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Synthesis, structure-activity relationship and \textit{in vitro} pharmacodynamics of A-ring modified caged xanthones in a preclinical model of inflammatory breast cancer

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\textbf{A B S T R A C T}

Inflammatory breast cancer (IBC) is a highly metastatic, lethal form of breast cancer that lacks targeted therapeutic strategies. Inspired by the promising cytotoxicity of gambogic acid and related caged xanthones in spheroids\textsuperscript{A-M}
\textsuperscript{RYX}, an \textit{in vitro} preclinical IBC model, we constructed a library of synthetic analogs and performed structure-activity relationship studies. The studies revealed that functionalizing the A-ring of the caged xanthone framework can significantly affect potency. Specifically, introduction of hydroxyl or fluorine groups at discrete positions of the A-ring leads to enhanced cytotoxicity at sub-micromolar concentrations. These compounds induce complete dissolution of spheroids\textsuperscript{A-M}
\textsuperscript{RYX} with subsequent apoptosis of both the peripherally- and centrally-located cells, proliferative and quiescent-prone (e.g. hypoxic), respectively. These results highlight the structural flexibility and pharmacological potential of the caged xanthone motif for the design of IBC-targeting therapeutics.

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1. Introduction

Tropical trees of the genus \textit{Garcinia} (Clusiaceae), grown mostly in Southeast Asia, India, Africa and Brazil, are widely known not only for their high value as sources of food but also for their role in ethnomedicine [1–3]. For instance, gamboge, the dried resin from \textit{Garcinia hanburyi}, has been used in traditional Eastern medicine for its anti-infective and anti-parasitic properties [4,5]. Efforts to isolate the bioactive constituents of gamboge led to the identification of gambogic acid (GBA, 1) (Fig. 1), a caged xanthone that displays potent anticancer activities both \textit{in vitro} and \textit{in vivo} [6–10] and presents a promising therapeutic window for clinical applications [11–14]. In fact, GBA has entered clinical trials in China for the treatment of non-small cell lung, colon and renal cancers [15–17].

By virtue of its unusual chemical architecture, GBA has become the archetype of a family of bioactive natural products that are commonly referred to as caged xanthones (CXs) [18–21]. Several members of this family exhibit potent activity against leukemia, glioblastoma, lung cancer and colorectal cancer cell lines [22–24]. Central to their structure is a xanthone framework in which the C-ring has been converted into a caged motif. Plant-specific oxidations and functionalizations of this framework contribute to the structural diversity of this family. The pharmacological promise of CXs has inspired several structure-function studies [25–30]. These efforts unveiled that cluvenone (2, CLV) represents the minimum pharmacophore of the CX family [31–33] and suggested that A-ring functionalization could lead to analogs with improved bioactivity [34–38]. Along these lines, MAD28 (3), the C6-hydroxylated cluvenone, was found to be approximately 3 times more active than MAD44 (4), the C18-hydroxylated cluvenone (Fig. 1), in inhibiting

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growth in a T-cell acute lymphoblastic leukemia (CEM) cell line [39]. More recently, MAD28 (1.25 μM) was shown to exhibit selective cytotoxicity against human epithelial breast cancer cells (MDA-MB-231 or MCF7) with no apparent detrimental effect in normal breast cells (MCF10A) [40].

The promising activity of MAD28 against breast cancer led us to evaluate its cytotoxicity in spheroids, a 3-dimensional multicellular system formed from a patient-derived xenograft of inflammatory breast cancer (IBC) [41]. IBC is a highly aggressive and often lethal subtype of breast cancer accounting for about 3–10% of diagnosed breast carcinomas [42–46]. Its high mortality rate is due to its metastatic nature and triple negative signature that renders IBC resistant to chemotherapeutic regimens [47–49]. Additionally, IBC lacks molecular markers making diagnosis and design of targeted therapeutic approaches very challenging. It is accepted that the lymphovascular embolus is a marker of cancer aggressiveness, recurrency, metastatic progression and therapeutic failure in many tumor types [50–53]. The spheroids maintain the 3-dimensional and pathophysiological features of the lymphovascular embolus [54]. As such, they provide a relevant model to test efficacy of therapeutics to IBC and metastatic disease in general. We found that both GBA and MAD28 induce spheroid dissolution and cell death in the spheroids assay with IC50 values of 0.42 and 0.66 μM, respectively [41]. Importantly, this activity was 20-fold greater than that observed with taxol (IC50 = 7.8 μM) while other FDA-approved drugs failed to induce any response [41].

