Editor's Summary

A Cancer Double Feature—3807

A traditional chemotherapeutic drug performs a one-act play: It enters and kills a dividing cancer cell and then takes its bow. However, some chemotherapeutics have a wider range—they not only kill individual cancer cells but also do so in such a way that the dead cells function as a vaccine that primes the immune system to attack other cancer cells. Menger et al. now identify cardiac glycosides as potent inducers of this so-called immunogenic cell death.

Using fluorescence microscopy to detect the hallmarks of immunogenic cell death, the authors identified cardiac glycosides, such as the heart drug digoxin, as immunogenic cell death inducers. They then verified that these drugs had anticancer effects in mice with intact immune systems but not in mice that lacked functional immunity. Cancer cells that died from digoxin exposure then effectively functioned as a vaccine—stimulating the immune system so that growth of future cancers is prevented. Indeed, human cancer patients on chemotherapy who happened to be taking the cardiac glycoside digoxin to treat other medical conditions had improved overall survival compared with patients who were not taking these drugs. Although efficacy in cancer patients remains to be formally tested, cardiac glycosides may augment chemotherapeutic response—forcing cancer to bow out.

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Cardiac Glycosides Exert Anticancer Effects by Inducing Immunogenic Cell Death

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Some successful chemotherapeutics, notably anthracyclines and oxaliplatin, induce a type of cell stress and death that is immunogenic, hence converting the patient’s dying cancer cells into a vaccine that stimulates antitumor immune responses. By means of a fluorescence microscopy platform that allows for the automated detection of the biochemical hallmarks of such a peculiar cell death modality, we identified cardiac glycosides (CGs) as exceptionally efficient inducers of immunogenic cell death, an effect that was associated with the inhibition of the plasma membrane Na+ and K+-dependent adenosine triphosphatase (Na+/K+-ATPase). CGs exacerbated the antineoplastic effects of DNA-damaging agents in immunocompetent but not immunodeficient mice. Moreover, cancer cells succumbing to a combination of chemotherapy plus CGs could vaccinate syngeneic mice against a subsequent challenge with living cells of the same type. Finally, retrospective clinical analyses revealed that the administration of the CG digoxin during chemotherapy had a positive impact on overall survival in cohorts of breast, colorectal, head and neck, and hepatocellular carcinoma patients, especially when they were treated with agents other than anthracyclines and oxaliplatin.

INTRODUCTION

Primary or transplantable tumors respond to chemotherapy with anthracyclines or oxaliplatin much more efficiently when they are implanted in syngeneic immunocompetent mice rather than in immunodeficient hosts (1–3). Along similar lines, clinical studies demonstrate that severe lymphopenia negatively affects prognosis in multiple distinct solid cancers subjected to chemotherapy (4). These findings suggest that immune responses elicited by chemotherapy may play a decisive role in the outcome of anticancer therapy (5, 6).

Although most chemotherapeutics fail to trigger immunogenic cell death (ICD), some (like anthracyclines and oxaliplatin) are able to do so (1, 2, 7). Cancer cells succumbing to ICD can vaccinate mice against a subsequent challenge with living cells of the same type (1, 2, 7) or elicit anticancer immune responses in patients (8). Extensive biochemical analyses have revealed the distinctive properties of immunogenic (as opposed to nonimmunogenic) cell death. These turned out to be the preapoptotic exposure of calreticulin (CRT) at the cell surface (2, 8, 9), the secretion of adenosine 5′-triphosphate (ATP) from stressed and dying cells (7, 10), and the cell death–associated release of the nuclear high mobility group box 1 (HMGB1) protein (2, 11). CRT stimulates the engulfment of dying cancer cells by dendritic cells (DCs) (2). HMGB1 acts on Toll-like receptor 4 (TLR4), hence eliciting a myeloid differentiation primary response gene 88 (MyD88)–mediated response that underpins optimal tumor antigen processing by DCs (2, 11). ATP binds purinergic P2RX7 receptors [thus activating the nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inflammasome] to stimulate the production of interleukin-1β (IL-1β), which is required for stimulating interferon-γ (IFN-γ) production by tumor-specific CD8+ T lymphocytes (7). Knockout of Tlr4 or P2rx7 (as well as that of genes coding for their downstream signal transducers including Myd88, Nlrp3, Il1r1, and Ifng) phenocopies the ablation of the cellular immune system, meaning that tumors growing in Tlr4−/−, P2rx7−/−, Myd88−/−, Nlrp3−/−, Il1r1−/−, Ifng−/−, Il1r1−/−, athymic, or CD8− T cell–depleted mice all fail to respond to immunogenic chemotherapeutic regimens. Along similar lines, single-nucleotide polymorphisms that compromise the function of human TLR4 or P2RX7 are negative predictors of the clinical response to adjuvant chemotherapy with anthracyclines (in breast cancer) or oxaliplatin (in colorectal cancer) (2, 7, 12). These results suggest the possible translational relevance of the data obtained in mouse models, implying the obligate contribution of anticancer immune responses to the success of ICD-inducing chemotherapies.

