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High aldehyde dehydrogenase activity does not protect colon cancer cells against $TPCS_{2a}$-sensitized photokilling

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Aldehyde dehydrogenases (ALDH) are detoxifying enzymes that are upregulated in cancer stem cells (CSCs) and may cause chemo- and ionizing radiation (IR) therapy resistance. By using the ALDEFLUOR assay, CD133 + human colon cancer cells HT-29, were FACSorted into three populations: $ALDH^{bright}$, $ALDH^{dim}$ and unsorted (bulk) and treated with chemo-, radio- or photodynamic therapy (PDT) using the clinical relevant photosensitizer disulfonated tetraphenyl chlorin ($TPCS_{2a}$/fimaporfin). Here we show that there is no difference in cytotoxic responses to $TPCS_{2a}$-PDT in $ALHD^{bright}$, $ALDH^{dim}$ or bulk cancer cells. Likewise, both 5-FU and oxaliplatin chemotherapy efficacy was not reduced in $ALDH^{bright}$ as compared to $ALDH^{dim}$ cancer cells. However, we found that $ALHD^{bright}$ HT-29 cells are significantly less sensitive to ionizing radiation compared to $ALDH^{dim}$ cells. This study demonstrates that the cytotoxic response to PDT (using $TPCS_{2a}$ as photosensitizer) is independent of ALDH activity in HT-29 cancer cells. Our results further strengthen the use of $TPCS_{2a}$ to target CSCs.

Aldehyde dehydrogenases (ALDHs) constitute a group of enzymes that have been associated with cancer progression and cancer therapy resistance.[1] ALDHs have diverse cellular activity, including vital role in detoxification of aldehydes to carboxylic acids, thereby preventing generation of reactive oxygen species (ROS) and lipid peroxidation.[1] In addition, ALDHs are involved in the synthesis of retinoic acid, which is important for cell survival, proliferation, embryogenesis and development of the immune system.[1] Overexpression of ALDH1 is used as a marker for both normal stem and progenitor cells and cancer stem cells (CSCs).[2,3] High ALDH1 activity provides a survival advantage of CSC as they are more equipped to resist accumulation of toxic aldehydes induced by increased metabolic activity, ionizing radiation or ROS-generating drugs.[1,4] In this communication, we present results obtained in fluorescence-activated cell sorted (BD FACS Aria II cell sorter from Becton Dickinson (BD Biosciences, San Jose, USA)) human colon cancer cells with high ($ALDH^{bright}$) and low ($ALDH^{dim}$) ALDH activity. We compared these populations with regard to cytotoxic responses to chemotherapy, ionizing radiation or photodynamic therapy (PDT). For PDT, we selected the photosensitizer disulfonated tetraphenyl chlorin ($TPCS_{2a}$/fimaporfin, PCI Biotech AS, Oslo, Norway) as $TPCS_{2a}$ is a clinical relevant photosensitizer used in the drug delivery technology photochemical internalization (PCI).[5,6]

The ALDEFLUOR assay (STEMCELL Technologies, Vancouver, Canada) was performed to evaluate ALDH activity and cell sorting. The assay is based on the use of BODIPY-aminoacetaldehyde (BAAA) which is a substrate of ALDH which convert BAAA into BODIPY-aminoacetate (BAA^-) that is highly fluorescent and retained in live cells due to its negative charge.[7] Thus, cells with high and low ALDH activity can be distinguished and sorted using flow cytometry based on the fluorescent signal from BAA^-. The ALDH inhibitor, *N*,*N*-diethylaminobenzaldehyde (DEAB), was included as a control providing adequate gating strategy for flow cytometry. By flow cytometry (BD LSR II, BD Biosciences), we screened a panel of eight cancer cell lines for ALDH activity which included; HT-29 (human colorectal adenocarcinoma, ATCC®HTB-38™), 5-FU-resistant and sensitive Panc 03.27-derived monoclonal cell lines (human pancreatic adenocarcinoma generated as previously described,[8] provided by Dr. Stephan Krauss), CT26.WT (murine undifferentiated colon carcinoma, ATCC®CRL-2638™) and 4T1 (murine triple negative mammary carcinoma, ATCC®CRL-2539™) (Fig. 1).

A shift in fluorescence was observed in both murine cell lines, CT26.WT and 4T1, and in three of the 5-FU-resistant Panc 03.27-derived cell lines, Panc 03.27R-B1L, Panc 03.27R-B1Q and Panc 03.27R-B1LV, indicating homogenous ALDH activity. Interestingly, the 5-FU-sensitive Panc 03.27S-Nt and Pan03.27S-Nw cell lines, displayed heterogeneous ALDH activity compared to Panc 03.27R-B1L, -B1Q and B1V.