In this study, we synthesized and evaluated a library of caged xanthenes in which the A-ring has been chemically modiﬁed and found that such functionalizations substantially modulate the observed cytotoxicity in the spheroids assay. Notably, attachment of electron withdrawing groups at the A-ring as well as introduction of a free hydroxyl group at C6 enhances cytotoxicity and results in complete dissolution with subsequent apoptosis of the spheroids at submicromolar concentrations. Spatial/temporal analysis of the multicellular IBC spheroid showed pyknotic nuclei throughout as well as activation of PARP (i.e. cleaved PARP) at 3 h, an indication of irreversible apoptosis. Fluorescent overlay analysis of cPARP and the proliferation marker Ki67 indicated that these compounds induce apoptosis irrespective of proliferation status. Specifically, they target both the peripherally-located proliferative cells as well as the centrally-located quiescent-prone (e.g. hypoxic) cells of the multicellular spheroid. The latter represents the often resistant and chemo-refractory cell population of this disease. Thus, the caged xanthene motif represents a promising platform for future preclinical development of IBC-targeted therapeutics.

2. Results and discussion

2.1. Synthesis of A-ring modified caged xanthenes


Treatment of [13] with tri(ethylene glycol) monomethyl ether tosylate resulted in the formation of compounds [14] and [15] in variable yields (Scheme 2) [58]. Monoalkylated compound [14] was isolated as the only product when the reaction was performed at room temperature, likely due to the higher reactivity of the C18 phenol. On the other hand, at 80 °C the reaction gave rise to dialkylated product [15] in 55% yield. Under similar conditions, caged xanthenes [3] and [4] [39] were converted to derivatives [16] and [17], respectively.

The synthesis of the caged xanthenes [23] and [25] is shown in Scheme 3. Treatment of commercially available 2-fluoro-1,4-dimethoxybenzene [18] with n-BuLi and methyl chloroformate
afforded, after ester deprotection, the corresponding carboxylic acid 19 (96% combined yield) [59]. A two-step condensation of 19 with pyrogallol (20) produced xanthone 21 in 30% combined yield. This compound was subsequently allylated with 9 under Pd (0) catalysis and the resulting adduct 22 was rearranged to caged xanthone 23 via a Claisen/Diels-Alder reaction cascade. Various conditions, e.g. Pd(O(OAc)2) or cerium ammonium nitrate, were evaluated for the in situ oxidative demethylation of 23 to 25 but they proved to be unsuccessful [60]. To overcome this issue, we sought to accomplish this conversion in two distinct steps [61,62]. Along these lines, we converted 21 to 25 via: (a) boron tribromide induced demethylation of the C6 methyl ether; (b) Pd (0) mediated allylation of the resulting xanthone with 9; (c) Claisen/Diels-Alder rearrangement; and (d) Pd(O(OAc)2)-mediated oxidation of the resulting caged xanthone 24 (19% combined yield).

The synthesis of A-ring fluorinated caged xanthones is described in Scheme 4. Xanthone 29 containing fluorine at C6 and C18 was prepared via: (a) conversion of 2,4,6-trifluoro benzoic acid (26) to its acyl chloride 27; (b) Friedel-Crafts acylation of 27 with pyrogallol (20); and (c) intramolecular nucleophilic aromatic substitution of the resulting benzophenone 28 (39% combined yield, Scheme 4). Pd (0)-catalyzed reverse prenylation of 29 with carbonate 30 gave xanthone 31. In the final step, heat-induced Claisen/Diels-Alder reaction cascade produced the regular caged xanthone 32 in 64% yield accompanied by a small amount of the neo cage isomer 33 (3% yield).

