Curcumin-based-fluorescent probes targeting ALDH1A3 as a promising tool for glioblastoma precision surgery and early diagnosis

Glioblastoma (GBM) is the most aggressive primary brain tumour for which both effective treatments and efficient tools for an early-stage diagnosis are lacking. Herein, we present curcumin-based fluorescent probes that are able to bind to aldehyde dehydrogenase 1A3 (ALDH1A3), an enzyme overexpressed in glioma stem cells (GSCs) and associated with stemness and invasiveness of GBM. Two compounds are selective versus ALDH1A3, without showing any appreciable interaction with other ALDH1 isoforms. Indeed, their fluorescent signal is detectable only in our positive controls in vitro and absent in cells that lack ALDH1A3. Remarkably, in vivo, our probe selectively accumulates in glioblastoma cells, allowing the identification of the growing tumour mass. The significant specificity of our compounds is the necessary premise for their further development into glioblastoma cells detecting probes to be possibly used during neurosurgical operations.
The term Glioma refers to a class of primary brain tumors that represent the 40% of all brain tumors. Glioblastomas are characterized by highly morphological heterogeneous neoplasms. The term “Glioblastoma” is a synonym of grade IV astrocytoma (GBM multiforme, or conventional GBM according to WHO classification). Two histologic variants of GBM has been recognized with different clinicopathologic properties: gliosarcoma and giant cell glioblastoma. Moreover, transcriptomic profiling has permitted to divide GBMs in four significant subtypes: proneural (PN), neural, classic, and mesenchymal (Mes). GBMs are very invasive and associated with a high ability to metastasize, with an extremely fast cell growth, a marked chemoresistance and a poor clinical outcome.

Their marked drug resistance is related to the presence of a high density of cancer stem cells (CSCs) that possess self-renewal ability and an unlimited proliferative potential, just like their physiological counterparts. Glioma stem cells (GSCs) also provide the tumor with an enhanced resistance to drugs, radiation and oxidative stress, thus increasing GBM resistance to treatments, and their presence is associated with metastasis and relapse. Moreover, mesenchymal GSCs (Mes-GSCs) proved to be significantly more radioresistant than proneural GSCs (PN-GSCs). It has been demonstrated that the radiation treatment can induce the phenotype shift from PN to Mes, set out by the loss of the PN marker SOX2 and by the expression of the Mes marker CD44. The tumors that are rich in Mes GSCs are the deadliest, the most dangerous and the most susceptible to relapse. As far as the tumorigenic behavior is concerned, Mes-GSCs showcased a higher growth potential under identical conditions compared to PN-GSCs both in vitro and in vivo. The only resolutive treatment is the surgical resection that, when possible, allows the complete resection of the main tumor. Even with this approach, though, the patient lifespan is around 5 years. This is the reason why the development of innovative tools for an early diagnosis and for the chemo treatment is of the utmost importance.

In a paper published by Zhang et al., a transcriptomic analysis highlights the enrichment of the cytoplasmatic enzyme aldehyde dehydrogenase 1A3 (ALDH1A3) in Mes-GSCs. ALDH1A3 belongs to an enzymatic superfamily of aldehyde dehydrogenases, composed of 19 different isoforms, and involved in the irreversible NAD+-dependent oxidation of a wide range of aldehydes. This superfamily is also involved in the reduction of oxidative stress and in the metabolism of several drugs, such as cyclophosphamide. ALDH1A3 belongs to the ALDH1A superfamily, that also includes ALDH1A1 and ALDH1A2. All three isoenzymes are involved in the oxidation of retinal to retinoic acid, a molecule that is essential for tissue differentiation and cellular development. ALDH1A1 and ALDH1A3 have been described as important markers and targets of CSCs in a wide variety of tumors. A several number of evidence acknowledge that ALDH1A3 can be considered a characteristic hallmark of the Mes-GSCs, which may play an important role in glioma malignancy, given that it is involved in stem cell viability drug resistance and cells maintenance arguing tumor invasion. Considering the well-known catalytic function of ALDHs, they are regarded as the key enzymes that can detoxify harmful aldehydes within the organism, and this could be the reason why so many cytotoxic antineoplastic molecules are inactivated by CSCs. As a result, ALDH1A1 and ALDH1A3 may protect CSCs from antineoplastic molecules, their levels could represent a prognostic factor that could anticipate the chemotherapy efficacy and their inhibition could make the tumor cells susceptible to medical treatments. To date, CSCs are considered as one of the key mechanisms used by the tumor to evade chemotherapy and radiation treatment. Based on these evidences targeting CSCs aiming to improve already-existing therapies, prevent the relapse of the tumor and facilitate an early diagnosis could improve already-existing therapies and prevent the relapse of the tumor.