We have previously shown that the 5-FU-resistant Panc 03.27R-B1L, -B1Q and -B1V are hypersensitive to $TPCS_{2a}$-PDT compared to the 5-FU sensitive clones.[9] Based on this, and the

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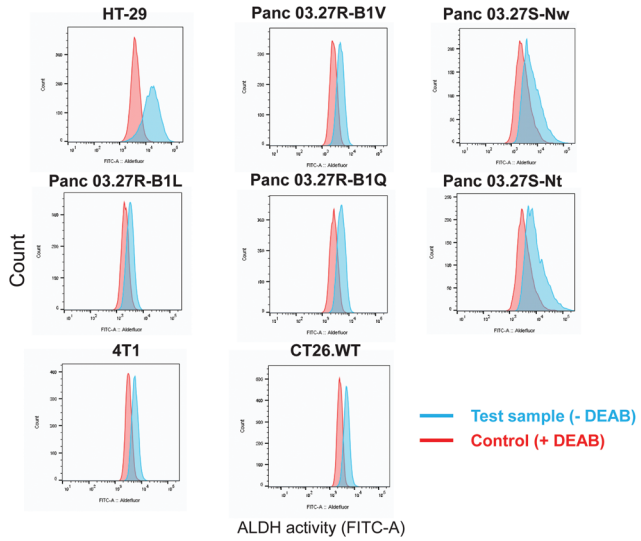

Fig. 1 ALDH activity in 8 different cancer cell lines measured by the ALDEFLUOR assay. Representative flow results from ≥3 independent experiments of both human (HT-29 and 5-FU-sensitive (Nt and Nw) and resistant (B1L, B1Q and B1V) sub-clones of Panc 03.27) and murine (4T1, CT26.WT) cancer cell lines.

lack of information regarding the PDT-effect on ALDH^{bright} *versus* ALDH^{dim} cancer cells in the literature, we wanted to explore the cytotoxic effect of TPCS$_{2a}$-PDT with regard to ALDH activity within the same cell line to exclude inter-cell line genetic/proteomic variations.

Of all cell line tested, the HT-29 cell line exhibited the highest heterogeneous mixture of ALDH activity, where the median fluorescence intensity in cells incubated with BAA$^-$ was more than 5-fold higher than the DEAB control (Fig. 1). Thus, HT-29 was selected for fluorescence activated cell sorting (FACS) and subsequent evaluation of responses to chemo-, radio-, and photodynamic therapy (PDT). By means of the ALDEFLUOR assay, HT-29 cells were FACSorted into three populations: (1) Cells that exhibited very high fluorescence intensity (near 10% of the cells gated with the highest BAA$^-$ signals), indicating high ALDH activity, were designated ALDH^{bright} (Fig. 2). (2) Correspondingly, cells that displayed very low fluorescence intensity (near 10% of the cells gated with the lowest BAA$^-$ signals) were defined as ALDH^{dim}. (3) Finally, unsorted cells were included to represent the bulk population. In all experiments, cells were sorted directly onto 96-well- or 6-well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) containing sterile filtered (0.22 μm) conditioned medium mixed with fresh McCoy's 5a medium (1 : 1). The culture medium was supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich). The sorted cells were allowed to attach overnight and subjected to treatment as indicated.

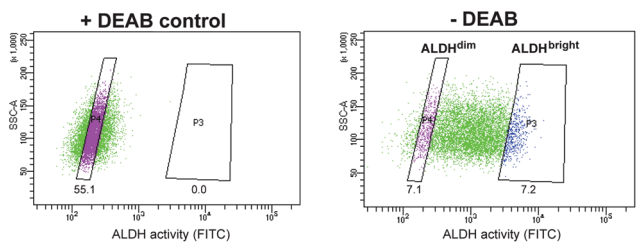

Fig. 2 Heterogeneous ALDH activity in HT-29 colon cancer cells stained with ALDEFLUOR. Representative dot-plot of live and single cells showing sorting gate for ALDH^{dim} and ALDH^{bright}. A control containing DEAB, an ALDH inhibitor, was used to set the gates. The number under each gate indicate percentage of parent population. The figure is representative of at least three independent experiments.