2.2. Screening of caged xanthones using the spheroidsMARY-X assay

The activity of the synthetic caged xanthones was determined using the spheroidsMARY-X assay. This system allows quick assessment of the drug response via brightfield microscopy and ImageJ circularity analysis (see Fig. S1, Supplemental Information), where spheroid dissolution is displayed as a deviation from the well-circumscribed spheroid edge which correlates with induction of apoptosis (i.e. drug response) [41,63]. Briefly, 30–50 spheroidsMARY-X were seeded in a multi-well plate and treated with increasing doses of each compound or DMSO (vehicle) for 24 h. All the compounds were tested at five different concentrations, ranging from 0.5 μM to 2.5 μM. CX analogs that showed no response (NR), low response (LR) and moderate response (MR) at the highest concentration (2.5 μM) are presented in Fig. 2. MAD69 and CR240C showed no response (NR) as there was no deviation of the well-circumscribed edge of the spheroidsMARY-X comparable to the vehicle only (DMSO, control) treated spheroidsMARY-X (Fig. S1). Analogs MAD44 and P67 showed low response (LR) while CR140, CR170B showed low to moderate response (LR-MR); both pairs of drugs resulted in only minor deviations from the well-circumscribed spheroid edge. In contrast, the extent of the deviation was increased for spheroidsMARY-X treated with CR170A and CR136 leading to a moderate response (MR).
Out of the thirteen caged xanthones tested, five exhibited complete response (CR) as evidenced by a total dissolution of the spheroids after 24 h treatment at 2.5 μM. Specifically, MAD28, MAD67, CLV, RA25 and RA25N induced complete response (CR) leading to a single cell suspension of the formerly intact, well-circumscribed spheroids. These analogs were subjected to a separate, more detailed screening, aiming to quantify their effect on spheroids and determine their IC50 values. During this second screening, nine concentrations from 0.25 μM to 2.25 μM at 0.25 μM dose intervals of each compound were generated by serial dilutions and were used to treat the spheroids for 24 h. The effect of these compounds in four representative concentrations (0.5, 0.75, 1.0 and 2.0 μM) is shown in Fig. 3 (the entire 9-concentration panel is shown in Supplemental Information; Fig. S2). The brightfield images acquired from this screening were further analyzed using ImageJ software in order to quantify the circularity/response of the spheroids and construct a dose-response graph that allows for calculation of IC50 values (Fig. 4).  

### 2.3. Structure-activity relationship study

Comparison of CLV (IC50 = 0.63 μM) and MAD28 (IC50 = 0.51 μM) indicates enhancement of cytotoxicity when there is a free OH group at the C6 center of the A-ring of the CX scaffold (Table 1). Alkylation of this group decreases the cytotoxicity as shown with CR136 (MR) and also with P67 (LR). These results may be due to an intramolecular hydrogen bonding between the C6-OH and the near carbonyl group that, in turn, increases the electrophility of the C10 enone. On the other hand, installation of a free OH group at the C6 center improves activity as shown by comparing CR170B (LR-MR) to MAD69 (NR) as well as MAD44 (LR) to CR140 (LR-MR). Moreover, when the C18 phenol is alkylated, the presence of a free OH group at the C6 center improves activity as shown by comparing CR170A (MR) to CR170B (LR-MR). In fact, MAD67 that contains a MOM-protected C18 phenol and a free C6 hydroxyl group induces complete response (CR; i.e. leads to a single cell suspension of the formerly intact, well-circumscribed spheroids) and has the highest cytotoxicity (IC50 = 0.38 μM).

Conversion of the A-ring phenol to a quinone leads to complete loss of activity as shown with CR240C (NR) indicating that the A-ring should maintain its aromatic character. On the other hand, replacement of hydrogens with fluorines at the A-ring maintains the CR phenotype but leads to a slight decrease of the cytotoxicity as shown by comparing the difluorinated analogs RA25 (IC50 = 1.12 μM) and RA25N (IC50 = 0.89 μM) to CLV (IC50 = 0.63 μM). The finding that both RA25 and RA25N induce complete response (CR) and have similar cytotoxicity (IC50 = 1.12 μM and 0.89 μM, respectively) indicates that both isomeric forms of the caged motif have comparable activity. Similar observations have been reported when comparing other caged xanthones that contain regular versus neo caged structures [65,66].