Curcumin is the most famous and most abundant congener of curcuminoids, a class of bioactive compounds isolated from turmeric (*Curcuma longa* L.) and commonly used for flavoring food in the Southeast Asian and Middle Eastern countries. Nowadays it is used in the food industry as a coloring agent known as E100. In the past decade, this natural dye became one of the best candidates for the development of new therapies against gliomas. The antineoplastic abilities of curcumin, such as the induction of apoptosis and the inhibition of proliferation and invasion, have been proved in several tumors, including gliomas. Curcumin is also capable of inducing reactive oxygen species (ROS) in a wide variety of cancers, so as to lead to the activation of the MAPK apoptotic pathway. Even though CSCs radical scavenging systems have been reported, several studies confirmed that curcumin-induced ROS can target CSCs. However, this plethora of bioactivities should be viewed with skepticism: curcumin is one of the most famous Pan-Assay Interference Compounds, exhibiting all known behaviors of this class of molecules and confining it to a mere academic curiosity. Besides the well-known health-promoting benefits, curcumin also possesses a strong intrinsic fluorescence and some of its derivatives have recently been acknowledged as optical probes for the in vivo studies of several diseases, such as Alzheimer and solid tumor.

To date, as the surgical resection of the tumor mass is the fundamental treatment for GBM, having a tool that could lead to an early diagnosis and improve the surgeon accuracy during the operation would be a great advantage. Therefore, a fluorescent probe that can detect a mass of CSCs within the brain of a patient with glioma could be extremely helpful and useful. In this paper, we present the first study of two different selective ALDH1A3 fluorescent probes, with a curcumin scaffold-based nature, that are able to inhibit the activity of the recombinant enzyme and can be detectable only in our positive controls, both in vitro and in vivo.

**Results**

**Chemistry.** In this project we propose the synthesis of a class of curcumin-based probes where a triazole moiety is used as connector between the fluorescent dye and the functional group needed for the interaction with the active site of the enzyme. Since we already worked on the curcumin scaffold, we identified five key synths as starting points: the hemi-curcuminoids 4 and 5, the O-propargyl-vanillin (3) and the azido derivatives 8 and 9. Compounds 4 and 5 were easily obtained condensing respectively vanillin (1) and 4-(dimethylamino)benzaldehyde (2) with acetylacetone under Pabon conditions. The replacement of the vanillic moiety with a dimethylamino group was done to modulate the fluorescence emission and, in the attempt to enhance the solubility of the final compound. A second Pabon condensation of hemi-curcuminoids 4 and 5 with O-propargyl-vanillin (3), furnished respectively compounds 6 and 7 that underwent to a copper catalyzed Huisgen [3 + 2] cycloaddition with 8 and 9 leading compounds 10, 11 and 12. Given the possible solubility problems due to the presence of a triazole moiety together with a curcumin sub-structure, compounds 10 and 11 were phosphorylated using diethylchlorophosphate in presence of triethylamine affording compounds 13 and 14 (Fig. 1 and Supplementary Fig. S1).

