Non-cardiotoxic Properties of Annamycin, a Clinically Evaluated Anthracycline and Potent Topoisomerase 2β Poison

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INTRODUCTION

Cardiotoxicity is an important side effect limiting the clinical use of all anthracyclines, including doxorubicin (DOX). DOX targets topoisomerase II-alpha (Top2α) and -beta (Top2β), two enzymes that regulate DNA topology. Top2β is present in cardiomyocytes and has been proposed as a major contributor to DOX-induced cardiotoxicity. Annamycin (ANN), a novel clinically evaluated DOX analog, and its liposome-formulated L-ANN displays no or significantly reduced cardiotoxic properties in preclinical *in vivo* experiments, while demonstrating superior activity in cancer models (example shown in Fig. 1) and high potency *in vitro* against tumor cell lines. Further, the active monitoring of potential cardiotoxicity of ANN in the ongoing clinical trials of ANN did not detect cardiotoxic events in treated patients.

Collectively, this surprising evidence of an apparent lack of cardiotoxicity of ANN despite its high *in vitro* potency and *in vivo* efficacy warrants further investigation of this phenomena and the role of Top2β in cardiotoxicity of anthracyclines.

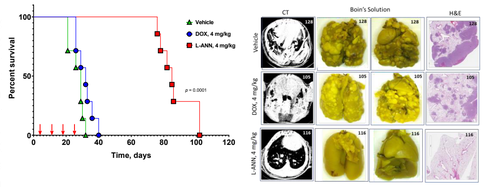


Figure 1. An example of anticancer activity of L-ANN and DOX in experimental metastatic model of fibrosarcoma

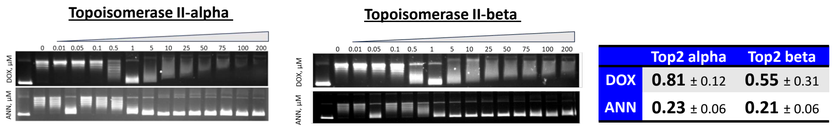
Female B6 mice received intravenous injection of 1 x 10^5 MCA205 cells. Treatment was started on day 4, when mice received four weekly injections of L-ANN or DOX at 4 mg/kg. Controls received vehicle. Survival and heart physiology was assessed by CT and histopathology.

The objective of this study was to directly assess and compare the potency of DOX and ANN against Top2α and Top2β in rat cardiomyocytes and tumor cell lines, and to determine DOX and ANN impact on physiology of human cardiomyocytes and mice hearts following chronic *in vivo* exposure.

RESULTS

I. Annamycin is an equally potent inhibitor of Topoisomerase II-alpha and Topoisomerase II-beta *in vitro*

An *in vitro* relaxation assay was performed to compare the potency of ANN and DOX to inhibit both isoforms of topoisomerase. Surprisingly, ANN showed a uniformly higher inhibitory effect towards the alpha and beta subunit with ED_{50} 0.23 and 0.21 nM, respectively, and was two to four times more potent than DOX (Fig. 2)



	Top2 alpha	Top2 beta
DOX	**0.81** ± 0.12	**0.55** ± 0.31
ANN	**0.23** ± 0.06	**0.21** ± 0.06

Figure 2. Inhibition of topoisomerase II-alpha and II-beta by Annamycin and Doxorubicin.

The inhibition of Topoisomerase II-alpha and II-beta was assayed in DNA relaxation assay. Briefly, 1 U of human Topoisomerase II-alpha and beta was incubated with 0.5 μg of supercoiled pBR322 DNA in a 30 μl reaction at 37°C for 30 minutes under the following conditions: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 50% (v/v) glycerol, and 50 μg/ml albumin in the presence of 1mM ATP and 2% DMSO. Each reaction was stopped by extraction of the reactions with 60 μL of water saturated butan-1-ol to remove the compounds. The samples were vortexed for 10 seconds, centrifuged 30 seconds, and the (upper) butanol phase removed. The reactions were then further treated by the addition of 30 μl chloroform/iso-amyl alcohol (24:1) and 30 μl Stop Dye (40% sucrose (w/v), 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5 μg/ml bromophenol blue), before loading 20 μL of the aqueous phase on a 1% TAE gel. The samples were run at 90V for 1.5 hours.
Bands were visualized by ethidium bromide staining for 10 minutes and de-staining for 20 minutes. Gels were scanned using documentation equipment (GeneGenius, Syngene, Cambridge, UK) and % inhibition levels (where appropriate) were obtained with gel scanning software (GeneTools, Syngene, Cambridge,UK).