### 2.4. Evaluation of the spatial and temporal induction of apoptosis in spheroids by selected caged xanthones

The spheroids is a spontaneously-forming 3-dimensional model system that accurately recapitulates the growth, behavior and microenvironment of solid tumors [67–72]. To investigate whether the lead compounds can target any cell of the multicellular spheroid or are restricted to targeting a specific cell population, we evaluated the status of proliferation and apoptosis within the spheroids using immunofluorescence. For this experiment, the spheroids were suspended in an extracellular matrix (ECM) plug which provides a scaffold that prevents dispersion of the cells during drug treatment. The ECM plug containing spheroids were then exposed to 2.5 μM of MAD28, MAD67 and RA25, or DMSO (control) and monitored using brightfield microscopy for loss of the well-circumscribed spheroid edge. Treatment was terminated after 3 h, when deviations from the well-circumscribed spheroid edge of all compound-treated spheroids were detected (Fig. 5; brightfield column, white arrowheads). The ECM plugs of vehicle only (DMSO) and compound-treated samples were then fixed and stained with the nuclear stain, 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), and immunostained with the apoptotic marker, cleaved fragment of poly (ADP-ribose) polymerase (cPARP) and the proliferative marker Ki67 (Fig. 5). Spheroids treated with vehicle only (DMSO) were found to have intact nuclei (i.e. viable cells) as shown with DAPI staining in sections corresponding to the middle of the spheroids. However, treatment for 3 h with 2.5 μM of MAD28, MAD67, and RA25 was sufficient to induce apoptosis, as shown by the appearance of pyknotic nuclei (Fig. 5; DAPI column, white arrowheads). These pyknotic nuclei correlate to the loss of well-circumscribed edges in all three compound-treated samples (Fig. 5; brightfield column, white arrowhead). Pyknotic nuclei were detected both in cells on the periphery and the center of the spheroid. This indicates that not only can the compounds effectively target the peripherally-located proliferative cells but also the centrally-located, quiescent-prone cells of the spheroids. Detection of cPARP after treatment with all three compounds, suggests that 3 h of treatment at 2.5 μM is adequate to activate caspases and irreversible initiation of apoptosis throughout the spheroid (Fig. 5; cPARP column). Staining with Ki-67, a commonly used proliferation marker, and fluorescent overlay analysis (i.e. induction of apoptosis vs. proliferation status) shows that MAD28, MAD67 and RA25 target all cells of the multicellular spheroid and thus are efficacious irrespective of proliferation status (Fig. 5; Ki67 column). This impartial cancer cell targeting is clearly advantageous over the effect of various FDA-approved anticancer drugs that mainly target proliferating cells [40,41,73,74].

### 3. Conclusions

We report here the synthesis of a library of caged xanthones and their in vitro evaluation in spheroids, a preclinical model of inflammatory breast cancer. The synthetic strategy starts with acid-
induced condensation of two appropriately functionalized aromatic rings to form the central xanthone motif that is then converted to the caged structure using the Claisen Diels-Alder reaction cascade. The overall strategy proceeds in 5–6 steps and delivers A-ring modified analogs in good yields. Structure-activity relationship studies reveal that the cytotoxicity in the spheroid assay can be regulated by selective functionalization of the A-ring. Notably, introduction of a free hydroxyl group at the C6 position leads to compounds that exhibit complete response in the spheroid assay at low micromolar concentrations. Specifically, MAD28 and MAD67 were found to induce complete dissolution of the spheroids with IC50 values of 0.51 and 0.38 μM, respectively. Conversely, introduction of a free hydroxyl group at C18 as well as further oxidation of the A-ring leads to a decrease in cytotoxicity as evidenced with MAD44, MAD69, CR240C and related structures. Exchange of hydrogen atoms with fluorines at the A-ring leads to compounds RA25 and RA25N that maintain the complete response phenotype albeit at a slight increase of the effective concentration (IC50 values 1.12 and 0.89 μM, respectively). Additional studies in ECM-immobilized spheroids showed that MAD28, MAD67 and RA25 effectively target both proliferative (peripherally located) and quiescent-prone (centrally located) cells of the multicellular spheroid. Therefore, these compounds hold promise for the treatment of resistant and chemo-refractory quiescent cancer cells [75]. Overall, these findings can be used to accelerate and streamline the development of IBC-based therapeutics based on the caged xanthone pharmacophore.