Biochemical characterization of the interaction of probe 10 and probe 11 with recombinant human ALDH1A3. All five
fluorescent compounds were firstly characterized for their solubility in physiological buffer and for their absorbance values. We then performed a 3D analysis using a TECAN SPARK to simultaneously evaluate the excitation and the emission wavelengths, that were supposed to be similar to the curcumin ones. Among all five molecules, the Probe 10 and 11 showed the highest solubility and their fluorescence values were similar to the not substituted fluorophore. For these reasons, we decided to continue the biochemical characterization on Probe 10 and 11 using the selected parameters (Fig. 2). The fluorescent signal changes for both molecules upon binding to the target, were firstly evaluated with recombinant human ALDH1A3. Prior to the interaction with the target protein, the two probes possess an intrinsic low fluorescence emission, but the subsequent addition of ALDH1A3 to the mix leads to a consistent increase in the intensity of the fluorescent signal of about 12 folds for Probe 10 and 8 folds for Probe 11 (Fig. 2). To better characterized our fluorescent compounds, we tested their potential cross-reactivity toward the other two isozymes of the ALDH1A subfamily. As shown in Fig. 2, Probe 10 exhibits low cross-reactivity with the other two isozymes, with a reduction of the fluorescence signal of about 2 folds for ALDH1A1 and 4 folds for ALDH1A2, compared to what observed with ALDH1A3. In the case of probe 11, 4 folds and 6 folds reduction of fluorescence was observed in the presence of ALDH1A1 and ALDH1A2, respectively, compared to what observed with ALDH1A3. Both probes therefore revealed selectivity for ALDH1A3. In addition, a wide range of biologically relevant analytes were tested at a concentration of 100 \( \mu \text{M} \) in complex with a fixed concentration of 10 \( \mu \text{M} \) of the probe (either probe 10 or probe 11), to make sure that no potential off-target signals could affect the analysis. More specifically, we checked if there were abnormal fluorescence signals that could have been contributed by the presence of biologically relevant compounds, (e.g., biologically relevant amino acids), to buffers and molecules commonly used in experimental procedures. None of the selected compounds generated significant fluorescent signals, compared with the positive controls (Fig. 2).

On the basis of these encouraging results, we conducted an in-depth biochemical analysis on the two probes in order to characterize their mechanism of action. For greater clarity we will first illustrate the results obtained for Probe 10 and then those for Probe 11. The affinity of Probe 10 to the different isoenzymes revealed highly similar \( K_a \) values for all the three forms (ALDH1A1 = 31.1 \( \mu \text{M} \), \( R^2 = 0.98 \), ALDH1A2 = 36.9 \( \mu \text{M} \), \( R^2 = 0.98 \) and ALDH1A3 = 38.2 \( \mu \text{M} \), \( R^2 = 0.96 \)). To clearly understand the nature of the interaction between Probe 10 and ALDH1A3 we tested our fluorescent compound as an inhibitor, using an already published protocol. As shown in Fig. 3a, ALDH1A3 resulted to be the only strongly inhibited isozyme, with a \( K_i \) value of 0.880 \( \mu \text{M} \) (\( R^2 = 0.97 \)) and a competitive mechanism of action. Neither of the two other isozymes were inhibited, even at the highest probe concentration used to test the catalytic activities of ALDH1A1 and ALDH1A2. Probe 10 was also tested as a possible substrate, due to the presence of a benzaldehyde on the lateral chain of the fluorophore (Fig. 3b). Indeed, after 1 h of incubation, Probe 10 was fully metabolized and converted to its carboxylic derivative (Probe 10-COOH) by both ALDH1A1 and in part by ALDH1A2, while no oxidation could be detected in the case of ALDH1A3 as unambiguously demonstrated by a LC-HRMS analysis (Fig. 4 and Supplementary Fig. S2.a). Our investigations clearly demonstrate that Probe 10 binds to the enzyme active site without undergoing oxidation. All the results obtained allow us to understand and explain why it shows the same affinity toward the three isoenzymes: it is a substrate for ALDH1A1 and ALDH1A2 and a potent competitive inhibitor for the isoform 1A3. In conclusion, the isoform 1A3 is inhibited by Probe 10 in the low micromolar range, shows selectivity vs. the other two isoforms and its binding to the enzyme active site results in a significant increase of the fluorescence signal.

As described above for molecule 10, the affinity of Probe 11 for the three isozymes has also been investigated by determining the \( K_d \) of the compound-enzyme complexes. Probe 11 preferentially interacts with ALDH1A1 and ALDH1A3, with similar \( K_d \) values, while is less affine to the ALDH1A2 isozyme (Supplementary Fig. S3). Surprisingly, Probe 11 has no inhibitory effect against any of the three ALDH1A isoenzymes and has not been shown to be a substrate. These results have been further confirmed by LC-HRMS experiments carried out on Probe 11 in the presence of the three different isoenzymes (Supplementary Fig. S2.b). Overall, our data suggest that Probe 11 does not display a strong
selectivity among the three enzymes but rather behaves as a pan-probe for the ALDH1A subfamily.

