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Compounds A1-A4

Anti-Proliferation and Anti-Viability

In Vitro Assays

Q-308-05

Experimental Report

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Executive summary

Study Objectives

- To evaluate the anti-proliferative and cytotoxic effect of A1-A4 compounds.

Main findings

- Compound A1 affected the metabolic rate of human breast cancer cells, resulting only 34% cell viability relative to the control. Moreover, A1 compound inhibited 95% of cell proliferation as compared to the control.
- Compound A4 has less potent anti-proliferation activity than A1 compound in human breast cancer cells (43% inhibition) and did not affect cell viability as compared to the control.
- Compounds A3 and A4 did not affect neither cell viability nor cell proliferation rate of human breast cancer cells.

Introduction

The alamarBlue™ assay is designed to measure quantitatively the viability of various human and animal cell lines, bacteria and fungi. The bioassay is also used to establish relative cytotoxicity of agents within various chemical classes[1], thus utilized to predict cytotoxic or necrotic effects of medical devices or materials.

The alamarBlue™ is a growth indicator that exhibits colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic reduction. It is also minimally toxic to living cells and has a clear, stable distinct change, which is easy to interpret. As cells grow and uptake alamarBlue™, their innate metabolic activity results in its chemical reduction. In its reduced form, alamarBlue™ displays pink fluorescent color, in contrast to being blue and non-fluorescent in its oxidized state.

One of the characteristics of a tumor cell is its ability to proliferate (multiply) endlessly. To determine this proliferation, a radioactive-labeled-DNA base (^{3}H-thymidine) is added to the culture medium and is incorporated in the DNA proportionally to proliferative activity. The higher the proliferative response the higher the radioactive counts in the cell's culture. ^{3}H-thymidine incorporation assay is considered to be the most sensitive method for cell proliferation. However, the ability to detect a minimum number of proliferating cells in a certain sample depends on the amount of ^{3}H-thymidine incorporated into the cells and thus on the labeling period. Hence, as long as ^{3}H-thymidine is present in the medium and DNA is synthesized new cells are labeled. In most cases, detection requires a labeling period of 2 to 24 h, in which one cycle of DNA synthesis in all the cells is achieved.

The assays that are described above discriminate between two basic activities of the cell – its viability and its proliferation ability. In order to assess the efficacy of therapeutic agents such as antibiotics and anti-cancer agents, the drug developer should employ them both.

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[1] Fields, RD and Lancaster, MV. (1993). Dual attribute continuous monitoring of cell proliferation/cytotoxicity. American Biotechnology Laboratory, 11(4), pp. 48-50.

Materials

Test item

The Sponsor supplied four compounds labeled A1 to A4 as dry powders. Prior to the assay the compounds were dissolved in Ethanol to 1mg/ml. The vials were sonicated and incubated at 40°C for 15 minutes. Compounds were diluted with medium to final concentration of 10^{-5}M, in eight repeats for each assay.

Reagents

[^{3}H]-methyl-Thymidine- 1mCi/ml, 6.7 Ci/mmol, *Amersham, Pharmacia*

Alamar blue™ *Serotec*, Cat. No. BUG012B.

Controls

Doxorubicin (*Sigma,* cat no. 44583) was used at final concentration of 10^{-5}M for viability and cell proliferation assay.

Since compounds A1 to A4 were dissolved in Ethanol and further diluted in medium, a vehicle control group, containing final concentration of 0.38% Ethanol was included.

Cell lines

MCF-7, originated from human breast cancer, was provided by ATCC (American Type Culture Collection) and propagated in HPBM.

Growth Media

MCF-7 were grown in DMEM medium supplemented with 5% Fetal Bovine Serum 1% penicillin-streptomycin and 1% L-glutamine.

Methods

Cell Culture

The cells were cultured in 25cm^2 culture flasks. The flasks were kept at 37°C in an atmosphere of 5% CO_2 and 100% humidity. The culture medium was changed every other day. For subculturing, the medium was removed and the cells were detached from the culture flasks with 0.25% Trypsin–EDTA. Culture medium with fetal bovine serum (FBS) was added to stop trypsinization.

Cells were seeded at a density of 5,000 cells per well in a 96-optical well plates.