The capacity of anthracyclines and oxaliplatin to induce ICD relies, at least in part, on off-target effects. These agents indeed trigger an endoplasmic reticulum (ER) stress response that leads to CRT exposure even in cytoplasts, that is, cells that have been enucleated and hence lack the best-known target of these chemotherapeutics (DNA) (2, 13). Although all anthracyclines that have been tested so far induce ICD (2), platinum compounds are heterogeneous in this regard. Thus, in contrast to oxaliplatin, cisplatin [cis-diaminedichloroplatinum(II) (CDDP)] is unable to induce an ER stress response followed by ICD (12, 14), meaning that there is no simple structure-function relationship that would predict...
the capacity to cause ICD. As a result, ICD inducers have thus far been identified empirically by determining whether mouse cancer cells responding to them would elicit a protective immune response in vivo (1, 2, 15).

To overcome this limitation, we designed an automated epifluorescence microscopy–based platform allowing for the detection of the known biochemical hallmarks of ICD in human cancer cells stably expressing fluorescent biosensors. Using this platform, which is suitable for high-content, medium-throughput analyses, we identified cardiac glycosides (CGs) as a class of agents that induce bona fide ICD and improve the clinical outcome of anticancer chemotherapies.

RESULTS

Measuring ICD parameters by fluorescent biosensors

The known parameters determining ICD are the co-translocation of CRT and the ER chaperon ERP57 at the cell surface (2, 16), the autophagy-dependent secretion of ATP (7, 10), and the release of the non–histone chromatin protein HMGB1 (2), which occur before, during, and after apoptosis, respectively (2, 7, 13).

