Combining Annamycin, a Non-cardiotoxic Potent Topo II Poison, with Azacitidine, Cytarabine, Gemcitabine, Ifosfamide, Trabectedin, or Vincristine to Synergize Anticancer Effects and Identify Potential Clinical Applications

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center
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INTRODUCTION

Annamycin (ANN) is a potent topoisomerase II poison structurally related to doxorubicin (DOX) but displaying superior biological properties, such as strong *in vivo* efficacy, reduced toxic side effects, and activity against cancers resistant to common therapeutic agents. ANN is currently clinically evaluated as a liposome formulated drug product, L-ANN. Importantly, L-ANN preclinical and clinical data clearly indicate a lack of cardiotoxicity, which is a common side effect and a dose-limiting factor in this class of therapeutics.

In addition, ANN and L-ANN display unique organotropism that significantly differs from that of DOX. This finding further increased our interest to study the potential synergistic or additive effects of combining ANN with different anticancer agents. The efficacy of L-ANN has been established in multiple tumor models including leukemia and solid tumors, further strengthening the rationale of this study.

OBJECTIVE

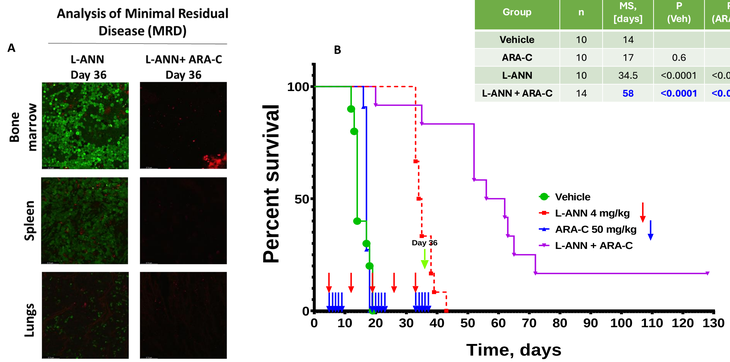
The objective of the study was to assess the efficacy of ANN in combination with approved anticancer agents in order to identify novel potentially highly efficacious clinical application of ANN alone and with a therapeutic partner.

METHODS

ANN (*in vitro*) and L-ANN (*in vivo*) were tested in combination with selected FDA approved drugs. Activity *in vitro* was assessed after 72 hours of treatment using a cytotoxicity assay followed by determination of the combination indexes. In separate *in vitro* experiments, ANN activity was tested against drug resistant cell lines, including cells resistant to cytarabine and venetoclax. The most efficacious drug combinations from the *in vitro* studies were then tested using well developed *in vivo* models of leukemia and solid tumors, including sarcoma and pancreatic cancer.

RESULTS

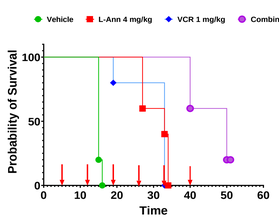
Annamycin (L-ANN) Synergizes with ARA-C in Increasing Survival in p53-null AML Model (AML-mTurq2)



Group	n	MS, [days]	P (Veh)	P (ARA-C)	P (L-ANN)
Vehicle	10	14			
ARA-C	10	17	0.6		<0.0001
L-ANN	10	34.5	<0.0001	<0.0001	-
L-ANN + ARA-C	14	58	<0.0001	<0.0001	<0.0001

Figure 1. Efficacy of L-ANN and Cytarabine in syngeneic AML model . Female C57B6 mice were injected intravenously with 1 x 10^5 AML-Turq2 cells. On day five animals were randomized into four experimental groups (n=10-12) receiving: vehicle, L-ANN, ARA-C and L-ANN plus ARA-C. On day 36 of the study distribution of leukemic blasts in selected animals was analyzed using ex vivo confocal microscopy . MS - Median Survival.