High ALDH activity has been associated with chemoresistance in different cancer types.[10–13] We assessed the chemotherapy response of FACSorted HT-29 cells to increasing concentrations of 5-FU or oxaliplatin (both from Sigma-Aldrich) (Fig. 3A and B).

Chemotherapy-induced cytotoxic responses were measured using the MTT viability assay (0.25 mg ml^{-1}, 4 hours incubation). Surprisingly, the cell viability was found to be similar in all FACSorted populations at all concentrations tested which indicate that ALDH activity does not significantly affect 5-FU and oxaliplatin sensitivity in the HT-29 cell line. Our results are in contrast with Kozovska *et al.* that reported inhibition of

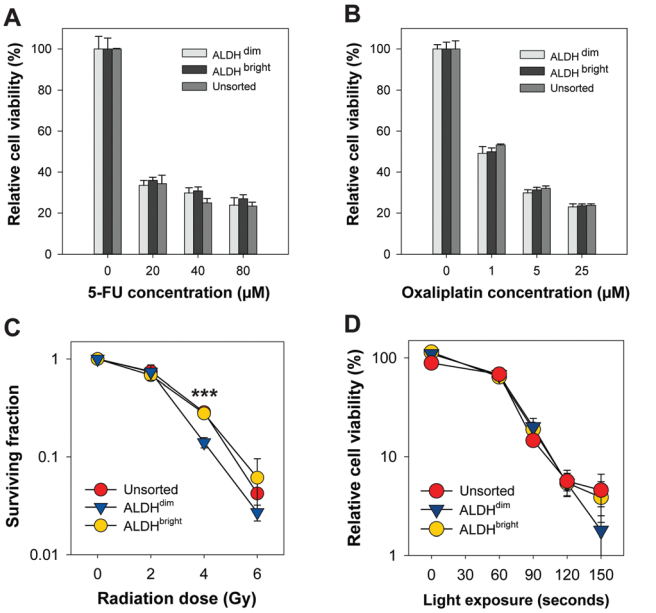

Fig. 3 Treatment sensitivity of ALDH^{dim}, ALDH^{bright} and unsorted cells after chemotherapy, ionizing radiation or TPCS$_{2a}$-PDT. Relative cell viability after 72 hours incubation with (A) 5-FU and (B) oxaliplatin, measured by MTT. Representative results of three independent experiments (mean ± S.D.). **(C)** Surviving fraction (SF) of HT-29 cells after increasing radiation dose, measured with clonogenic assay. Up to 14 days post-irradiation, the colonies were fixed and stained. To determine SF, count were normalized using plating efficiency of corresponding control. Mean of three independent experiments ± S.E. *** = $p < 0.001$, two-tailed p-value (Student's t-test). (D) Relative cell viability measured 72 hours post-TPCS$_{2a}$ PDT, measured with MTT assay. 60 seconds light exposure = 0.58 J cm^{-2}, MTT data are normalized to untreated controls. Representative results of three independent experiments (mean ± S.D.).

ALDH using DEAB in combination with 5-FU or cisplatin significantly reduced cell viability in HT-29 cells.[14] On the other hand, Prasmickaite *et al.* demonstrated similar sensitivity of the anti-melanoma drug dacarbazine in ALDEFLUOR-sorted cells isolated from malignant melanoma patients which indicate that ALDH alone might not be sufficient to select for chemoresistant malignant melanoma cells.[15] In 5-FU- and oxaliplatin-resistant HT-29 cells, a 16-to-30 fold enrichment of the cancer stem cell marker CD133 was observed which may indicate that CD133 alone or in combination with ALDEFLUOR may be more suitable to select for resistant HT-29 cells.[16] Moreover, CD133 + cells were found to be highly resistant to 5-FU and oxaliplatin in human colon cancer cells derived from patients.[17] Data from our lab[18] indicate that HT-29 exhibit high CD133 expression. As we did not include CD133 expression as a parameter for gating in our FACS, we cannot exclude that sorting based on a combination between ALDH and CD133 would have resulted in isolation of a chemo-resistant population. Therefore, the HT-29 cytotoxicity data obtained after 5-FU or oxaliplatin chemotherapy and the ALDEFLUOR assay results showing reduced ALDH activity in the 5-FU-resistant Panc 03.27 cell lines (Fig. 1) suggests that resistance to 5-FU may not be directly linked to ALDHs.[9] As this is in conflict with existing literature, we suggest that more experimental research on the role of ALDH in response to 5-FU treatment is important, *e.g.* including ALDH knock-out models and evaluations in other cancer cell lines with heterogeneous mixture of ALDH activity.