Human osteosarcoma U2OS cells were used to screen a chemical library encompassing all the 120 anticancer drugs that are currently used in the clinic (final concentration, 1 μM). The drugs were exposed to human osteosarcoma U2OS cells expressing CRT-GFP and H2B-RFP (histone 2B–red fluorescent protein) or HMGB1-GFP and H2B-RFP as well as wild-type (WT) U2OS cells. Results (means, n = 3; each point represents one agent) have been sorted according to mean Z score for each agent to highlight the nonlinear time dependency of the appearance of distinct ICD characteristics. (C) Hierarchical clustering of drug effects. (D) Hits. Green and red values indicate positive and negative effects (Z score), respectively.
concentrations, 1 and 10 μM), and ICD-related parameters were measured (time points, 4, 12, and 24 hours) by robotized epifluorescence microscopy followed by automated image analysis (Fig. 1A). The translocation of a CRT-GFP (green fluorescent protein) fusion protein (14) from the perinuclear ER to the cellular periphery paralleled a similar relocalization of ERp57-GFP (16), as well as the exposure of a CRT-HaloTag fusion protein on the cell surface (detected with a plasma membrane impermeant HaloTag ligand) (14) (Fig. 1B). The staining obtained with an ATP-sensitive fluorophore, quinacrine (17), was comparable to the ATP-dependent signal given by a fluorescence resonance energy transfer (FRET)–based biomarker relying on the ε subunit of the bacterial F$_{1}$F$_{0}$–ATP synthase flanked by cyan and yellow fluorescent proteins (18). The reduction of intracellular ATP could be detected with quinacrine as well as by means of a luciferase-based assay to measure extracellular ATP concentrations. Moreover, the loss of quinacrine-dependent fluorescence was paralleled by the aggregation of a GFP-LC3 fusion protein in cytoplasmic puncta (autophagosomes or autophagolysosomes) (19) (Fig. 1C), in accord with the observation that autophagy is required for ATP release from dying tumor cells (10). HMGB1 release was detected as a loss in the nuclear fluorescence of an HMGB1-GFP chimera (20) or as an accumulation of extracellular HMGB1 [detected by enzyme-linked immunosorbent assay (ELISA)] and occurred in conjunction with plasma membrane breakdown [as measured with the vital dye propidium iodide (PI)], in turn reflecting primary or secondary (postapoptotic) necrosis (21) but not with apoptotic chromatin condensation (Fig. 1D). Among the seven compounds that most effectively triggered the known hallmarks of ICD, as determined by the hierarchical clustering of data on CRT translocation, nuclear HMGB1 loss, and cellular ATP release (Fig. 2, A and B), were several known ICD inducers (Fig. 2, C and D), namely, three anthracyclines [daunorubicin, doxorubicin, and mitoxantrone (MTX)] (1, 2), as well as bortezomib (22). In addition, this list included two vinca alkaloids with known immunogenic side effects (vincristine and vinorelbine) (23, 24), as well as the tyrosine kinase inhibitor crizotinib, for concentrations, 1 and 10 μM), and ICD-related parameters were measured (time points, 4, 12, and 24 hours) by robotized epifluorescence microscopy followed by automated image analysis (Fig. 1A). The translocation of a CRT-GFP (green fluorescent protein) fusion protein (14) from the perinuclear ER to the cellular periphery paralleled a similar relocalization of ERp57-GFP (16), as well as the exposure of a CRT-HaloTag fusion protein on the cell surface (detected with a plasma membrane impermeant HaloTag ligand) (14) (Fig. 1B). The staining obtained with an ATP-sensitive fluorophore, quinacrine (17), was comparable to the ATP-dependent signal given by a fluorescence resonance energy transfer (FRET)–based biomarker relying on the ε subunit of the bacterial F$_{1}$F$_{0}$–ATP synthase flanked by cyan and yellow fluorescent proteins (18). The reduction of intracellular ATP could be detected with quinacrine as well as by means of a luciferase-based assay to measure extracellular ATP concentrations. Moreover, the loss of quinacrine-dependent fluorescence was paralleled by the aggregation of a GFP-LC3 fusion protein in cytoplasmic puncta (autophagosomes or autophagolysosomes) (19) (Fig. 1C), in accord with the observation that autophagy is required for ATP release from dying tumor cells (10). HMGB1 release was detected as a loss in the nuclear fluorescence of an HMGB1-GFP chimera (20) or as an accumulation of extracellular HMGB1 [detected by enzyme-linked immunosorbent assay (ELISA)] and occurred in conjunction with plasma membrane breakdown [as measured with the vital dye propidium iodide (PI)], in turn reflecting primary or secondary (postapoptotic) necrosis (21) but not with apoptotic chromatin condensation (Fig. 1D). Among the seven compounds that most effectively triggered the known hallmarks of ICD, as determined by the hierarchical clustering of data on CRT translocation, nuclear HMGB1 loss, and cellular ATP release (Fig. 2, A and B), were several known ICD inducers (Fig. 2, C and D), namely, three anthracyclines [daunorubicin, doxorubicin, and mitoxantrone (MTX)] (1, 2), as well as bortezomib (22). In addition, this list included two vinca alkaloids with known immunogenic side effects (vincristine and vinorelbine) (23, 24), as well as the tyrosine kinase inhibitor crizotinib, for
we exposed human osteosarcoma U2OS cells for 4, 12, or 24 hours to a To identify novel agents endowed with the capacity to stimulate ICD, we used a high-throughput screening approach. Suitable ICD inducers yielded high scores and were identified on the basis of their capacity to induce ICD characteristics through their effects on cell surface-exposed CRT, luciferase-based detection of extracellular ATP, and ELISA for the quantification of HMGB1 in culture supernatants. Our screening approach confirmed that DIG and DIGT stimulate the three ICD hallmarks as efficiently as MTX, which we used as a positive control (Fig. 3E). Moreover, in experiments in which the hallmarks of ICD were measured by alternative methods (immunofluorescence staining of cell surface-exposed CRT, luciferase-based detection of extracellular ATP, and ELISA for the quantification of HMGB1 in culture supernatants) corroborated the capacity of DIG and DIGT to induce manifestations of ICD in several human cancer cell lines (Figs. 3, F to H, and 4). Moreover, CGs induced the exposure of heat shock protein 90 kD (Hsp90), yet another hallmark of ICD (22), on the cell surface (Fig. S1). Together, these results suggest that CGs might constitute novel inducers of ICD, prompting us to investigate the underlying molecular mechanisms and the in vivo relevance of our observations.