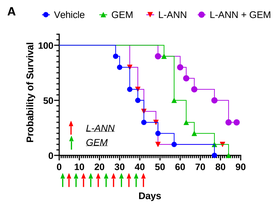
Efficacy of combination of liposome formulated Annamycin (L-ANN) and vincristine (VCR) in mouse acute myeloid leukemia model



Group	n	MS, [days]	Survival, %	P vs (Veh)	P vs (L-ANN)	P vs (VCR)
Vehicle	5	15	100			
L-ANN, 4 mg/kg	5	33	220	0.0016	0.0025	
VCR 1 mg/kg	5	33	220	0.0016	-	0.0035
Combination	5	50	>293	0.0016	0.0025	0.0035

Figure 2. Efficacy of L-ANN and VCR in murine acute myeloid leukemia model . Female C57B6 albino mice received bolus, IV injection of AML-Turq-2 cells. Day five following tumor inoculation, the mice were randomized into four experimental groups (n=5) receiving i) vehicle, ii) L-ANN 4 mg/kg, iii) VCR 1 mg/kg and iv) combination of L-ANN and VCR. Arrows indicate the administration of L_ANN and/or VCR. Survival was analyzed using Long-rank (Mantel-Cox) test in GraphPad Prism software. MS - Median Survival.

Efficacy of combination of liposome formulated Annamycin (L-ANN) and gemcitabine (GEM) in pancreatic ductal adenocarcinoma model

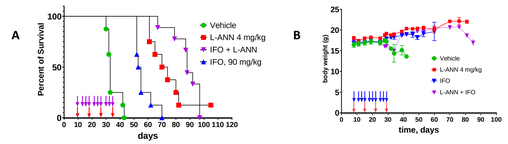


B

Group	n	Dose/Schedule	Route	MS [days]	P vs vehicle	P vs L-ANN	P value vs GEM
Vehicle	10	Saline	IP/IV	40.5	-	0.59	0.0088
GEM 50 mg/kg	10	50 mg/kg once a week	IP	60	0.0088	0.0129	-
L-ANN 2 mg/kg	10	2 mg/kg once a week	IV	42		-	0.0129
L-ANN 2 mg/kg + GEM 50 mg/kg	10	Same as single agents	IV/IP	80.5	0.0003	0.0017	0.028

Figure 3. Efficacy of L-ANN and GEM in orthotopic MDA PATC53 pancreatic cancer model. Female nude athymic mice were orthotopically implanted with 1 x 10^6 human MDA PATC53 PDAC cells in mixture with Matrigel (50%, 50 µl). On day 8, the animals were randomized into four experimental groups (n=10) receiving i) vehicle, ii) gemcitabine 50 mg/kg , iii) L-ANN at 2 mg/kg and iv) combination of gemcitabine and L-ANN. Both agents were administered on a once-a-week schedule for six cycles. Body weight and survival was tracked daily. Arrows indicate injection of the drug. Survival was analyzed using log-rank (Mantel-Cox) test in GraphPad Prism software. The distribution of BL signals was analyzed using nonparametric one-way ANOVA (Kruskal-Wallis test).

Efficacy of combination of liposome formulated of Annamycin (L-ANN) and alkylating agent ifosfamide (IFO) in fibrosarcoma lung metastasis model



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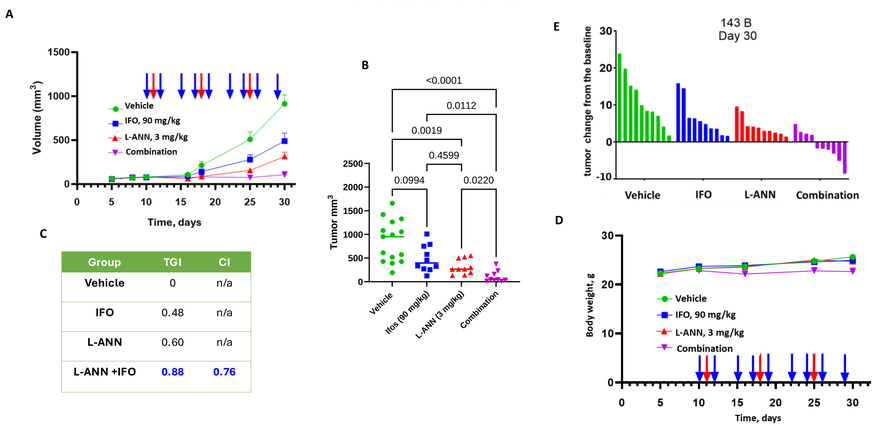
Group	n	Schedule	Dose [mg/kg]	Route	MS, [days]	P vs Veh.	P vs L-ANN	P vs IFO	P vs L-ANN+ IFO
Vehicle	8	As combination	n/a	IP/IV	33	-	<0.0001		<0.0001
L-ANN	8	Once a week x 4	4	IV	72	<0.0001	-	0.003	0.078
IFO	8	Three times a week x 3	90	IP	54	<0.0001	0.0029	-	<0.0001
L-ANN+IFO	9	Same as single agents	Same as single agents	IP/IV	88	<0.0001	0.0332	0.0001	-

