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Original article

Chemical screening identifies the β-Carboline alkaloid harmine to be synergistically lethal with doxorubicin

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ABSTRACT

Despite being an invaluable chemotherapeutic agent for several types of cancer, the clinical utility of doxorubicin is hampered by its age-related and dose-dependent cardiotoxicity. Co-administration of dexrazoxane as a cardioprotective agent has been proposed, however recent studies suggest that it attenuates doxorubicin-induced antitumor activity. Since compounds of natural origin present a rich territory for drug discovery, we set out to identify putative natural compounds with the view to mitigate or minimize doxorubicin cardiotoxicity. We identify the DYRK1A kinase inhibitor harmine, which phosphorylates Tau that is deregulated in Alzheimer's disease, as a potentiator of cell death induced by non-toxic doses of doxorubicin. These observations suggest that harmine or other compounds that target the DYRK1A kinase my offer a new therapeutic opportunity to suppress doxorubicin age-related and dose-dependent cardiotoxicity.

1. Introduction

Anthracyclines are a class of cancer chemotherapeutics considered to be one of the most efficacious anti-tumor drugs ever developed (Minotti et al., 2004). Doxorubicin is one of the first anthracyclines to be discovered and is currently used clinically for several solid and liquid tumors (Arcamone et al., 1969). It is the first line treatment for breast cancer and also used for soft tissue sarcomas and aggressive lymphomas (Minotti et al., 2004; Carvalho et al, 2014). The anti-tumorogenic properties of doxorubicin are attributed at least in part due to targeting Topoisomerase II (TOP2); an essential cellular enzyme that is required for regulating DNA topology during various cellular processes such as transcription, replication and recombination (Tewey et al., 1984; Wang 2002; Corbett and Berger, 2004; Vos et al., 2011). The mammalian genome encodes two isoenzymes of TOP2: TOP2α which is highly expressed in rapidly dividing cells and TOP2β which resides in quiescent cells such as cardiac cells (Pommier et al., 2010; Vos et al., 2011). Notably, doxorubicin possesses other TOP2-independent tumoricidal mechanisms such as DNA intercalation, mitochondrial targetting, and the induction of histone eviction from open chromatin (Nitiss, 2009; Pang et al., 2013). Doxorubicin exhibits its antitumor activity primarily by targetting TOP2α in cancer cells which leads to an increase in DNA double-stranded breaks (Tewey et al., 1984).

Nevertheless, age-related cardiotoxicity in the form of chronic cardiomyopathy and congestive heart failure has been strongly associated with doxorubicin treatment (Lefrak et al., 1973; Steinherz et al., 1991; Hequet et al., 2004). Intially, doxorubicin-associated cardiac damage was attributed to the induction of mitochondrial dysfunction (Wallace, 2003) and increased ROS production in cardiac cells (Tokarska-Schlattner et al., 2006). Accumulation of mitochondiral DNA damage is an established cause for age-related degenerative diseases Doroshow, (1983); Keizer et al., (1990) (El-Khamisy, 2011; Akbari et al., 2015). However, a recent study demonstrated that these effects are secondary to TOP2β isoenzyme inhibition in the heart muscle (Zhang et al., 2012).

Development of doxorubicin-induced cardiotoxicity is largely dose-dependent (Lefrak et al., 1973), but it may also occur at low doses in the presence of other risk factors especially increasing age (Von Hoff et al., 1979; Carvalho et al., 2014). It has been shown that 5%, 26% and 48% of patients developed congestive heart failure after receiving cumulative doses of 400 mg/m^2, 550 mg/m^2 and 700 mg/m^2 of doxorubicin respectively (Swain et al., 2003). Several

