***p≤0.001, **p≤0.01, *p≤0.05 vs Dox.

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GSH-responsive nanoparticles in the treatment of tumor cells

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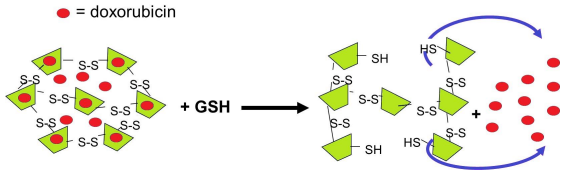
1.Background

New generations of drug-delivery nanovehicles, such as nanosponges, sensitive to intracellular concentration of glutathione (GSH-responsive-nanosponges, **GSH-NSs**), have shown to possess a great antitumor potential. Detoxifying systems have been recognized as an ideal and ubiquitous internal stimulus for a quick intracellular nano-carriers destabilization, to accomplish a proficient drug release. Since the chemo-resistance in tumor cells is generally accompanied by an increased GSH amount, the GSH-NSs should carry drugs preferentially in resistant cells, which show a higher GSH level than sensitive cells.

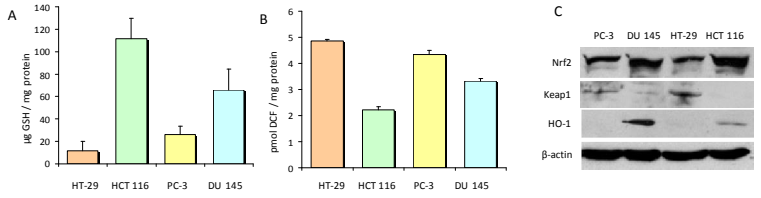


2.Materials and Methods

We employed doxorubicin incorporated in GSH-responsive nanosponges (**DOXO-GSH-NSs**) to test the drug toxicity, with respect the free form of doxorubicin (**DOXO**). For this purpose, two lines of human colorectal cells with different GSH content (**HT-29** and **HCT 116**) and two lines of prostate cancer cells (**PC-3** and **DU 145**) have been used. Cells were maintained in RPMI medium supplemented with 10% FBS and antibiotics; flow cytometric analysis were made on a FACScan with the Cyflogic software. Confocal images were acquired under a Fluoview laser-scanning microscope. Fluorescence microscope was an inverted Axiovert 35. Antibodies were from Santa Cruz, Abcam or Sigma-Aldrich (β-actin). Human Topoisomerase II assay kit was purchased from Topogen.

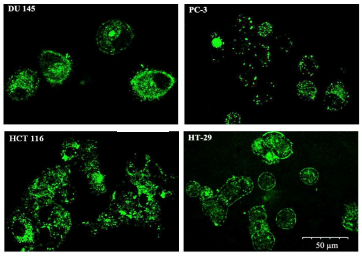
3.Our cell models show different intracellular GSH level (A), ROS content (B), and protein expression (Nrf2, Keap1 and heme oxygenase-1) (C).

HCT 116 and DU 145 display higher GSH levels, lower ROS content and more elevated Nrf2/Keap1 ratio and HO-1 expression than HT-29 and PC-3, suggesting a potential mechanism of resistance to pro-oxidant therapy.

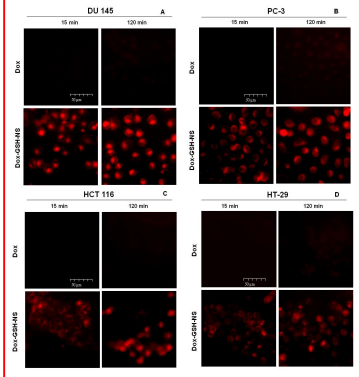


4. Rapid cell uptake of GSH-NSs

To detect the internalization of GSH-NSs into the cells, we performed a confocal microscopy analysis by using 6-coumarin-loaded GSH-NSs. The fluorescent nanoparticles were internalized within 10 minutes into all types of cells, with a slight difference for some PC-3 cells in which the fluorescence was evident, at this time point, mainly at the plasma membrane level.



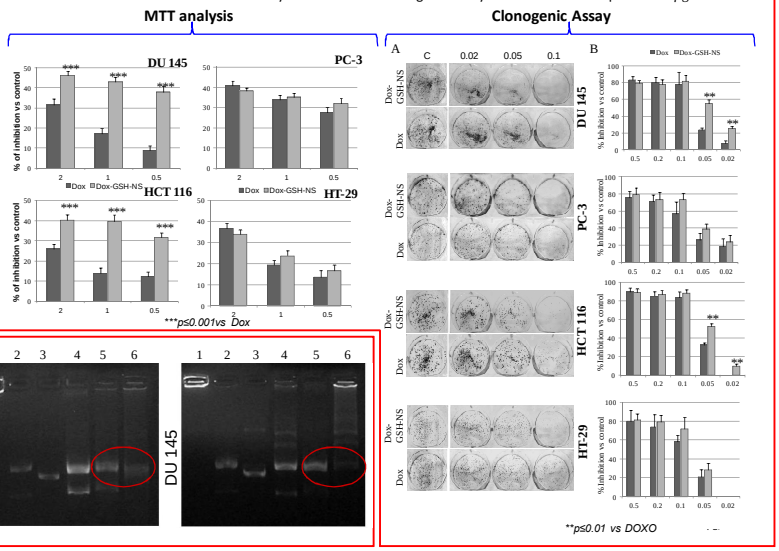
5. Internalization of DOXO is enhanced when the drug is loaded into the GSH-responsive nanoparticles



Internalization of doxorubicin (Dox) and doxorubicin loaded GSH-NS (Dox-GSH-NS). DU 145, PC-3, HCT 116 and HT-29 cells were collected after 15 and 120 min from the treatment with 0.5 μg/ml of Dox or Dox-GSH-NS. Red fluorescence of doxorubicin was examined by using a fluorescence microscopy (580 nm).