Figure 4. Efficacy of L-ANN and IFO in MCA205 fibrosarcoma lung metastatic model. Female C57B6 mice were injected intravenously with 1 x 10^5 mouse MCA205 fibrosarcoma cells. On day 10 animals were randomized into four experimental groups (n=8-9) receiving (i) vehicle (n=8), ii) L-ANN at 4 mg/kg (n=8), iii) IFO at 90 mg/kg (n=8) and iv) combination of both (n=9). Body weight and survival was tracked daily. Arrows indicate injection of the drug. MS - Median Survival.

Efficacy of combination of liposome formulated Annamycin (L-ANN) and ifosfamide (IFO) in human osteosarcoma model



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Group	TGI	CI
Vehicle	0	n/a
IFO	0.48	n/a
L-ANN	0.60	n/a
L-ANN +IFO	0.88	0.76

Figure 5. Efficacy of L-ANN and IFO in human osteosarcoma model. Human osteosarcoma cells (143B, 1 mln/mouse) were injected subcutaneously into female athymic mice. On day ten when the average tumor volume reached ~100 mm^3, the mice were randomized into four groups (n=10) receiving i) vehicle, ii) IFO 90 mg/kg, iii) L-ANN 3 mg/kg and iv) combination of both. L-ANN was administered once a week and IFO was given on a three-times-a-week schedule (Mon-Wed-Fri). The tumor growth was monitored based on tumor volume, estimated using ellipsoid equation based on tumor diameter measurement. Panels B,C and E show the signal distribution at the study termination on day 30. Combination index (CI) was calculated as follows: CI = ((cA + cB) - cA × cB) / cAB where cA represents the inhibitory rate of compound A, cB represents the inhibitory rate of compound B, and cAB represents the inhibitory rate of combination treatment of compound A and B. Synergy is defined as CI lower than 1.0, and antagonism is defined as CI significantly greater than 1.

Effects of combination of Annamycin (ANN) and trabectedin (TRB) in pancreatic cancer cell lines

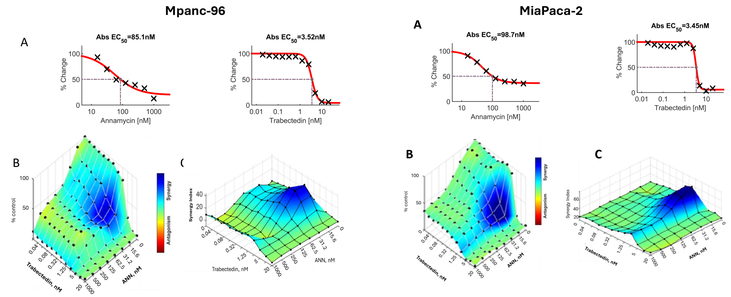
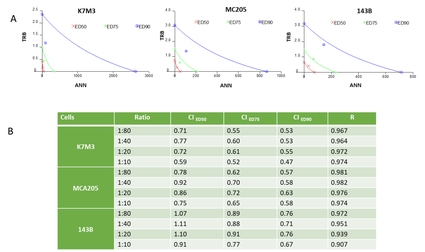


Figure 6. In vitro analysis of synergy between ANN and trabectedin in Mpanc-96 and MiaPaCa-2 cell lines.

