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# Self-assemblies of azacitidine prodrugs: an innovative therapy against myelodysplastic syndromes

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## INTRODUCTION

5-azacitidine, a cytidine analogue and a hypomethylating agent, is one of the main drugs being used for the treatment of myelodysplastic syndromes [1]. However, after administration, it exhibits several limitations including restricted diffusion and cellular internalization due to its hydrophilicity, and rapid enzymatic degradation. Thus, to improve the drug diffusion and to protect it from metabolic degradation it is formulated into an amphiphilic prodrug and its self-assembly into a nanoparticle. Following the conjugation of the fatty acid to the azacitidine, the obtained prodrug was solubilized in acetone and mixed with water at different ratios to obtain self-assemblies by nanoprecipitation, thus protecting the active molecule from enzymatic degradation. This prodrug should be cleaved by cathepsin B [2], an enzyme overexpressed in cancerous cells [3], thus increasing the specificity of the drug.

## MATERIALS AND METHODS

### Prodrug synthesis

The prodrugs were obtained following a three-step synthesis reaction: first, the azacitidine was protected using

tert-butyldimethylsilyl chloride (TBDMSCl) in basic conditions, second the fatty acid chains of interest (oleic acid, docosahexaenoic acid and eicosapentaenoic acid) were conjugated using ethyl chloroformate separately, finally the protecting group was removed using tetra-n-butylammonium fluoride (TBAF). Shown in figure 1. All the obtained molecules were purified using traditional column chromatography and characterized using <sup>1</sup>H-NMR spectra and mass spectrometry.

### Determination of critical aggregation concentration

Pyrene, a fluorescent probe, was used to determine the critical aggregation concentration (CAC). Measurements were done by a spectrophotometer (Fluoromax-4), pyrene was excited at a wavelength of 336 nm. The emission spectra were recorded in the range 350-500 nm.

### Flow cytometry Analyses

Apoptosis was analyzed by a double-staining method using annexin-v /propidium iodide labeling solution according to the manufacturer's instructions (Roche), to differentiate cells with membrane damage, PI solution was added.

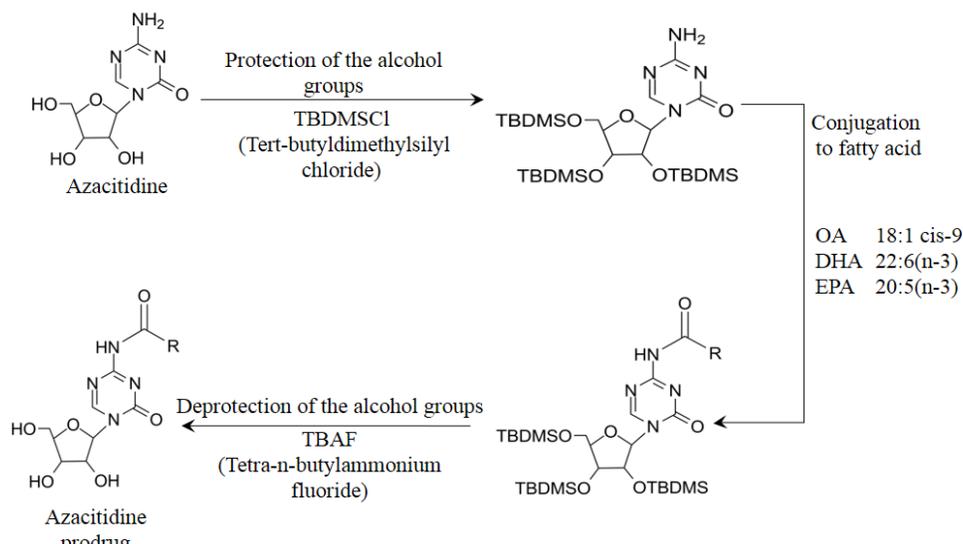


Figure 1: The three-step synthesis reaction of the prodrugs

## RESULTS AND DISCUSSION

### Metabolic activity determination

The metabolic activity of the separate molecules that make-up the prodrugs was evaluated at 6, 24 and 48 h post treatment on HL60 cell line, the metabolic activity was evaluated by performing MTT proliferation assay.

The results are presented in figure 2 (a-c) and show that there was no synergistic effect brought by the co-treatment with azacitidine and the different fatty acids before conjugation.

### Apoptosis assay

In order to determine the apoptotic effects of the specific concentrations of our drugs chosen around the  $IC_{50}$  of azacitidine, and in equimolar amounts, an apoptotic assay based on the double-staining method using annexin V /propidium iodide was performed. Results present in figure 2 (d-g) confirm that while azacitidine shows higher apoptosis levels at 5 times the  $IC_{50}$  concentration ( $\sim 1\mu M$ ), the other fatty acids have no apoptotic effect at these concentrations.