Mechanisms of CG-induced ICD

ICD inducers are known to activate a pre-mortem ER stress response (2, 12, 13), and CGs did activate three arms of the ER stress response (that is, phosphorylation of eIF2α (eukaryotic initiation factor 2α), nuclear translocation of ATF6 (activating transcription factor 6), and splicing of XBP1 (X-box binding protein 1)) at least as efficiently as MTX (fig. S2). The broad-spectrum caspase inhibitor Z-VAD-fmk (N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) as well as the antioxidants N-acetylcysteine and reduced glutathione inhibited not only the cytotoxicity of CGs but also CRT exposure and ATP release (Fig. 5A and fig. S3). Thus, the induction of ICD hallmarks by DIG or DIGT is intimately linked to their cytotoxic potential. CGs are known to target the plasma membrane Na⁺,K⁺-ATPase subunit, thus reducing the Na⁺ and K⁺ gradients and stimulating Ca²⁺ influx (25). Taking advantage of the fact that DIG and DIGT have a far lower affinity for the murine Na⁺,K⁺-ATPase subunit (a) than for its human counterpart (26, 27), we determined whether ICD induction by CGs is mediated by on-target or off-target effects. Human U2OS cells transfected with mouse a1 (but not with human a1 or another mouse subunit, a2) were indeed partially protected from CG cyto toxicity (Fig. 5B and fig. S4) and exhibited reduced CRT exposure (Fig. 5C), ATP secretion (Fig. 5D), and HMGB1 release (Fig. 5E). These results suggest that CGs induce ICD characteristics through their effects on Na⁺,K⁺-ATPase-regulated ion fluxes. Accordingly, the Ca²⁺ ionophore A23187 (but neither the K⁺ ionophore nigericin nor the protonophore
carbonyl cyanide m-chlorophenylhydrazone) was able to induce CRT exposure, ATP secretion, and HMGB1 release (Fig. 5A and fig. S5A), and these effects were prevented by both intracellular and extracellular Ca$^{2+}$ chelators (fig. S5B). To further investigate the mechanisms through which CGs induce manifestations of ICD, we reverse-transfected U2OS cells with an array of 500 siRNA (small interfering RNA) duplexes, corresponding to 250 target genes involved in cell death signaling, and monitored their response to MTX and DIG (the most widely used CG in the clinic) (28) in terms of CRT exposure, ATP secretion, and HMGB1 release. As revealed by unsupervised hierarchical clustering, the signaling pathways that regulate CRT exposure, ATP secretion, and HMGB1 release in response to DIG and MTX are overall relatively dissimilar, yet present consistent overlaps (Fig. 5F and table S2). For instance, DIG- and MTX-induced CRT exposure, the prime ICD characteristic, was disrupted by the knockdown of a largely overlapping cluster of target genes (Fig. 5G). Among the siRNAs that most effectively prevented DIG- and MTX-stimulated CRT exposure, two targeted death domain–containing proteins (FADD and CRADD), and two others targeted proteins that have been previously implicated in MTX-induced CRT exposure, namely, Erp57 (16) and BAK1, a proapoptotic multidomain protein from the BCL-2 family (13). Although the interpretation of this data set is limited by the suboptimal efficacy of reverse transfection arrays, these results underscore that CGs and anthracyclines stimulate overlapping pathways of ICD signaling.
ICD induced by CGs in vivo

In tumor vaccination experiments, cancer cells were exposed to cytotoxic chemotherapeutics in vitro until 70 ± 10% of the cells expose phosphatidylserine on the outer leaflet of the plasma membrane, as assessed by means of annexin V binding. Dying cancer cells were then injected subcutaneously into mice, and 1 week later, the animals were rechallenged with live tumor cells of the same kind, injected into the opposite flank. The absence of tumor growth is interpreted as the sign of a productive anticancer immune response (1, 2, 14). In a first series of experiments involving DIG- or DIGT-exposed cancer cells, mice developed cancers at the primary injection site, presumably because the washing out of CGs allowed for the rapid recovery of viable cells and their growth in vivo. In contrast, cells treated with the DNA-damaging agents CDDP and MitoC (mitomycin C) failed to form tumors at the primary injection site. In this assay, however, CDDP and MitoC were unable to induce ICD, meaning that tumor cells killed by these agents and injected into mice failed to trigger a protective anticancer immune response (1, 2, 14). Accordingly, CDDP and MitoC were unable to induce preapoptotic CRT exposure in vitro, although they did trigger ATP secretion and HMGB1 release. This defect in CRT exposure was corrected in the presence of DIG or DIGT (Fig. 6A). Accordingly, when murine cancer cells were treated with a combination of CDDP or MitoC plus DIG or DIGT, such cells lost their growth potential and acquired the capacity to stimulate a productive immune response in vaccination experiments (Fig. 6, B and C). This immune response was partially lost upon antibody-mediated depletion of CD8⁺ T cells (fig. S6A). After systemic treatment of tumor-bearing mice with MitoC plus DIG, inguinal lymph node cells secreted higher amounts of IFN-γ compared to cells isolated from mice receiving MitoC alone, as shown upon recovery of draining popliteal lymph node cells and stimulation with anti-CD3 plus anti-CD28 antibodies (fig. S6B). Thus, CGs restored the capacity of dying cells to stimulate anticancer immune responses in conditions in which nonimmunogenic chemotherapeutics alone failed to do so.