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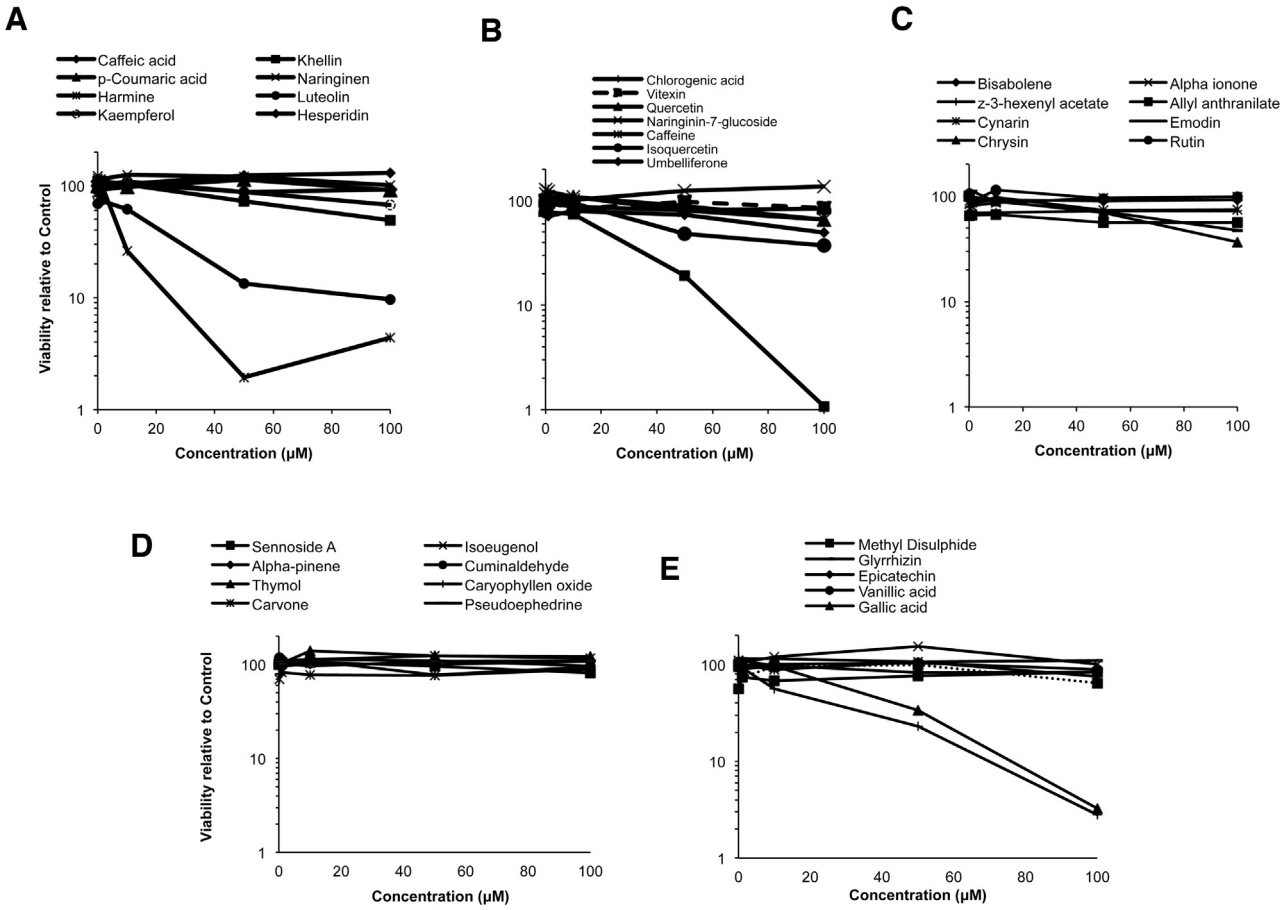

Fig. 1. Determination of the cytotoxicity of natural compounds on the breast cancer cell line, MCF-7. MCF-7 cells were treated with the indicated compounds at 0.1, 1, 10, 50 and 100 μM for 48 h followed by the determination of cell viability using the CellTiter-Blue® Cell Viability Assay (Promega). Viability at a given concentration is determined as a percentage relative to that of a DMSO control.

studies pointed towards age being one of the most significant risk factors in developing doxorubicin-mediated cardiac damage with children and elderly patients being the most suceptible to developing this side effect (Von Hoff et al., 1979; Kremer et al., 2002; Swain et al., 2003; Doyle et al., 2005; Lipshultz, 2006). For elderly patients, reduced doxorubicin clearance caused by a decline in the regional blood flow may be the primary reason for their increased susceptibility to cardiotoxicity (Robert and Hoerni, 1983; Li and Gwilt, 2003). Interestingly, postmortem analysis of patients treated with doxorubicin exhibited high accumulation of the drug in the cardiac muscle (Hong et al., 2002).

One proposed strategy to minimize this deleterious side effect is the co-administration of dexrazoxane, an FDA-approved cardioprotective agent (Speyer et al., 1988; Speyer et al., 1992). Dexrazoxane acts by complexing the TOP2β ATPase domain and thus blocking doxorubicin binding to the cardiac TOP2. However, the ATPase domain is identical in both TOP2α and TOP2β which means that dexrazoxane administration could diminish doxorubicin's tumoricidal activity (Lyu et al., 2007; Vejpongsa and Yeh, 2014). Moreover, studies regarding the combined anticancer activity of doxorubicin and dexrazoxane have reported contradictory results (Wadler et al., 1986; Hasinoff et al., 1996; Pearlman et al., 2003). The problem of doxorubicin-induced cardiotoxicity still poses a serious unmet challenge limiting its clinical utility.

Natural products have been historically the most rich source of drug leads owing to the fact that they exhibit unparalleled structural diversity compared to combinatorial chemistry (Mishra and Tiwari, 2011; Dias et al., 2012). Indeed, nature presents an untapped reservoir of chemical structures with potential biological activities (Dixon, 2001). Plant extracts such as that of *Taxus baccata* (European yew) tree and *Catharanthus roseus*, also known as *Vinca rosea*, were found to exhibit cytotoxic effects (Mantle et al., 2000). Current regimens for the treatment of breast cancer also include that of vinblastine, another indole alkaloid from *vinca* plant and a known component of a number of chemotherapies (Ospovat et al., 2009). The hunt for other potential cytotixic drug entities from natural origin will continue to grow. In the current study, we aimed to identify a synthetic lethal interaction between doxorubicin and a natural product using the breast cancer cell line MCF-7 with the view to obtain preliminary evidence to supress cardiotoxicity. For this purpose, compounds from a library of natural product isolates derived mostly from plant origin were assessed in combination with doxorubicin at sub-toxic concentrations. We identify harmine, a β-carboline alkaloid, to be synergistically toxic in short-term viability assays and additively toxic in long-term clonogenic survival assays, with doxorubicin. We propose that co-adminisstration of harmine and doxorubicin will permit the use of lower doses of the latter and thus help reduce its associated dose-dependent cardiac damage.