6. DOXO-GSH-NSs are more effective in affecting cell viability and cell proliferation vs free DOXO.

All cell lines are sensitive to the free form of doxorubicin, in a dose-dependent manner; however, when they are treated with the drug loaded in GSH-NSs, toxic and inhibitory effects are greater in the cells with a higher level of GSH, HCT 116 and DU 145. These results were obtained with the MTT analysis and with the Clonogenic Assay at 24h. Doses are expressed in μg/ml.



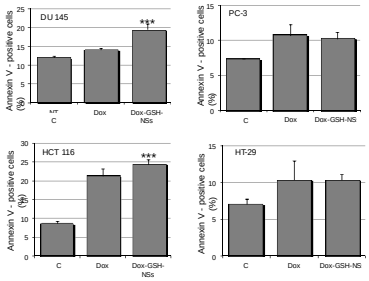
7. DOXO-GSH-NSs affects Topoisomerase II activity

The induction of topoisomerase II-mediated DNA strand breaks plays a key role in the doxorubicin-induced cytotoxicity. Doxorubicin binds the topoisomerase II cleavable complex, resulting in double-strand DNA breaks. Resistant cells (HCT 116 and DU145) were treated with 0.5 μg/ml of DOXO-GSH-NSs or free DOXO for 18h and topoisomerase II activity was evaluated. Nuclear extracts were added to KDNA (kinetoplast DNA) and the amount of DNA decatenation was examined. Decatenation of DNA was more inhibited in both cell lines when treated with DOXO-GSH-NSs vs free DOXO.

Lane 1, KDNA without topoisomerase II (catenated form);
lane 2, KDNA with topoisomerase II (decatenated form);
Lane 3: linear DNA;
line 4: control;
lane 5: Cells treated with DOXO;
lane 6: cell treated with DOXO-GSH-NSs.

8. The amount of cell death induced by DOXO-GSH-NSs was higher than that induced by free DOXO, in cells with high GSH content.

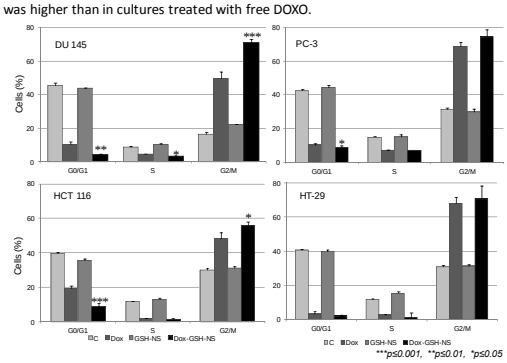
Annexin V-positive cells were analyzed 24 hours after the treatment with the drugs at the concentration of 2 μg/ml. While, the amount of annexin-positive cells was similar in PC-3 and HT-29 cells after treatment with free DOXO or DOXO-GSH-NSs, in DU 145 and HCT 116 cells the amount of annexin V-positive cells, after treatment with DOXO-GSH-NSs was higher than that obtained after treatment with free DOXO.



***: p < 0.001 vs Dox,

9. Cell cycle is significantly impaired by DOXO-GSH-NSs

Treatments with DOXO and DOXO-GSH-NSs were performed at the concentration of 0.5 μg/ml and the cell cycle analysis was performed 24 hours after the treatment. DOXO induced a reduction of G0/G1 cells and an increase of cells in the G2/M phase of the cell cycle. However, in cells with high GSH (DU145 and HCT-116) treated DOXO-GSH-NSs, the amount of G2/M cells was higher than in cultures treated with free DOXO.



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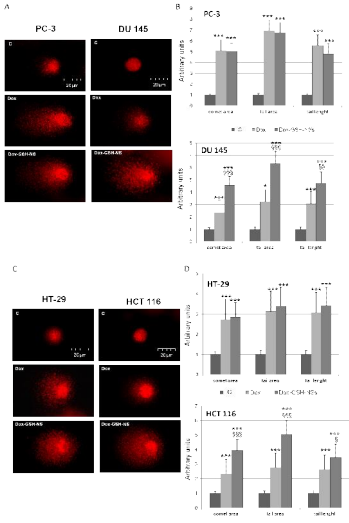
10. DOXO-GSH-NSs cause more DNA damage than free DOXO

DNA damage was assessed by comet assay 24 hours after treatment with 1 μg/ml of DOXO or DOXO-GSH-NSs. Results confirmed the higher effect displayed by DOXO-GSH-NSs, with respect to free doxorubicin, in DU 145 and HCT 116 cells, with high content of GSH.



*** p ≤0.001 vs. C; *p≤0.05 vs. C; §§§ p≤0.001 vs. Dox; § p≤0.05 vs. Dox

11.Conclusions

Doxorubicin loaded- GSH-NSs are rapidly internalized by cancer cells of different origins and, by targeting cells with high antioxidant potential, showed, in these cells, a higher cytotoxic activity than the free drug. Moreover, this delivery system can maintain cell toxicity, for a longer time than the drug in free form, thus allowing a reduction of the effective doses and, of consequence, a reduction of drug systemic adverse effects. All these characteristics suggest that GSH-NSs can be a suitable drug delivery carrier for future applications in cancer therapy.