated and reconstituted in 0.1% formic acid in acetonitrile/water 70:30. PBS was used as the surrogate matrix for calibration standards and quality-control samples. The calibration ranges were 0.25–250 ng ml⁻¹ (homogenate) and 0.05–50 ng ml⁻¹ (formol). The analytical batches included quality-control samples at low, medium and high concentrations. The analysis was performed on a Shimadzu Nexera X2 UPLC coupled to a ScieX TripleTOF 6600 mass spectrometer. The extracted samples were injected onto a Phenomenex Kinetex EVO C18, 1.7 µm, 50 × 2.1 mm analytical column, at 35 °C. Gradient elution was performed using 0.1% formic acid in water and an increasing percentage of 0.1% formic acid in acetonitrile at 0.5 ml min⁻¹, with a total run time of 0.5 min. A detectable product ion was observed for each precursor ion. The total ion chromatograms were scanned in the Hamamatsu NanoZoomerSQ (20X for heart, lung, liver, spleen, kidney and primary tumour; and 10X for the central nervous system).

Quantum mechanical calculations. Full geometry optimizations were carried out with Gaussian 16 (ref. 17), using the M06-2X hybrid functional and 6-31+G(dp) basis set. Vibrational analysis was run with 6-311G* basis set. Vibrational frequencies were calculated and the force constants were converted to a 3D vibrational grid. The vibrational frequencies were calculated using the IEF–PCM polarizable continuum model (ref. 18). The possibility of different conformations was taken into account for all structures. All stationary points were characterized by a frequency analysis performed at the same level used in the geometry optimizations from which thermal corrections were obtained at 313.15 or 298.15 K. Frequency analyses
were carried out at the same level used in the geometry optimizations, and the nature of the stationary points was determined in each case according to the appropriate number of negative eigenvalues of the Hessian matrix. The quasiharmonic approximation reported by Truhlar and co-workers was used to replace the harmonic oscillator approximation for the calculation of the vibrational contribution to enthalpy and entropy\(^\mathrm{a}\). Scaled frequencies were not considered. Mass-weighted intrinsic reaction coordinate calculations were carried out using the Gonzalez and Schlegel scheme\(^\text{b}\) to ensure that the TSs indeed connected the appropriate reactants and products. Single-point energies were alternatively calculated on the optimized geometries using combinations of different density functionals (M06-2X and \(\omega\)-B97x-D\(^9\)) and basis sets (6-31+G(dp) and 6-31+G+(2d,p)) and implicit solvation models (IEF-PCM and SMID\(^\text{g}\)) (Supplementary Table 4). Gibbs free energies (\(\Delta G\)) were used for the discussion on the relative stabilities of the considered structures. The lowest-energy conformer for each computed stationary point was considered in the calculation of the elimination activation barriers; all the computed structures can be obtained from authors upon request. Computed molecular structures were depicted using open-source PyMol 2.3 (https://pymol.org). Electronic energies, entropies, enthalpies, Gibbs free energies and lowest frequencies of the calculated structures are summarized in Supplementary Table 5. Cartesian coordinates of the lowest-energy structures calculated with PCM(H2O)/M06-2X/6-31+G(dp) are shown in Supplementary Table 6. Theoretical kinetic and equilibrium constants derived from fitting of experimental and computed data were calculated using Microcal Origin Pro 2020b.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** Data supporting the findings of this study are available within the paper and its Supplementary Information. The Supplementary Information reports experiments described within the manuscript in greater detail, and describes synthetic procedures and characterization data. Requests for materials should be addressed to G.L.J.B. and G.I.O. Source data of HPLC traces used to calculate the kinetics of PAB-PbQ, PAB-BL and PAB-DN elimination in aqueous solution are provided with the manuscript. All computed geometries, energies and fitting data can be accessed through the Zenodo repository (https://doi.org/10.5281/zenodo.6525898). No restrictions on data availability apply. Source data are provided with this paper.

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**Author contributions**

L.D. and G.J.L.B. conceived the study. L.D. performed chemical syntheses, aqueous kinetics studies, kinetic NMR experiments, antibody conjugations, LC-MS analysis and in vitro and cellular tests with protected quinone models, prodrugs and ADCs. C.D.N. and G.I.-O. performed molecular simulations of protected \(\text{ortho}\)-quinones. A.G. performed in vivo studies. E.G.D.M. performed chemical synthesis, NMR analysis and antibody conjugations. J.B. performed chemical synthesis, NMR analysis and aqueous kinetics studies. L.B. and V.Z. performed expression of antibodies. E.M. performed LC-MS analysis of unmodified antibodies. E.S. performed bio-layer interferometry binding studies. E.H. performed native-MS and assisted with kinetic NMR spectroscopy experiments. S.M., L.D. and I.B. performed 5-LO shRNA knockdown experiments. J.C. and I.B. performed 5-LO CRISPR-Cas9 knockout experiments. S.L., performed 5-LO inhibition experiments. P.R., G.V., O.W., T.R. and F.P.d.C. contributed to analysis and discussions. L.D. and G.J.L.B. wrote the manuscript with contributions from all authors.

**Competing interests**

P.R., V.Z., E.S. and E.M. are or were formerly employed by AstraZeneca plc. L.D., J.B., E.G.d.M. and G.J.L.B. are inventors on a patent application (GB2020791.3) that incorporates discoveries described in this manuscript. T.R. and G.J.-O. are inventors on a patent that describes the discovery of the allosteric inhibition of 5-LO by \(\beta\)-lapachone (PCT/PT2018/050029), which is related to parts of the work related in this manuscript. The authors of this article declare that V. Cantrill, who was involved with the editing of this article, is employed by G.J.L.B. as a research coordinator at the University of Cambridge. V. Cantrill is married to S. Cantrill, who is the Chief Editor of *Nature Chemistry*. All other authors declare no competing interests.