Short-term assay – Alamar blue

In order to avoid quenching signal caused by the compounds, 24 hours following exposure of cells to the compounds the medium was removed and Alamar blue was added in fresh medium. The fluorescent signal (Excitation 544nm/Emission 590nm) was counted after additional incubation for 24 hours at 37^{0}C.

Long-term assay – Thymidine incorporation assay

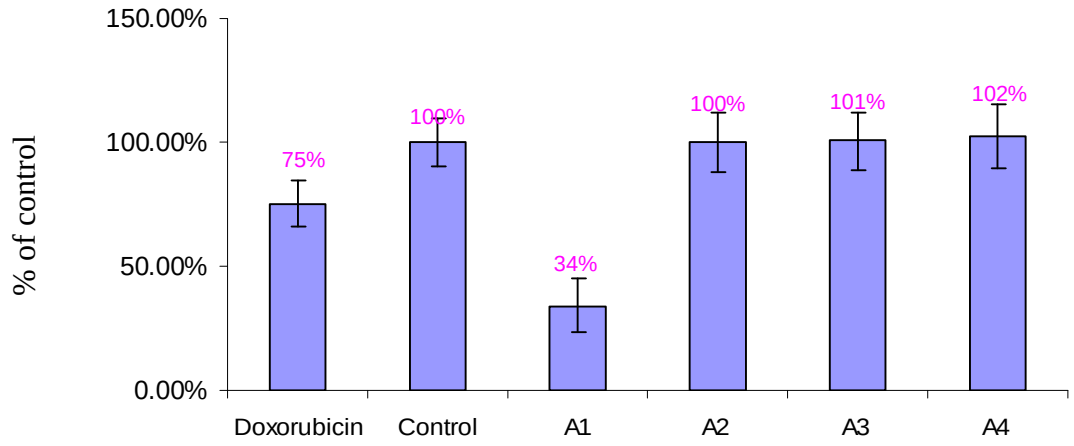
After three incubation days with the tested compounds, ^{3}H-Thymidine was added to final concentration of 0.4µCi/well. Plates were returned to the incubator for additional 48 hours. Then, medium was removed and cells were washed twice with PBS at 4^{0}C. Cells were incubated with 5% TCA at 4^{o}C for 20 minutes. Lysis was achieved by the addition of 10M NaOH solution and lysis buffer for 30 minutes with shaking. Radioactivity was determined after addition of 350µl scintillation liquid (MicroScint 40^{TM}, Packard).

Results

Short-Term Metabolic Rate Effect

The assay was performed as described in Methods. Briefly, the cells were seeded (5000/well) and the day after the tested compounds and the controls were added at concentration of 10^{-5}M each. Following 24 hours of incubation with the compounds, media were removed, Alamar blue was added and its fluorescence was measured.

Figure 1 **The effect of compounds A1-A4 on the metabolic rate of MCF-7 cells**
MCF-7 cells were treated with 10µM A1-A4 compounds, 10µM Doxorubicin positive control and vehicle control (0.38% Ethanol in medium) for one day. Alamar blue fluorescent signal at excitation/emission 544nm/590nm was measured after 24hours. Results (Mean value ±SD) were expressed as % of vehicle control. Raw data is presented in Appendix 1.