4. Experimental section

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**Fig. 3.** Dose-dependent response of spheroids MARY-X upon treatment with MAD28, CLV, MAD67, RA25 and RA25N. Representative brightfield images of spheroids MARY-X 24 h post drug treatment at 0.5, 0.75, 1.0 and 2.0 μM concentrations (bar 100 μm).

**Fig. 4.** Dose-response curves and IC50 values of CX analogs inducing complete response. Circularity values obtained via ImageJ analysis were used to calculate the IC50 values for MAD28, MAD67, CLV, RA25 and RA25N. The non-linear fit curves and calculation of IC50 value for each analog were performed using GraphPad Prism.

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**General information for chemical purification and compounds characterization.** Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F–254) and visualized under UV light and/or by treatment with a solution of CAM or KMnO4 stain followed by heating. Preparative thin-layer chromatography separations were carried out on 0.25 or 0.50 mm E. Merck silica gel plates (60F–254). Flash
column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh) using hexane/ethyl acetate or hexane/ethyl ether as standard eluents. $^1$H NMR and $^{13}$C NMR spectra were recorded on a 400 or 500 MHz Varian or a 500 JEOL instrument. Chemical shifts ($\delta$) are quoted in parts per million (ppm) referenced to the appropriate residual undeuterated solvent peak, with the abbreviations s, bs, d, t, q, dd, m, denoting singlet, broad singlet, doublet, triplet, quartet, doublet of doublets, multiplet, respectively. J is a coupling constant given in Hertz (Hz). High resolution mass spectra (HRMS) were recorded on a VG7070HS mass spectrometer under chemical ionization (CI) conditions, on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions, or on a Bruker microTOF mass spectrometer under electrospray ionization (ESI) conditions. Specific information on the synthetic/analytical protocols as well as copies of the spectroscopic data for all compounds are shown in the Supporting Information.

**General procedure for Pd(0)-catalyzed reverse prenylation.** A solution of starting xanthone (1 equiv) in anhydrous THF (approximate 0.2 mM) was degassed by argon and was placed in an ice bath. To this homogeneous solution was added tert-butyl 2-methylbut-3-en-2-yl carbonate (9) or bis(2-methylbut-3-en-2-yl) carbonate (30) (10 equiv) via syringe, followed by 10% mol Pd(PPh$\ce{3}$)$_4$. The mixture was stirred under argon at 0–5 °C and the reaction progress was monitored by TLC. Upon completion the reaction mixture was quenched with water and extracted with EtOAc. The combined organic layers were dried over MgSO$_4$, filtered and concentrated by rotary evaporation. The crude material was purified through flash column chromatography (silica, EtOAc in hexane).

**Table 1**

<table>
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<tr>
<th>CX analogs</th>
<th>R$\ce{1}$</th>
<th>R$\ce{2}$</th>
<th>Response</th>
<th>IC$\ce{50}$ ($\mu$M)</th>
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<tbody>
<tr>
<td>CLV</td>
<td>H</td>
<td>H</td>
<td>CR</td>
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<tr>
<td>MAD28</td>
<td>OH</td>
<td>H</td>
<td>CR</td>
<td>0.21</td>
</tr>
<tr>
<td>MAD44</td>
<td>H</td>
<td>OH</td>
<td>LR</td>
<td></td>
</tr>
<tr>
<td>MAD67</td>
<td>OH</td>
<td>OOMOM</td>
<td>CR</td>
<td>0.38</td>
</tr>
<tr>
<td>MAD69</td>
<td>OH</td>
<td>OH</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>CR170B</td>
<td>OH</td>
<td>O(CH$_2$CH$_2$O)$_3$Me</td>
<td>MR</td>
<td></td>
</tr>
<tr>
<td>CR136</td>
<td>O(CH$_2$CH$_2$O)$_3$Me</td>
<td>H</td>
<td>MR</td>
<td></td>
</tr>
<tr>
<td>CR140</td>
<td>H</td>
<td>O(CH$_2$CH$_2$O)$_3$Me</td>
<td>LR-MR</td>
<td></td>
</tr>
<tr>
<td>P67</td>
<td>OMe</td>
<td>OMe$^a$</td>
<td>LR</td>
<td></td>
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<tr>
<td>CR340C</td>
<td>C=O</td>
<td>C=O$^b$</td>
<td>NR</td>
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<tr>
<td>RA25</td>
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<td>F</td>
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<td>RA25$^a$</td>
<td>F</td>
<td>F</td>
<td>CR</td>
<td>0.89</td>
</tr>
</tbody>
</table>

$^a$ R$\ce{2}$ is located at the C17 center.