Mpanc-96 and MiaPaca-2 (from ATCC) cells were plated in 96-well plates at 3 x 10^3 cells/well and incubated overnight at 5% CO_2 at 37°C. The next day, the cells were exposed to increasing concentrations of ANN and Trabectedin in stable ratio 50:1 and constant vehicle (0.5% DMSO). After 72 hours, the viability of the cells was assessed using Resazurin assay. Viability was normalized to vehicle and no cell treated controls, and expressed as a percentage, The viability matrix was analyzed using Bliss algorithm in Combenefit Software to identify potential regions of synergy.

Effects of combination of Annamycin (ANN) and trabectedin (TRB) in a panel of sarcoma cell lines



B

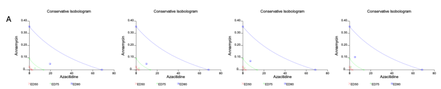
Cells	Ratio	CI ED50	CI ED75	CI ED90	R
K7M3	1:80	0.71	0.55	0.53	0.967
	1:40	0.77	0.60	0.53	0.964
	1:20	0.72	0.61	0.55	0.972
	1:10	0.59	0.52	0.47	0.974
MCA205	1:80	0.78	0.62	0.57	0.981
	1:40	0.92	0.70	0.58	0.982
	1:20	0.86	0.72	0.63	0.976
	1:10	0.75	0.65	0.58	0.974
143B	1:80	1.07	0.89	0.76	0.972
	1:40	1.11	0.88	0.71	0.951
	1:20	1.10	0.91	0.76	0.939
	1:10	0.91	0.77	0.67	0.907

Figure 7. *In vitro* analysis of synergy between ANN and trabectedin in a panel of sarcoma cell lines.

Established murine sarcoma cell lines (K7M3 (osteosarcoma) and MCA205 (fibrosarcoma)) and human osteosarcoma cells (143B) were plated at 500 cells/well in 384- well plates. After overnight attachment, the cells were treated with a combination of TRB, and ANN at fixed ratio of 1:80, 1:40, 1:20, and 1:10. The viability of the cells following 72h drug exposure, was assessed using CellTiterGlo assay. The data was normalized to vehicle-treated control and no-cell control and expressed as fraction control viability. The obtained matrices were analyzed using CaluSyn Version 2.11 to compute the combination indexes (CIs). CI below 1 indicates synergistic nature of interaction, 1.0 – additive effect and CI above 1.0 indicates antagonism between tested agents.

CI_{ED50}, CI_{ED75}, CI_{ED90} ,-combinatory index calculated of fraction effect ED50, ED75, ED90. R – linear correlation coefficient

In vitro analysis of synergy between ANN and Azacitidine (AZA) in MOLM-13 human myeloid leukemia model



B

Cell line	Ratio	CI ED50	CI ED75	CI ED90	R
Molm-13	1:400	0.69	0.53	0.42	0.951
	1:200	0.42	0.34	0.28	0.951
	1:100	0.32	0.31	0.31	0.976
	1:50	0.36	0.36	0.37	0.985

Figure 8. *In vitro* analysis of synergy between ANN and Azacitidine in MOLM-13 human myeloid leukemia model

MOLM-13 cells were plated at 3500 cells/well on 96-well plates. The cells were treated with a combination of AZA, and ANN at fixed ratio of 1:400, 1:200, 1:100, and 1:50 (ANN to AZA). The viability of the cells following 72h drug exposure, was assessed using Resazurin assay. The data was normalized to vehicle-treated control and no-cell control and expressed as fraction of control viability. The obtained matrices were analyzed using CaluSyn Version 2.11 to compute the combination indexes (CIs). CI below 1 indicates synergistic nature of interaction, 1.0 – additive effect and CI above 1.0 indicates antagonism between tested agents. CI_{ED50}, CI_{ED75}, CI_{ED90} ,-combinatory index calculated of fraction effect ED50, ED75, ED90. R – linear correlation coefficient.

CONCLUSIONS

Annamycin (ANN) is a highly versatile drug capable of working synergistically with numerous mechanistically different FDA approved anticancer agents both *in vitro* and *in vivo*. Our studies are working towards identifying new efficacious clinical applications of L-ANN drug combinations with the long-term goal of developing novel therapeutic strategies for treatment resistant cancers.

ACKNOWLEDGEMENTS

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