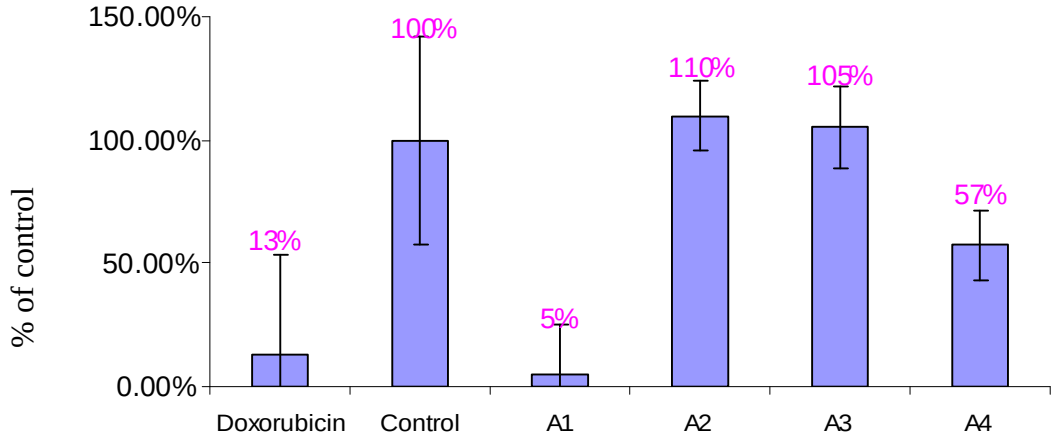


As can be seen in Figure 1, metabolic rate of MCF-7 was decreased following treatments with A1 compound (66% relative to the control). The reduction was even higher than for Doxorubicin, the Positive control substance that showed 25% decrease relative to the control. The other tested compounds (A2-A4) did not affect the metabolic rate of MCF-7. Thus, A1 compound affects MCF-7 cells viability more than Doxorubicin at the same concentration.

Long-Term Anti-Proliferative Effect

The long-term assay detects DNA synthesis, a marker for cell proliferation, by using radiolabeled Thymidine. The results of the long-term assay are depicted below.

Figure 2 **Anti proliferative effect of A1-A4 compounds on MCF-7 cells**
MCF-7 were treated with 10µM compounds A1-A4, positive control (Doxorubicin), and vehicle control (0.38% Ethanol in medium) for 3 days. Incorporation of ^{3}H-Thymidine to proliferating cells is measured in CPM and results (Mean value ±SD) are presented % of vehicle control. Raw data is presented in Appendix 1.



As depicted in Figure 2, following treatments with Doxorubicin (positive control) and A1 tested compound a significant decrease in cells proliferation is observed. The tested compound A4 also demonstrates a mild decrease in cells proliferation. Specifically, MCF-7 cell proliferation was decreased in ~87% following Doxorubicin treatment, 43% following A4 treatment, and up to 95% cell proliferation inhibition was obtained following A1 compound treatment.

MCF-7 cell proliferation was not affected by A2 and A3 compounds treatment.

To summarize, compounds A1 had major anti-proliferative activity in MCF-7 breast cancer cells. A4 compound was less potent for MCF-7 cells.

Summary

The utilization of two different assays, one that measures metabolism and the other that measures proliferation, allows us to discriminate between A1-A4 compounds activities on MCF-7 breast cancer cells. These activities are summarized in Table 1.

Table 1 **Activities Summary of Compounds A1-A4**
Summary of the results described above. + indicates activity, – indicates no activity.

Compound	MCF-7	
	Anti-Metabolic Activity	Anti-Proliferation Activity
A1	+	+
A2	–	–
A3	–	–
A4	–	+

The results above suggest that for MCF-7 cells,

- Compound A1 is toxic to the cells (anti-metabolic effect) and has anti proliferation activity.
- Compound A4 has anti-proliferative effect, without being toxic to cells.
- Compounds A2 and A3 do not effect cells viability or cell proliferation.

Appendix 1

Plan plate cultured with MCF-7 cells

Column	1	2	3	4	5	6
Treatment	Doxorubicin	Medium +Ethanol	A1	A2	A3	A4

AlamarBlueTM Signal (Short-Term Assay)

Measurement count:1 Ex: 544 Em: 590 Scaling Factor : 1/1

	1	2	3	4	5	6
A	441.7	588.9	214.3	604	596.1	611.9
B	458.8	575.4	210.9	592.2	581.8	589.3
C	439.8	576.3	190.8	572	586.4	591.5
D	382	598.1	176.2	582.7	602.8	590.1
E	473.5	571	207.9	579.4	579.8	585.7
F	450.3	574.6	187.1	576.9	578.9	589.3
G	474.8	578.8	206.5	570.1	573.3	581.2
H	367.5	583.2	196.9	571.4	579	615.9

Thymidine Incorporation (Long-Term Assay)

	1	2	3	4	5	6
A	358	3055	88	1930	2464	735
B	517	1675	142	2153	1487	1588
C	316	2548	124	2124	1828	1115
D	211	2655	82	3481	2767	1495
E	264	1264	117	1661	1797	1031
F	152	2413	100	2039	1259	171
G	226	1743	164	1965	2816	2235
H	215	2073	101	3758	3922	1643