Subsequently, we evaluated the capacity of DIG to improve the response to therapy of established cancers growing on immunocompetent or immunodeficient mice. In conditions in which systemic chemotherapy with MitoC, CDDP, or DIG alone had minor effects on tumor growth, the combination of MitoC or CDDP plus DIG induced a significant therapeutic benefit (Fig. 6, D and E). This effect was observed when tumors grew on immunocompetent mice, yet was lost when the tumors proliferated on athymic (nu/nu) mice (Fig. 6, F and G). These results underscore the obligate contribution of the immune system to the chemotherapeutic activity of DIG. Indeed, the administration of MitoC plus DIG induced tumor infiltration by IL-17A–producing γ/δ T cells and IFN-γ–producing CD4⁺ or CD8⁺ T cells as
efficiently as MTX did (fig. S6C), demonstrating the elicitation of a local anticancer immune response.

Next, we used a text-based research algorithm to identify all carcinoma patients who, between 1981 and 2009, received CGs (in particular, DIG) during conventional carcinoma therapies, as documented in the computerized archives held at Institut Gustave Roussy. We compared the overall survival of 145 patients treated with CGs with that of 290 patients who did not receive CGs. Patients were matched (1:2) for tumor type, stage, anticancer therapy, gender, and major prognostic parameters [such as estrogen receptor and human epidermal growth factor receptor 2 (HER2) expression for breast cancer, etiology and α-fetoprotein for hepatocellular cancer, and Gleason score for prostate cancer] (Fig. 7A and figs. S7 to S9).

DIG-treated patients exhibited a significantly enhanced overall survival (Fig. 7B) compared to age- and sex-matched patients with similar tumor characteristics (tumor type and stage) and receiving similar treatment but no CGs. Patients who did not receive CGs presented a 52% (45 to 58%) survival at 5 years, whereas patients treated with CGs presented a 65% (56 to 73%) overall survival at 5 years (log-rank \( P = 0.002 \)). The hazard ratio for death was 0.62 [95% confidence interval (95%CI), 0.46 to 0.84] for patients treated with CGs. DIG was efficient in ameliorating the overall survival of patients treated with chemotherapy alone but not of those treated with radiotherapy alone (fig. S7). Moreover, DIG had a significant positive impact on the overall survival of patients with breast, colorectal, head and neck, and hepatocellular cancers, yet failed to improve the overall survival of lung and prostate cancer patients (Fig. 7C and figs. S8 to S10). Further subgroup analyses revealed that DIG failed to affect the overall survival of patients who received chemotherapy with immunogenic agents, namely, anthracyclines for breast cancer and oxaliplatin for colorectal cancer. In contrast, DIG had a significant positive impact on patients who received treatment with agents other than anthracyclines or oxaliplatin (Fig. 7, D and E). Together, these results suggest that DIG can ameliorate the efficacy of nonimmunogenic anticancer therapies.

**DISCUSSION**

The present study describes a fluorescent biosensor–based platform for the identification of ICD inducers. Three major hallmarks of ICD (CRT exposure, ATP secretion, and HMGB1 release) were measured by robotized fluorescence microscopy coupled to automated image analysis on cancer cells expressing appropriate biosensors, using a setup that is suitable for high-content, medium-throughput screening and that can be scaled up for high-throughput purposes.
Using a collection of FDA-approved and experimental drugs, we identified CGs as candidate ICD inducers and then validated their capacity to induce ICD in preclinical models and in clinical settings, hence providing a proof of principle of the approach.