2. Materials and methods

2.1. Chemicals

Caffeic acid, p-coumaric acid, naringinen, chlorogenic acid, quercetin, isoquercetin, umbelliferone, harmine, luteolin, caf-

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Table 1
Summary of the natural compounds used in the study.

Compound	Name	CAS Number	Class	Sub-lethal Concentration (μM)
1	Caffeic acid	331-39-5	Phenolic acid	100
2	Khellin	82-02-0	Chromone	10
3	p-Coumaric acid	7400-08-0	Phenolic acid	100
4	Naringenin-7-O-glycoside	529-55-5	Flavonoid	10
5	Allyl anthranilate	7493-63-2	Terpenoid	0.1
6	Cynarin	1182-34-9	Phenolic acid	0.1
7	Caffeine	58-08-2	Alkaloid	10
8	Isoquercitrin	482-35-9	Flavonoid	10
9	Umbelliferone	93-35-6	Coumarin	0.1
10	2,4,4′ trihydroxy chalcone	961-29-5	Flavonoid	0.1
11	Emodin	518-82-1	Aanthraquinone	10
12	Chrysin	480-40-0	Flavonoid	10
13	Sennoside A	81-27-6	Aanthraquinone	50
14	Isoeugenol	97-54-1	Phenylpropanoid	100
15	Pseudoephedrine	90-82-4	Alkaloid	100
16	Glycyrrhizin	1405-86-3	Saponin	100
17	Epicatechin	490-46-0	Flavonoid	50
18	Rutin	153-18-4	Flavonoid	100
19	Vanillic Acid	121-33-5	Phenylpropanoid	100
20	Gallic acid	149-91-7	Phenolic acid	10
21	Xanthohumol	6754-58-1	Flavonoid	1
22	Beta-glycyrrhetinic acid	471-53-4	Terpenoid	100
23	Naringenin	67604-48-2	Flavanoid	100
24	Harmine	442-51-3	Alkaloid	0.1
25	Luteolin	491-70-3	Flavooid	50
26	Kaempferol	520-18-3	Flavonoid	50
27	Hesperidin	520-26-3	Flavonoid	100
28	Bisabolene	17627-44-0	Terpenoid	100
29	Alpha-ionone	127-41-3	Terpenoid	100
30	z-3-Hexenyl acetate	3681-71-8	Terpenoid	0.1
31	Alpha-Pinene	80-56-8	Terpenoid	100
32	Cuminaldehyde	122-03-2	Phenylpropanoid	100
33	Thymol	89-83-8	Phenylpropanoid	100
34	Caryophyllene oxide	17627-43-9	Terpenoid	10
35	Carvone	99-49-0	Terpenoid	0.1
36	Methyl Disulphide	624-92-0	Sulphur compound	10
37	Amentoflavone	1617-53-4	Flavonoid	50
38	Sarcophine	55038-27-2	Terpenoid	50
39	Chlorogenic acid	327-97-9	Phenolic acid	10
40	Vitexin	3681-93-4	Flavonoid	1
41	Quercetin	117-39-5	Flavonoid	50

feine, 2,4,4′-trihydroxy chalcone, naringinin-7-glucoside, pseudoephedrine, glycyrrhizin, epicatechin, vanillic acid, gallic acid, β-glycyrrhetenic acid, xanthohumol and amentoflavone were purchased from Sigma Aldrich (St. Louis, MO, USA). Bisabolene, α-ionone, (Z)-3-hexenyl acetate, allyl anthranilate, isoeugenol, α-pinene, cuminaldehyde, thymol, caryophyllene oxide, carvone and methyl disulphide were provided from Bedoukian Research (Danbury, CT USA). Cynarin, emodin, rutin and sennoside A were purchased from Chromadex (Wesel, Germany). Khellin was a gift from Prof. Dieter Treutter (Tech Univ. of Munich, Germany). Sarcophine was isolated from *Sarcophyton ehrenbergi* soft coral whereas vitexin and chrysin were isolated from *Passiflora edulis* leaf in Dr. Farag laboratory, Cairo University. All compounds were dissolved in Dimethyl sulfoxide (DMSO).

2.2. Cell culture

Michigan Cancer Foundation (MCF-7) cells were obtained from Dr. El-Khamisy's laboratory at the University of Sheffield, UK and cultured in DMEM media (Lonza) supplemented with 10% Fetal Bovine Serum (Gibco), 100 units of Penicillin/Streptomycin (Lonza) and 100 U/ml L-Glutamine (Lonza) at 37 °C and 5% CO_2.