$^b$ RA25 $^N$ contains a neo caged motif.

**Fig. 5.** Analysis of induction of apoptosis in spheroids of CX analogs after 3 h of treatment with MAD28, MAD67 and RA25 at 2.5 μM. Brightfield microscopy panels show the loss of well-circumscribed spheroid edge upon treatment in comparison to the control (DMSO). The immunofluorescence microscopy panels show optical slices from the middle of the spheroids and present DAPI stained nuclei (pyknotic nuclei indicated by arrowheads), localization of c-PARP, Ki-67 and the merged pictures. (bar 20 μm).
1-hydroxy-3-(methoxymethoxy)-5,6-bis(2-methylbut-3-en-2-yl)oxy)-9H-xanthene-9-one (10): 89%, a colorless oil. Rf = 0.57 (25% EtOAc–hexane); 1H NMR (400 MHz, CDCl3) δ 7.82 (d, J = 9.0 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 6.56 (d, J = 2.2 Hz, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.29–6.14 (m, 2H), 5.25 (s, 2H), 5.21–5.15 (m, 3H), 5.03 (d, J = 10.9 Hz, 1H), 3.51 (s, 3H), 1.57 (s, 9H), 1.57 (s, 17H); 13C NMR (100 MHz, CDCl3) δ 180.1, 164.0, 163.5, 157.5, 152.5, 143.7, 143.5, 120.4, 116.8, 114.4, 113.9, 98.8, 94.9, 94.4, 83.9, 82.5, 56.7, 27.4, 27.1; HRMS calc for C25H29O7 (M + Na)+ = 441.1988, found 441.1907.

1,2-dihydro-1,5-methanofuro[2,3-\(d\)]xanthene-4,7(3H,5H)-dione (24): 72%, yellow solid. Rf = 0.50 (50% EtOAc–hexane); 1H NMR (400 MHz, CDCl3) δ 115.5 (s, 11H), 7.49 (d, J = 7.0 Hz, 1H), 7.12 (dd, J = 8.9, 1.8 Hz, 1H), 6.47 (dd, J = 8.9, 1.8 Hz, 1H), 4.43–4.39 (m, 1H), 3.84 (s, 3H), 3.55–3.52 (m, 1H), 2.64 (d, J = 9.5 Hz, 2H), 2.47 (d, J = 9.6 Hz, 1H), 2.36 (dd, J = 13.6, 4.2 Hz, 1H), 1.73 (s, 3H), 1.36 (s, 3H), 1.31 (s, 3H), 1.00 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 202.8, 181.6, 156.2, 149.5, 140.7, 135.4, 130.0, 124.1, 119.0, 107.6, 106.5, 90.4, 84.7, 84.0, 57.9, 49.0, 47.1, 29.8, 29.4, 29.3, 25.7, 24.9, 16.8; HRMS calc for [C24H28O7Na]+ (M + Na)+ = 433.1622, found 433.1623.

1.68 (s, 3H), 1.38 (s, 3H), 1.32 (2H), 4.45 (J = 135.4, 121.3, 117.2, 114.5, 113.4, 101.1 (d, JCF = 25.1 Hz), 101.0 (t, JCF = 24.2 Hz), 84.0, 82.6, 77.4, 72.7; HRMS calc for C23H22F2O4Na (M + Na)+ = 423.1378, found 423.1377.

General procedure for Claisen/Diels-Alder reaction. A solution of allylated compound (1 equiv) in DMF was heated at 120°C. The onset of a yellow color indicated the formation of the caged xanthene. The reaction progress was monitored by TLC. Upon completion, the yellow reaction mixture was cooled to room temperature and the solvent was removed under vacuum by rotary evaporation. The crude material was purified by flash column chromatography (silica, EtOAc or Et2O in hexane) to yield the caged xanthene.