Although the initial discovery and pharmacological development of CGs are unrelated to oncology, several investigators have advocated the use of CGs for the treatment of cancer (29, 30). Thus, CGs can effectively reduce the growth of xenografted human cancer cells in mice (30–32), a finding that, however, has been criticized because CGs have a higher affinity for the human Na⁺, K⁺-ATPase than for its murine equivalent (26). Nonetheless, it has been reported that, as opposed to normal cells, human cancer cells express a combination of Na⁺, K⁺-ATPase subunits that has a particularly high affinity for CGs, rendering them selectively susceptible to CG cytotoxicity (29). CGs are widely used in the general population, in particular, elderly patients (28), and several clinical studies have suggested that CGs may affect the clinical course of cancer. Thus, long-term use of CGs can increase the incidence of breast cancer (33) and reduce that of prostate cancer (33), perhaps because CGs are weak agonists of estrogen receptors.

Here, we provide evidence that clinically used CGs such as DIG and DIGT can stimulate ICD in vitro through their inhibitory effect on Na⁺, K⁺-ATPase and consequent Ca²⁺ influx through the plasma membrane, in line with the documented proapoptotic and proinflammatory effects of raised cytoplasmic Ca²⁺ levels (14, 34). According to one recent study, CGs can inhibit the ATPase activity of the pattern recognition receptor RIG-1 (retinoic acid–inducible gene 1), a sensor of viral RNA, thus reducing IFN-β expression, and may inhibit tumor necrosis factor (TNF) signaling, at least in part by interfering with the nuclear translocation of the transcription factor NF-κB (nuclear factor κB) (27). These anti-inflammatory effects are likely not to affect the anticancer immune responses, because neither the knockout of the common receptor for class I IFNs nor that of TNF has a major negative impact on the antitumor effects of anthracyclines in mice (7). Moreover, it appears plausible that the doses of CGs that are effective in human cancer cells (29, 30) are lower than those that mediate anti-inflammatory outcomes in normal cells of the immune system.

The clinical data supporting a positive impact of CGs on the outcome of some chemotherapeutic regimens have been obtained by retrospective analyses of patient cohorts who received CGs in one single hospital. Although this design facilitated the identification of matched controls (who shared biological and clinical parameters and were treated by the same department in the same period), it requires further validation. Prospective analyses must confirm that the administration of CGs along with chemotherapy ameliorates overall survival in cancer patients within the disease categories identified in this paper. We surmise that prospective clinical trials would have high chances to unravel beneficial effects for CGs in patients with locally advanced head and neck cancer who are treated with CDDP (which does not induce ICD on its own).

The fluorescent biosensor–based platform that we describe here has multiple potential applications. First, it can be used in its present setup to screen other drug collections. Thus far, we have only investigated a limited collection of agents, among which about 200 had a clear cytotoxic effect on the cells used in this screen, namely, human U2OS osteosarcoma cells. Results from previous functional screens suggest that only a minority (namely, 4 of 28 tested) of cytotoxic agents can stimulate ICD (1, 2, 12), indicating that this is an exceptional event that differs from nonimmunogenic apoptotic death because it occurs in normal development and adult tissue homeostasis. In accord with this concept, we observed that most apoptotic inducers used in this screen failed to concomitantly stimulate all the hallmarks of ICD. It will be interesting to determine the ICD-inducing capacity of large collections of cytotoxic agents to identify new drugs that elicit an immunological bystander effect. Moreover, in the pipeline of drug discovery, it might be advisable to decide on the clinical development of compounds that share target and mechanism of action based on their (perhaps differential) ICD-stimulatory capacity.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig. S1. Cardiac glycosides induce CRT exposure.
Fig. S2. ER stress induction by cardiac glycosides.
Fig. S3. Cardiac glycosides induce CRT exposure.
Fig. S4. Human cells expressing murine Na⁺, K⁺-ATPase subunit α₁ are less sensitive to cardiac glycosides.
Fig. S5. A role for calcium fluxes in cardiac glycoside–induced immunogenic cell death.
Fig. S6. T cell populations and cytokines involved in the digoxin-stimulated anticancer immune response.
Fig. S7. Comparison of digoxin effects on the survival of cancer patients treated with chemotherapy alone or radiotherapy alone.
Fig. S8. Effect of digoxin on the survival of patients with non–small cell lung cancer (NSCLC) or prostate cancer.
Fig. S9. Effect of digoxin on the survival of patients with breast cancer or colorectal cancer (CRC).
Fig. S10. Effect of digoxin on the survival of patients with head and neck cancer or prostate cancer.

Table S1. Drug screen identifies cardiac glycosides as immunogenic cell death inducers.
Table S2. RNA interference arrays identify overlapping signaling pathways toward immunogenic cell death.

**REFERENCES AND NOTES**

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