II. Annamycin demonstrates potent anticancer activity *in vitro* while remaining inactive against established rat cardiomyocyte cultures

Figure 3 is an example of the *in vitro* anticancer potential of ANN and DOX in a panel of sarcoma cell lines and an established culture of rat H9c2 myoblasts. This cell line was isolated from isolated from ventricular tissue of myocardium and is currently used *in vitro* as a mimetic for skeletal and cardiac muscle due its biochemical, morphological and electrical/hormonal signaling properties.

Cell line	IC_{50} (nM) ANN	Average IC_{50} for ANN	IC_{50} (nM) DOX	Average IC_{50} for DOX	Ratio IC_{50}DOX/ IC_{50}ANN
M63.2	**8.2** ± 4.3	**15.8** nM	**41.9** ± 7.0	**54.2** nM	**5.2**
MCA205	**17.4** ± 7.7		**51.1** ± 14.0		**2.9**
K7M3	**14.9** ± 9.8		**62.7** ± 27.4		**4.2**
143B	**22.7** ± 10.0		**60.1** ± 15.0		**2.6**
H9c2 (Rat cardiomyocytes)	**1,264.4** ± 131.2		**88.8** ± 15.3		**0.07**

Figure 3. Annamycin is more cytotoxic *in vitro* than DOX against cancer cell lines, yet in contrast to DOX, ANN shows low toxicity (Resistance Index RI~80) against established cardiomyocyte cultures.

Examples of *in vitro* efficacy of ANN and DOX in a panel of sarcoma cell lines and established culture of rat cardiomyocytes at 72h exposure (cells were exposed to ANN or DOX for 72 h, followed by determination of the viability using Cell Titer Glo).

III. xCELLigence RTCA CardioECR analysis confirms no toxic effect of ANN on human cardiomyocytes

To compare the effect of ANN and DOX on human cardiomyocytes derived from induced pluripotent stem cells (iPSCs), we employed RTCA CardioECR. The assay enables probe-free determination of viability (cell index, impedance), contractility and electric potential of the cells.

Figure 4. Analysis of impedance of human cardiomyocytes exposed to ANN or DOX

A. Viability of established culture of human cardiomyocytes (iCell, Fiji film) were analyzed using xCELLigence RTCA CardioECR (Agilent Technologies). **B**. Cell index (normalized to the time-of-treatment) was calculated based on impedance reading. **C**. Brightfield images of cells performed 72h after exposure to ANN or DOX at 1 μM.

IV. Analysis of contractility and electric potential of cardiomyocytes exposed to ANN and DOX

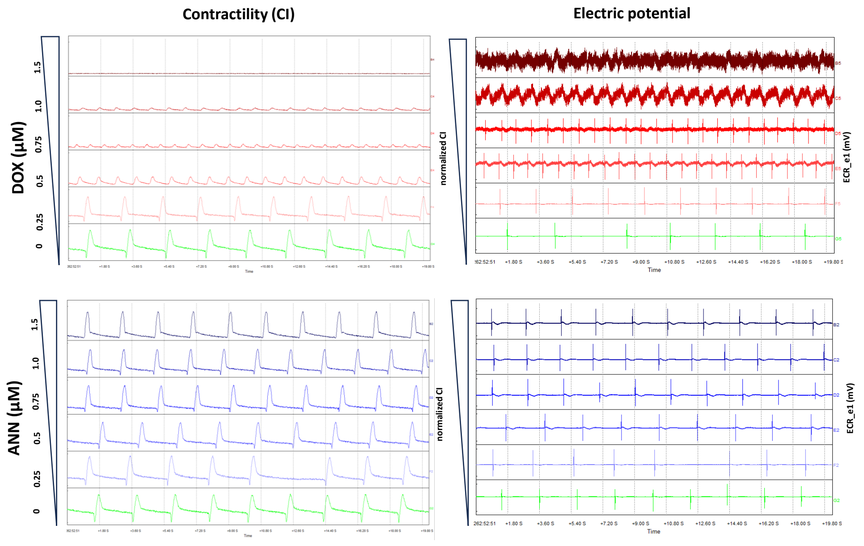


Figure 5. Analysis of contractility of human cardiomyocytes exposed to ANN or DOX.