2.3. Cell viability assays

MCF-7 cells were plated at a density of 5000 cells in triplicates in a 96-well plate. Next day, cells were treated with the indicated compounds (Table 1) at the indicated concentrations in a final volume of 100 μl. Cell viability was assessed after 48 h using CellTiter-Blue®Cell Viability Assay (Promega). 20 μls of the reagent were added to each well, the plate was incubated for 3 h and fluorescence was subsequently measured (490 nm) using a plate reader. The viability was calculated relative to a DMSO control.

2.4. Clonogenic assay

MCF-7 cells were plated at a density of 2000 cells in 10-cm dishes followed by treatment with the indicated compounds (Table 1) on the next day. After 12 days, colonies were fixed, stained with 1% Giemsa Stain in Methanol and the colonies containing more than 50 cells were counted and the survival was calculated relative to counts in DMSO controls.

3. Results

3.1. Synthetic lethal screen of natural compounds with doxorubicin using the breast cancer cell line MCF-7

We assembled a library of forty-one natural products of plant origin belonging to various chemical classes including alkaloids viz. harmine, phenylpropanoids viz. isoeugenol, terpenoids viz. caryophyllene oxide & sarcophine, flavonoids viz. xanthohumol, anthraquinones viz. sennossoide A, saponins viz. glycyrrhizin and phenolic acids viz. gallic acid (Suppl. Fig. 1). We first assessed

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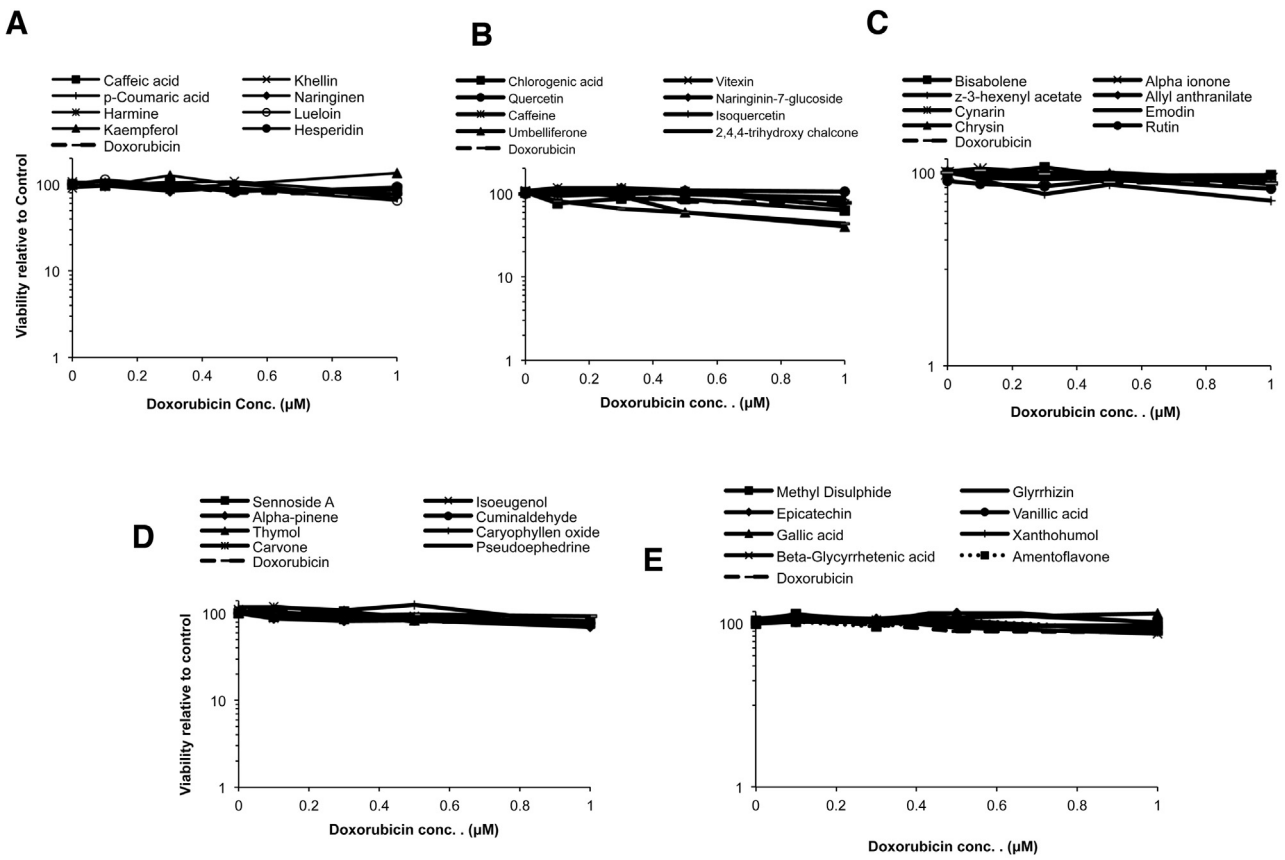