### Determination of critical aggregation concentration

The CAC was determined as previously described for Azacitidine-Oleic acid conjugate, demonstrating that the self-assembly of this conjugate starts at a concentration of  $38\mu M$  and thus no treatment should be considered below this concentration to ensure that the amphiphilic prodrugs are self-organized. The nanoparticle was further

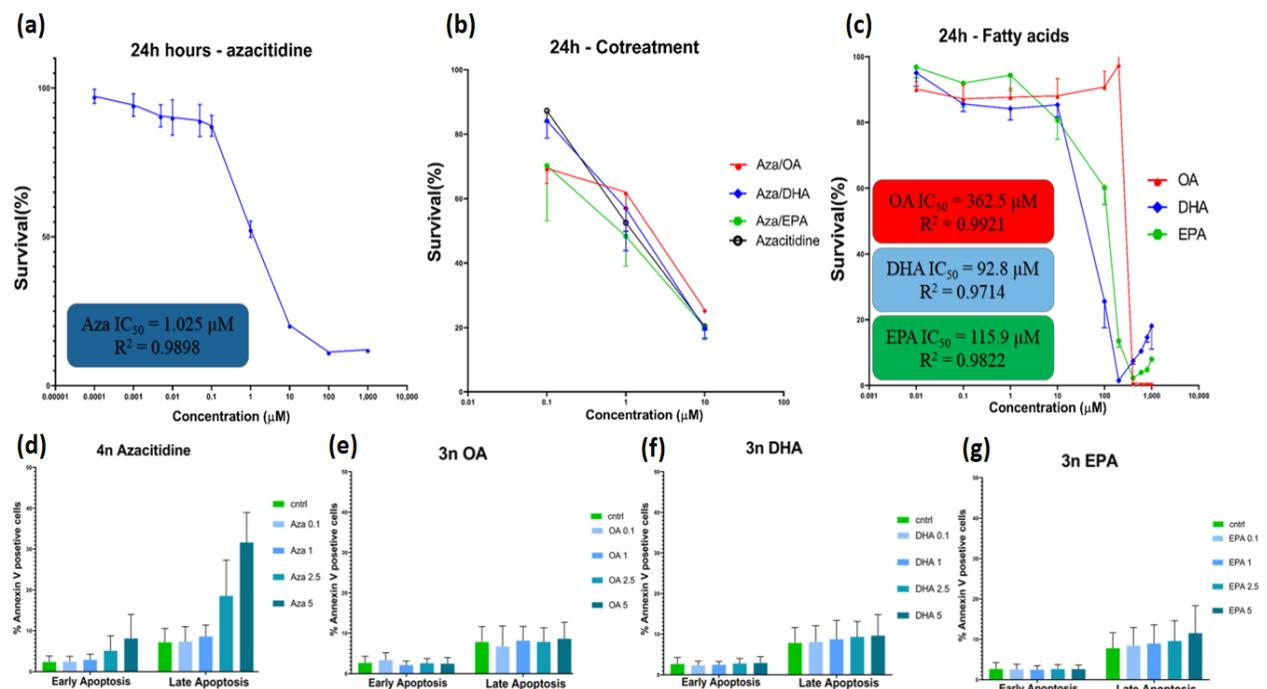
characterized using DLS (Zetasizer Nano ZS – Malvern) with a Z avg of  $97.54\text{ nm} \pm 2.34\%$ , PDI of  $0.227 \pm 0.032\%$  and a zeta potential of  $-15.5\text{ mV} \pm 6.32\%$ .

## CONCLUSION AND PERSPECTIVE

In this project, in order to advance azacitidine as a myelodysplastic syndrome treatment, azacitidine was conjugated to three different fatty acids and nanoprecipitated into nanoparticles. The obtained results showed that no additional synergistic apoptotic effect was obtained from the physical co-treatment of the drugs. Thus, any synergistic results that might be obtained ahead are due to the prodrugs/nanoparticles in their conjugated form, rather than physical proximity/presence. Further studies will be performed to characterize the nanoparticles obtained from the three conjugates and to investigate their anti-MDS effect in vitro (SKM1, HL-60 and K562 cell lines) and in vivo (NSJ mice, PDX model, IV injection).

## REFERENCES

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**Figure 2:** a-c: MTT cell metabolic activity assay on HL-60 cell line (acute myeloid leukemia) to determine the  $IC_{50}$  at 24 hours of azacitidine, the co-treatments and the fatty acids respectively ( $\mu M$ ) d-g: Apoptosis assay on HL-60 cell line to determine the percentage of Annexin V-positive cells at 24 hours of azacitidine, oleic acid, docosahexaenoic acid and eicosapentaenoic acid respectively; post treatment with concentrations around the  $IC_{50}$  of azacitidine ( $\mu M$ )