8-hydroxy-10-(methoxymethoxy)-2,2-dimethyl-3a-(3-methylbut-2-en-2-yl)-1,2-dihydro-1,5-methanofuro[2,3-\(d\)]xanthene-4,7(3H,5H)-dione (12): 69%, yellow solid. Rf = 0.24 (25% EtOAc–hexane); 1H NMR (400 MHz, CDCl3) δ 12.38 (s, 1H), 7.43 (d, J = 7.1 Hz, 1H), 6.20 (d, J = 2.1 Hz, 1H), 6.17 (d, J = 2.1 Hz, 1H), 5.19 (s, 2H), 4.45–4.41 (m, 1H), 3.51–3.48 (m, 1H), 3.47 (s, 3H), 2.61 (d, J = 8.8 Hz, 2H), 2.43 (d, J = 9.6 Hz, 1H), 2.33 (dd, J = 13.6, 4.7 Hz, 1H), 1.68 (s, 3H), 1.38 (s, 3H), 1.32–1.31 (m, 1H), 1.29 (s, 1H), 1.09 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 174.3, 166.8, 164.3 (dd, JCF = 15.4 Hz, 164.3 (dd, JCF = 15.0, 8.1 Hz), 161.7 (d, JCF = 15.6 Hz), 158.0 (d, JCF = 15.9 Hz), 157.4, 151.8, 143.7, 143.5, 135.4, 121.3, 117.2, 114.5, 113.4, 101.1 (d, JCF = 25.1 Hz), 101.0 (t, JCF = 24.2 Hz), 84.0, 82.6, 77.4, 72.7; HRMS calc for C23H22O7Na (M + Na)+ = 423.1378, found 423.1377.

Culture of spheroids [\(\text{MARY}^X\)]. The spheroids [\(\text{MARY}^X\)] were cultured in Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in an air–5% CO2 atmosphere at constant humidity.

Compound screening and drug response using the spheroids [\(\text{MARY}^X\)] assay. Spheroids [\(\text{MARY}^X\)] ranging in size from 40 to 100 μm were seeded at a density of 30–50 spheroids/well in a multi-well plate and treated in replicates with different drug concentrations or vehicle only (DMSO) as a control. Brightfield images were acquired 24 h post treatment and the response to the drugs was evaluated based on deviations from the circularity of intact, well-circumscribed spheroids [\(\text{MARY}^X\)]. Circularity measurements for compounds that led to complete dissolution, turning the spheroids [\(\text{MARY}^X\)] to single cell suspension were obtained using ImageJ. The parameters of the image analysis are set so that ‘1’ indicates a perfect circle (i.e. intact spheroid; no response) and numbers approaching ‘0’ indicate loss of circularity (i.e. dissolve spheroid; response). Response to drug treatment is ranked as no response, low response, moderate response or complete response, based on the degree of deviation from a well-circumscribed spheroid edge. The circularity measurements were then transferred into GraphPad Prism v. 7.04 in order to construct a dose-response curve and calculate the EC50 values of each drug.

Immunofluorescence in extracellular matrix plugs. Extracellular matrix (Matrigel; Corning LifeSciences, cat.# 356237) was thawed at 4°C overnight and kept on ice at all times during use. A mixture of matrigel with spheroids [\(\text{MARY}^X\)] in media at a 2:1 ratio was
prepared and added on top of a glass coverslip into each well of a plate. The plate with the matrigel plugs were then incubated at 4°C for 24 h with Alexa Fluor 488 conjugated anti-Ki67 (BD Pharmingen, #558616) and cPARP (Promega, #G7341) at 1:10 and 1:100, respectively. Incubation with Cy5 conjugated goat anti-rabbit (Invitrogen, A10523) secondary at 4 μg/ml followed at 4°C for 24 h. After three washes in 1× PBS, the treated cells were blocked with 3% normal goat serum in 1× PBS for 30 min at room temperature, followed by PBS washes and 15 min permeabilization PBS, a drop of Vectashield mounting medium with DAPI (Vector Laboratories, H-1200) was applied on top of the Matrigel plugs with the treated cells and then the coverslips were flipped and sealed on slides. The slides were visualized and subjected to z-stacking using a Zeiss AxioObserver Z1 with ApoTome2 inverted microscope.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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References