**Publisher note**

The editorial team of *Nature Chemistry* declare that Stuart Cantrill has had no involvement in the editorial handling of this article.

**Additional information**

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Correspondence and requests for materials should be addressed to Gonzalo Jimenez-Oroz or Gonzalez J. L. Bernardes.

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Extended Data Fig. 1 | Additional in vitro assays of the benzyl ketol protection unit. a, (i) Concentration-dependent in vitro methaemoglobin generation by 1, (ii) appearance of methaemoglobin peak at 630 nm for samples at 100 μM. Line represents mean values, with dotted borders representing ± SEM (n=3). This experiment was repeated once with similar results. b, Toxicity of 1, 24 and 25 to cancer cell lines: HL-60 (acute myeloid leukaemia), HeLa (endocervical adenocarcinoma) and HCT-116 (colon carcinoma). Data are presented as mean values fit to 4-PL model with error bars representing ± SEM (n=3). These experiments were replicated once with similar results. c, Viability of leukaemia cell line MOLM-13 at 24 h timepoints following treatment with peptide prodrugs at 5 μM compared to compounds 1 and 29. Values are mean normalised cell viability from three biological replicates ± SEM (n=9). d, Lysosome deacidification and inhibition assay in HL-60 over 24 h. Significant difference marked: paired two-tailed t test for 26, untreated vs. NH4Cl 30 mM treated viability; p=0.0199. Bar height displays mean values from one representative experiment with error bar displaying ± SEM (n=3).
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about: availability of computer code

Data collection
Quantum Mechanical transition state geometries, energies and vibrational frequencies were computed with Gaussian 16 Rev C01 (www.gaussian.com).

Data analysis
Computed molecular structures were depicted using Open-Source PyMol 2.3 (https://pymol.org). Theoretical kinetic and equilibrium constants derived from fitting of experimental and calculated data were calculated using Microcal Origin Pro 2006b. Mestrenova version 14.2.0 was used for NMR analysis. The MaxEnt algorithm in MassLynx version 4.1 was used for mass spectrometry analysis. Analysis of data was also performed with GraphPad Prism version 8.0 and Origin Pro 2020.

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Supplementary Information is available for this paper and details experiments described within the manuscript in greater detail in addition to describing synthetic procedures and characterisation data. Source data of I/PLC traces used to calculate the kinetics of PAB-PHQ, PAB-BI, and PAB-DN elimination in aqueous solution are provided with the manuscript. All computed geometries, energies and fitting data can be accessed through the Zenodo repository (DOI: 10.5281/zenodo.6325898). No restrictions on data availability apply.
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| Sample size | HPLC assays and in vitro biological experiments contained a minimum of n=3 replicates at each sampling point to allow for calculation of mean result value and an estimation of the standard error of the result value. For the pH-dependent elimination rate analysis experiments performed by HPLC, for some time-points fewer than n=3 replicates are available due to HPLC instrument errors. In the in vivo study, n=5 animals were tested for each test condition. |
| Data exclusions | No data exclusions apply. |
| Replication | The number of times each experiment was repeated with similar results is detailed in the figure caption for each experiment described within the Supplementary Information. |
| Randomization | Randomization was not applicable to this study, as the focus of this study was proof-of-concept of a protection chemistry. |
| Blinding | Blinding was not applicable to this study. It was not possible within the conditions of the research laboratories this work was performed in to blind the scientists performing the experiments to the samples they were testing. |

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| Materials & experimental systems | Methods |
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| n/a | Involved in the study |
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| □ | Dual use research of concern |
| □ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Antibodies

Antibodies used: Gem-igG1, Gem-LC-V205C, Gem-HC-S442C and Gem-HC-239IC are IgG1 antibodies expressed at AstraZeneca, Cambridge containing the variable region originally found in gemtuzumab, and hence the antibodies bind to CD33. Gem-LC-V205C, Gem-HC-S442C and Gem-HC-239IC are identical to Gem-igG1 with the specified cysteine mutations on the light chain (LC) or heavy chain (HC) respectively. Control antibody NIP228-HC-239IC was also expressed at AstraZeneca. NIP228-HC-239IC does not bind to CD33 and contains an heavy chain cysteine mutation in an identical position to Gem-HC-239IC.

Validation: QC data available for the antibodies used in this work is described within the Supplementary Information.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

HeLa, SKBr3 and HCT-116 were purchased from ATCC. HL-60 was donated from the group of Prof. Bruno Silva-Santos, IMM Lisbon. MCF-7 was donated from the group of Dr. Sérgio Almeida, IMM Lisbon. MOLM-13 was donated from the group of Prof. Tony Kouzarides, Gurdon Institute, University of Cambridge. HEL was either donated from the group of Prof. Bruno Silva-Santos from IMM, Lisbon (in vivo studies), from the group of Dr. Isaha Barbieri, Department of Pathology, University of Cambridge (in vitro ADC studies and shRNA 5-LO Kd study), or donated from the group of Prof. George Vassiliou, Wellcome-MRC Cambridge Stem Cell Institute, Department of Haematology, University of Cambridge (CRISPR 5-LO KO study).
### Animals and other organisms

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| Category                  | Description                                                                 |
|---------------------------|-----------------------------------------------------------------------------|
| Laboratory animals        | 8 week old female NOD-SCID mice.                                            |
| Wild animals              | No wild animals were used in this study.                                    |
| Field-collected samples   | No field collected samples were used in this study.                         |
| Ethics oversight          | Instituto de Medicina Molecular João Lobo Antunes & Direção-Geral de Alimentação e Veterinária, Portugal |

Note that full information on the approval of the study protocol must also be provided in the manuscript.