Fig. 2. Synthetic viability screen of natural compounds and doxorubicin on MCF-7. MCF-7 cells were treated with the indicated natural compounds at their sub-toxic concentration (Table 1) in combination with doxorubicin at 0.1, 0.3, 0.5 and 1 μM. Doxorubicin was determined to be non-toxic to MCF-7 cells at 0.1, 0.3 and 0.5 μM. The treatments were performed for 48 h followed by the determination of cell viability using the CellTiter-Blue®Cell Viability Assay (Promega). Viability at a given concentration is determined as a percentage relative to that of a DMSO control.

the toxicity of the natural compounds on MCF-7 cells by measuring their viability following treatment at five doses spanning from 0.1 to 100 μM (Fig. 1A–E). The sub-toxic concentration for each compound was determined, which we defined as the concentration which could incur less than 15% cytotoxicity (Table 1). The established IC values or sub-toxic doses were used as the dependent variables in the cluster analysis to determine similarities in the cytotoxic activity of the tested compounds. Hierarchical cluster analysis identified four distinct groups based on the level of their cytotoxic activity against MCF-7 cell line (Suppl. Fig. 2). The groups obtained were: (**1a**) Pseudoephedrine, naringinen, hesperidin, rutin, caffeic acid, p-coumaric acid, vanillic acid, bisabolene, α-ionone, isoeugenol, α-pinene, cuminaldehyde, thymol, β-glycyrrhetenic acid and glycyrrhizin with an IC value of 100 μM; (**1b**) Sennoside A, kaempferol, quercetin, epicatechin, luteolin, amentoflavone, sarcophine, caffeine, emodin, khellin, isoquercetrin, chrysin, chlorogenic acid, gallic acid, naringinin-7-*O*-glucoside, caryophyllen oxide and methyl disulphide with an IC value of 10–50 μM; (**1c**) Umbelliferone, 2,4,4′-trihydroxy chalcone, cynarin, (*Z*)-3-hexenyl acetate, allyl-anthranilate, carvone, harmine, xanthohumol and vitexin with an IC value of 0.1–1 μM and being most active among all examined chemicals. It should be noted that no segregation of compounds could be observed based on its class group i.e. alkaloids, flavonoids etc. Among the examined compounds, we observed that harmine, luteolin, 2,4,4-trihydroxy chalcone, gallic acid and xanthohumol exhibited the highest cytotoxicity on MCF-7 cells. This is in line with the reported effect of the dietary flavonoids luteolin (Sato et al., 2015) and xanthohumol (Blanquer-Rossello et al., 2013) on the MCF-7 human breast cancer cell line. Next, we performed the subsequent screen where we combined the compunds at their sub-lethal concentration (Table 1) with four concentrations of doxorubicin: three of which were sub-lethal (0.1, 0.3 and 0.5 μM) and one was lethal (1 μM) (Fig. 2A–E). However, this screening scheme failed to yield a combination which could result in a significant synergistic cytotoxic effect. We reasoned that it may have been due to an inherent short outcome in the viability assay or the concentrations used of both agents were not optimal to produce detectable synergism. Thus, we decided to re-examine the top five compounds observed in the primary screen to display the strongest cytotoxic effect on MCF-7 cells but now with the additional presence of varying concentrations of doxorubicin.

3.2. *The β-carboline alkaloid harmine potentiates doxorubicin-induced cytotoxicity on MCF-7 cells*

We examined the effect of the compounds at varying concentrations alone or in combination with two fixed concentrations of doxorubicin (0.5 and 1 μM) on MCF-7 cell viability. Out of the five compounds, harmine demonstrated a dose-dependent cytotoxicity on MCF-7 cells (Fig. 3A) and the most potent inhibition of cell viability when combined with doxorubicin (Fig. 3B). It should be noted that such synergism appeared at low doses of both agents (1 μM for harmine and 0.5 μM for doxorubicin). Harmine was previously found to exhibit cytotoxic effects though used at much higher concentrations ranging from 10 to 50 μM (Ma and Wink, 2010; Dai et al., 2012; Zhao and Wink, 2013). Interestingly, luteolin identified among the 5 structural hits is known to attenuate doxorubicin-induced cytotoxicity to MCF-7 cells (Sato et al., 2015) and that could account for the lack of a synergistic effect with doxorubicin in this study. To further confirm the observed synergism,