Contractility of established culture of human iPSCs cardiomyocytes (iCell², Fiji film) was analyzed using xCELLigence RTCA CardioECR (Agilent Technologies). The impedance was monitored for 72 h following the treatment with DOX or ANN.

V. ANN is well tolerated by animals at levels exceeding therapeutic doses

Annamycin was scrutinized in animal models and showed no apparent toxicity. In one study, animals were dosed side-by side with DOX at 8 mg/kg for 7 weeks (this dose significantly exceeds therapeutic schedules). Mice in the DOX arm developed acute toxicity that manifested with significant body weight reduction (Fig. 6A), and mild cardiotoxicity demonstrated by mild cytoplasmic vacuolation of cardiac myocytes (Fig. 6C) that was also associated with increased LDH levels in blood serum (Fig. 6B). None of these symptoms and pathologic changes were observed in animals treated with 8 mg/kg of ANN. Even mice treated for 12 weeks with therapeutically highly effective dose of 4 mg/kg of ANN had no targeted toxicity of heart/myocardium and lung, except a mild lymphotoxic and hematotoxic effect.

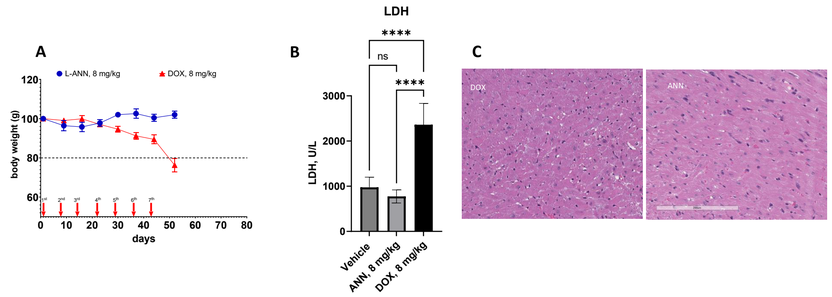


Figure 6. Evaluation of toxicity in L-ANN and DOX treated naïve CD-1 mice.

A. Female CD-1 mice were exposed to DOX or L-ANN for 7 weeks (once a week injection, marked with red arrows) for a total cumulative dose of 56 mg/kg. The DOX –treated arm showed a consistent decrease of body weight and morbidity after the last dose administration. **B.** Serum chemistry analysis revealed significantly upregulated LDH levels which might be associated with minimal cytoplasmic vacuolation of cardiac myocytes and a few occasional cardiac myocytes with swollen or eosinophilic cytoplasmic change as revealed by pathology examination. **C.** The lesions were not observed in L-ANN treated mice.

VI. Clinical data shows no cardiac events for patients enrolled in phase 1 clinical trial

L-ANN as monotherapy in r/r AML patients demonstrated an overall response rate (ORR) of 80% (240 mg/m², administered on three consecutive days (MB-105 study, NCT03388749, Gil et al. 2023)) with no cardiotoxicity noted in any patient. Since preclinical animal data have shown that L-ANN in combination with cytarabine demonstrated a significant improvement in median overall survival (mOS) when compared to both L-ANN as a single agent (68% improvement) and to cytarabine alone (241% increase), the MB-106 trial (NCT05319587) has been initiated. To date, this trial revealed an CR/Cri rate of 67% in the TPP population with mOS not being reached so far (Wadolowska et al., EHA #2139).

CONCLUSIONS

Annamycin (ANN) is a novel doxorubicin (DOX) congener formulated in multilamellar liposomes (L-ANN) currently undergoing clinical trials (NCT03388749; NCT05319587), which shows high anti-cancer activity in multiple models *in vitro* and *in vivo* and no cardiotoxicity.

- ANN is a more potent topoisomerase II-alpha and II-beta poison than DOX.
- In contrast to DOX:
  - ANN does not affect viability of established culture of human iPSCc cardiomyocytes (tested up to 1.5 μM)
  - ANN does not affect contractility or the electric potential of the cardiomyocytes
  - Rat cardiomyocytes (H9c2) appear to be resistant to ANN while sensitive to DOX

- ANN is well tolerated by the animals even at schedules exceeding the therapeutic dosage while *ex vivo* pathology examination of the mice confirmed no toxicity to the heart/myocardium.
- These data are clearly aligned with the lack of drug-related cardiotoxic events in ANN-treated patients in ongoing clinical trials.
- The role of topoisomerase II-beta in cardiotoxicity of anthracyclines should be further investigated.

ACKNOWLEDGEMENTS

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