Clonogenic assay was used to determine cell survival/death after ionizing radiation treatment of $ALDH^{bright}$, $ALDH^{dim}$ and unsorted HT-29 cells in 6-well culture plates (Nunc). The cells were treated with a single fraction irradiation up to 6 Gy (160 kV, 6.3 mA, X-ray generator, Faxitron CP160, Tuscon, AZ, USA). When sufficiently large colonies in control plates were formed (10–14 day post-treatment), colonies were ethanol fixed, methylene blue stained and counted manually. A colony was defined to consist of at least 50 cells.[19] Interestingly, based on three independent biological replicates, a slightly higher plating efficiency of $ALDH^{bright}$ (53.3 ± 2.5%) was observed compared to $ALDH^{dim}$ (43.0 ± 5.3%, not significant, $p = 0.152$). The plating efficiency of $ALDH^{dim}$ cells was also slightly lower compared to unsorted cells (49.3 ± 5.3%, not significant, $p = 0.404$). $ALDH^{bright}$ and unsorted HT-29 cells tended towards a higher ionizing radiation resistance than $ALDH^{dim}$ cells but only showed a significant difference after irradiation with 4 Gy (Fig. 3C). The surviving fraction (SF) of $ALDH^{dim}$ cells was significantly lower (~2-fold) at 4 Gy (SF: 14.1 ± 0.98%, $p < 0.001$) compared to $ALDH^{bright}$ (SF: 27.9 ± 1.2%) and unsorted cells (SF: 28.7 ± 4.1%, $p < 0.001$). This observation is in agreement with existing studies which reported radioresistance in cells with high ALDH activity as well as in CSCs selected using other markers.[20,21]

PDT is based on the use of a light sensitive drug (photosensitizer) that is nontoxic in the dark and which accumulates in tumour tissues. Light exposure of the tumour tissue results in excitation of the photosensitizer leading to energy transfer from the photosensitizer to molecular oxygen (O_2) or to other cellular components, resulting in generation of cytotoxic concentration of reactive oxygen species (ROS), of which singlet oxygen ($^1O_2$) is the most abundant. PDT-induced ROS-generation results in peroxidation of vital cellular components and initiation of cell death mechanisms such as apoptosis, necrosis or autophagy.[22]

In this work, we used the clinical relevant PCI photosensitizer $TPCS_{2a}$ (fimaporfin[23]) to compare PDT efficacy in HT-29 colorectal adenocarcinoma cells with either very high or very low ALDH activity. Cells were incubated with 0.4 μg ml^{-1} $TPCS_{2a}$ (PCI Biotech AS) for 18 hours, washed twice with PBS and chased for 4 hours in drug-free medium to remove plasma membrane-bound $TPCS_{2a}$ to mimic a PCI protocol. The cells were subjected to broadband blue light irradiation (λ_{max} = 435 nm) with an output of 9.6 mW cm^{-2} (LumiSource, PCI Biotech AS). Cell viability was evaluated 72 hours post-light exposure by using the MTT assay, which is widely accepted in the field of PDT and has been used for 30 years to assess cell viability.[24] Furthermore, we have also shown that there is a good consistency between this assay and the clonogenic cell assay.[25,26] Of high interest, no statistical significant differences ($p > 0.1$ at all light exposure times) in cell viability was found between $ALDH^{dim}$ and $ALDH^{bright}$ cells treated with $TPCS_{2a}$-PDT (Fig. 3D). Of relevance, we previously demonstrated that $TPCS_{2a}$ is not a substrate for the CSC markers ABCG2 and ABCB1 (P-gp) transporter[26–28] which may explain why no difference in $TPCS_{2a}$ sensitivity was observed in ALDH-sorted cells. As this study is on the importance of ALDH activity and its influence on $TPCS_{2a}$-PDT efficacy in only the HT-29 cell line, this should be verified in other cell lines in future studies. In addition, further investigation to establish the role of ALDH activity in PDT using other photosensitizers is warranted. In conclusion, we show that $ALDH^{dim}$ cells are more sensitive to ionizing radiation at 4 Gy compared to bulk and $ALDH^{bright}$ populations, which is in line with the literature. However, we report that $TPCS_{2a}$-PDT is equally efficient in both $ALDH^{bright}$ and $ALDH^{dim}$ HT-29 cancer cell populations. Our data further strengthen the use of $TPCS_{2a}$-based PCI of CSC-targeting therapeutics.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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