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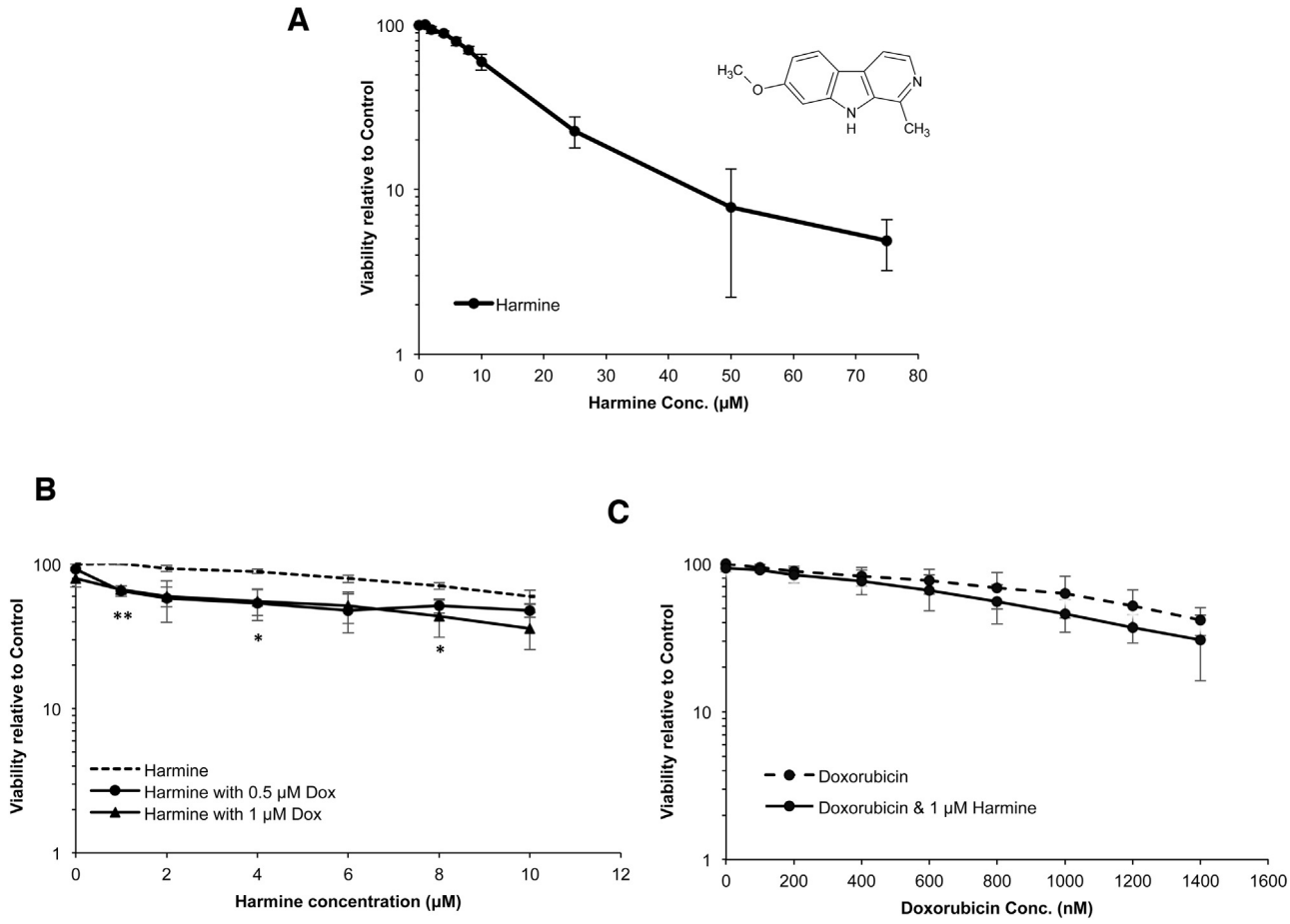

Fig. 3. Harmine potentiates doxorubicin-induced cytotoxicity in MCF-7 cells. (A) Viability of MCF-7 cells following treatment with the indicated concentrations of Harmine. (B) MCF-7 cells were treated with the indicated concentrations of harmine alone or in combination with 0.5 or 1 μM doxorubicin. (**C**) MCF-7 cells were treated with different concentrations of doxorubicin with or without 1 μM of harmine. In all assays, treatments were performed for 48 h followed by the determination of cell viability using the CellTiter-Blue®Cell Viability Assay (Promega). Viability at a given concentration is determined as a percentage relative to that of a DMSO control. Data are the mean ± s.d. from three independent biological replicates. Asterisks denote statistical significance (*$P < 0.05$ and **$P < 0.01$ by student *t*-test).

MCF-7 cells were treated with increasing doses of doxorubicin in the absence or presence of 1 μM harmine and evaluated for cell viability (Fig. 3C).

3.3. Harmine does not exhibit cytotoxic synergism with other topoisomerase poisons

Considering that both harmine and doxorubicin displayed cell killing effects on MCF-7 cells, we wondered whether similar effects would occur with other topoisomerases poisons widely used in the clinic (Gilbert et al., 2012; Alagoz et al., 2012). MCF-7 cells were treated with the TOP1-poison camptothecin (Fig. 4A) or with the TOP2-poison etoposide (Fig. 4B) in the absence or presence of 1 μM harmine, and assessed for cell viability. The addition of harmine did not enhance the cytotoxicity of either poison, suggesting that the observed synergistic effect is specific to doxorubicin.

3.4. Harmine and doxorubicin inhibit MCF-7 survival as measured by colony formation assays

The clonogenic survival assay determines the ability of single cells to form a colony where a colony is formed of at least *ca.* 50 cells. This assay measures the ability of every cell to undergo limitless division and is considered to be a more accurate readout for cytotoxicity, providing more insight on the long-term effects of a certain treatment compared to cell viability assays (Franken et al., 2006). We determined the ability of MCF-7 to form colonies after 12-day treatments of different concentrations of harmine with or without 10 nM doxorubicin (Fig. 4C). Harmine on its own reduced MCF-7 colony formation ability by ca. 35% at a concentration of 800 nM and the additional presence of doxorubicin further reduced colony formation in an additive manner. Notably, harmine was identified as a high affinity inhibitor of DYRK1A, a kinase that phosphorylates the tau protein, which is deregulated in Alzheimer's disease (Gockler et al., 2009; Frost et al., 2011). We therefore examined whether other reported DYRK1A inhibitors would simillarly potentiate the cytotoxic effect of doxorubicin on MCF-7 cells. Consistent with this notion, the DYRK1 inhibitor INDY exhibited a simillar but milder additive cytotoxic effect with doxorubicin, suggesting that inhibiting DYRK1 kinase may provide a plausible mechanism for the observations reported in this study (Fig. 4D).

4. Discussion

Antineoplastic agents with varying degree of efficacy and accompanying side effects target several cancer-driving signaling pathways. Topoisomerases types IIA (TOP2α and TOP2β) are elegant enzymes which resolve DNA topological entanglements during several cellular processes and are the molecular targets of the commonly used and one of the most effective antineoplastic agents; doxorubicin (Ashour et al., 2015). Despite being one of the most efficacious anticancer drugs (Weiss, 1992), it is well

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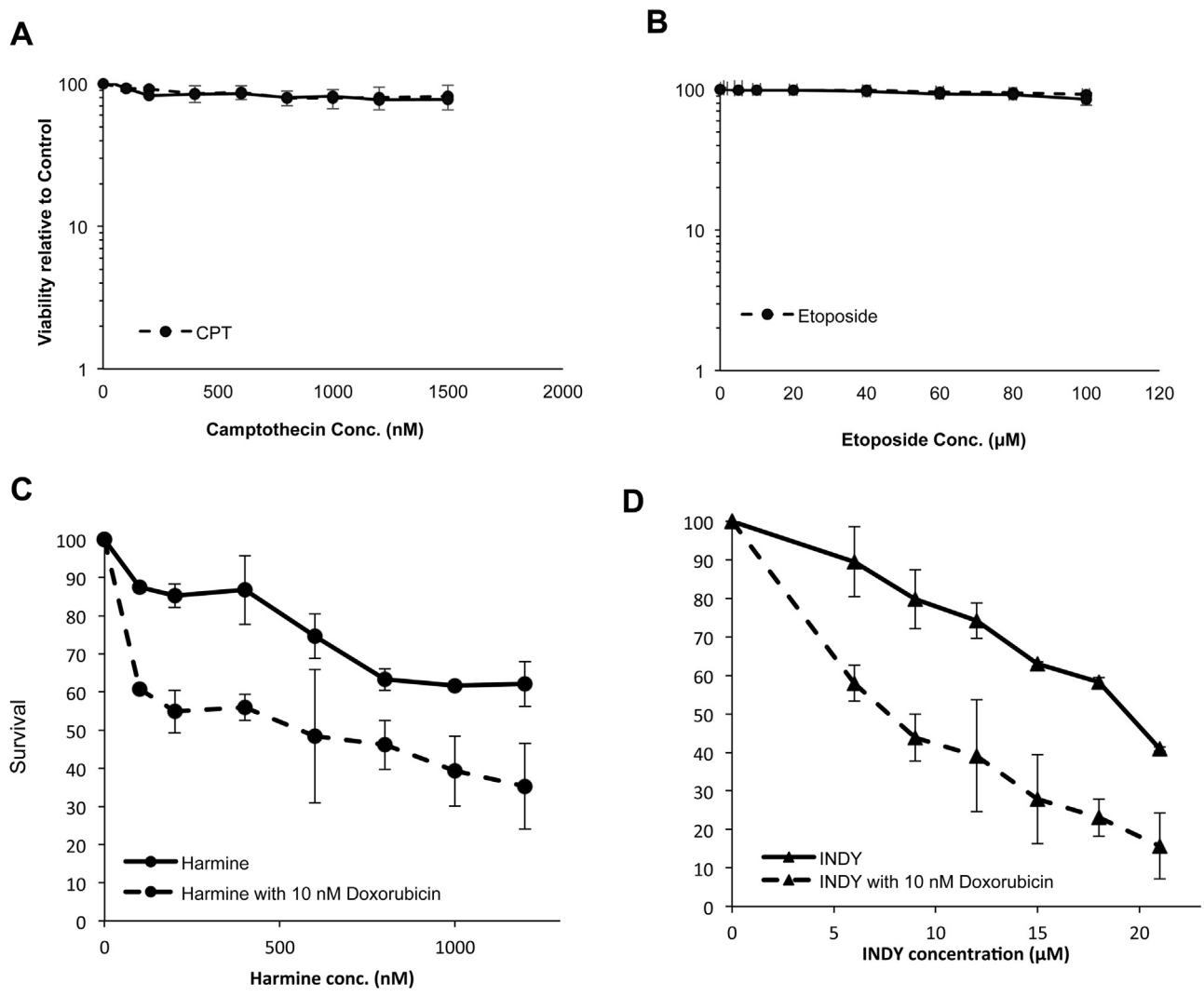

Fig. 4. Harmine does not enhance the cytotoxic effect of camptothecin or etoposide cells but potentiates the cytotoxic effect of doxorubicin as measured by clonogenic survival assays. MCF-7 cells are treated with increasing concentration of camptothecin (A) or etoposide (B) with or without 1 μM Harmine for 48 h and assessed for cell viability using CellTiter-Blue® Cell Viability Assay (Promega). Percentage of cell viability at a given concentration is determined relative to a DMSO control. Data represents the mean ± s.d. of three independent experiments. MCF-7 cells were treated with either Harmine (C) or INDY (D) alone or in combination with 10 nM doxorubicin continuously for 12 days. Cells were assessed for their ability to form macroscopic colonies Percentage survival was calculated from three biological replicated and presented as the average ± s.d.

established that doxorubicin causes chronic cardiomyopathy and congestive heart failure related to the cumulative dose of the drug where each dose adds an incremental injury to the heart (Von Hoff et al., 1979; Swain et al., 2003; Wouters et al., 2005; Yeh and Bickford, 2009; Vejpongsa and Yeh, 2014). Several risk factors underpin the probability of developing cardiac damage following doxorubicin treatment but notably, age was found to be one of the most significant among them. Doxorubicin-associated cardiotoxicity has been observed to be more frequent in older patients compared to their younger counterparts (Von Hoff et al., 1979; Swain et al., 2003; Hequet et al., 2004; Hershman et al., 2008). Interestingly, cardiac damage was found to increase with age. A study of the long-term effects of anthracycline chemotherapy on 201 patients showed that 23% of the cohort developed cardiac side effects up to 10 years after the cessation of treatment (Steinherz et al., 1991). Another study showed that pediatric cancer patients who received anthracycline treatment had a high incidence of developing cardiotoxicity later in life (Steinherz and Steinherz, 1991). The cause behind this long-term cardiotoxicity is not well understood. This serious side effect has significant impact on the overall prognosis and survival of cancer patients.

Such serious side effects warrant the hunt for novel interventions that can help prevent anthracycline-induced cardiotoxicity. Anticancer drug research in the last 20 years has utilized novel strategies to identify cancer-specific traits that can be exploited for selective targeting. One promising strategy is synthetic lethal screens, which is based on the interaction of two or more genes that contribute to the same vital process (Chan and Giaccia, 2011). Here, we hypothesized that cancer killing efficiency of doxorubicin could be achieved by sub-lethal non-toxic doses if combined with novel compounds targeting previously unidentified genetic pathways. In a hunt for such compounds we performed a synthetic lethal screen using doxorubicin and a library of natural products of plant origin. Naturally derived drugs appear more rewarding than synthetic agents since they are less likely to display toxic side effects. Our screen revealed the β-carboline alkaloid harmine as a putative potentiator of doxorubicin cytotoxicity.

Harmine is isolated from several medicinal plants including *Peganum harmala* and *Passiflora edulis*. It has been reported to exhibit anti-viral (Hudson et al., 1986), anti-leishmanial (Lala et al., 2004) and anti-neoplastic activities (Hamsa and Kuttan, 2010). Furthermore, recently, its anti-diabetic effect via the induction of

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pancreatic beta-cell proliferation holds promise for its use in diabetes therapy (Wang et al., 2015). Herein, we identify a novel function of harmine as a potentiator of doxorubicin cytotoxicity on the breast cancer cell line MCF-7. This combination even at low doses reduces cell viability compared to that of either agent alone (Figs. 3 B, 4 C). The mechanism by which harmine exerts its potentiating effect with doxorubicin could be due to improving doxorubicin reported inhibitory effect on TOP2. It could also be attributed to the reported effect of harmine as a DYRK1A kinase inhibitor, which may modulate mitochondrial function and the dynamics of reactive oxygen species. We favor the latter possibility for two reasons. First, harmine did not display detectable synergistic or additive activity with etoposide, a specific TOP2 poison. Second, the DYRK1 kinase inhibitor INDY similarly displayed additive cytotoxicity with doxorubicin in colony formation assays (Fig. 4C and D).

Further studies are required to determine the anti-cancer effect of the combination proposed here, in other breast cancer cell lines and in xenograft mouse models. Importantly, follow-up studies will compare the cardiotoxicity of doxorubicin alone and with the additional presence of harmine, which is predicted to permit the use of lower non-, or less, cardiotoxic doses of doxorubicin. Analyzing biological structure-activity relationships among other structurally related β-carboline alkaloids, e.g. harmane, may identify more potent synergism with doxorubicin. Similarly, assessing other DYRK1 inhibitors for their combined effects with doxorubicin may present a worthwhile investigation. These studies could serve as a guide for the rational design of further antitumor drugs and to help identifying structural motifs crucial for cytotoxic activity, with the view to minimize cardiotoxicity.

Author contribution

R.A. and E.I initiated the project. R.A. and M.A. performed the experiments and analyzed the data. M.F. assembled the compound library. R.A. and S.E-K wrote the manuscript. All authors interpreted the data and reviewed the manuscript. S.E-K conceived the study and coordinated the project.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2016.04.012.

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