Taken together, and from a biochemical perspective, these results suggest that both compounds are suitable for our aims with Probe $\text{10}$ that emerges as the best performer. Yet, a much deeper in vitro analysis is needed to confirm the potential selectivity of Probe $\text{10}$ and Probe $\text{11}$ and to better investigate the possible cytotoxic effects.

ALDH1A3 detection in vitro. To better characterized the selectivity fluorescence profile of Probe $\text{10}$, we selected four cell lines based on their different ALDH1As expression profile, as described in The Human Protein Atlas [https://www.proteinatlas.org/], in order to validate the in vitro behavior of the two probes. Human U87MG glioblastoma cells are labeled as ALDH1A3$^+$ cell line, HEK293T as ALDH1A2$^+$ cell line, human fetal astrocytes (hASTRO) as ALDH1A1$^+$ cell line and 4T1 mammary carcinoma as triple negative ALDH1As subfamily. Moreover, we have chosen two different patient-derived glioblastoma cell lines (3054 and 3060) according to their increased expression levels of ALDH1A3 compared to the other ALDH1A isoforms (ALDH1A3 fold change of 8.58 and 7.34, ALDH1A2 fold change 5.8 and 5.6, ALDH1A1 4.1 and 4.5, respectively, as reported in HGCC site [https://www.hgcc.se/]). We determined the ability of Probe $\text{10}$ to enter the cells and verified its fluorescence in ALDH1A3 positive cells. As depicted in Fig. 5a, b, Probe $\text{10}$ can determine fluorescence in patient-derived glioblastoma cell lines 3054, 3060 and in the immortalized line U87MG, but not in HEK293T, hASTRO and 4T1 cell lines (Fig. 5c–e). The probe is localized to the cytoplasm confirming the specific and exclusively cytosolic binding to ALDH1A3, as confirmed with fluorescence quantification (Fig. 5g). At the same time, Probe $\text{10}$ is unable to induce cell mortality (Fig. 6a). These data were corroborated by the results of flow cytometry analysis (Fig. 6b–d and Supplementary Fig. S4), showing that positivity to Probe $\text{10}$ was limited to ALDH1A3$^+$ cells. Positivity reverted with pre-treatment by the pan-ALDH inhibitor DEAB. Moreover, the fluorescence has been observed using Probe $\text{11}$ only in U87MG cell lines (Supplementary Fig. S5a–d).

Probe $\text{10}$ is able to selectively define glioblastoma cells in vivo. Based on the excellent results obtained from the in vitro analyses, we decided to verify the potential of our probes also in vivo. The presence of a mouse ortholog of our target enzyme, ALDH1A3, having a sequence homology equal to 98%, allowed us to set up an in vivo experiment. To evaluate the fluorescent signal of both probes in an in vivo model of orthotopic transplantation of glioblastoma cells, we decided to use the GL261, a murine transplantable high grade glioma cell line positive to ALDH1A3. We initially measured the mRNA of
the enzyme of our interest and checked whether the GL261 cells show the same permeability to our fluorescent compounds (Fig. 7a and Supplementary Fig. S6). Figure 7b, c and Supplementary Fig. S6 show that also in GL261 cells both Probes 10 and 11 were able to enter and label the murine glioblastoma cells. In particular, Probe 11 in vitro displays a higher intrinsic fluorescence, but it is less selective due to the presence of background signals that are non-specific for cancer cells internalization, as already suggested by the biochemical experiments. Nevertheless, even if the fluorescent signal has turned out to be lower in vitro, Probe 10 appears to be more promising, due to its ability to accumulate in tumor cells with a lower background signal. Subsequently, we induced glioblastoma in mice brain by stereotactically injecting 1 × 10⁵ GL261 cells into the left striatum of adult mice. All mice were injected i.p. with an equivalent dose of the 2 probes. The in vivo experiments showed that only Probe 10 was able to selectively label the growing tumor. As shown in Fig. 8a–c, Probe 10 accumulates in GL261 cells outlining the tumor growing in the left striatum and invading the adjacent areas of the brain, without significant interference from the adjacent tissue. Scattered tumor cells were also visualized infiltrating the adjacent areas of the mouse brain. In addition, it is possible to appreciate that in tumor the fluorescence of Probe 10 is mostly contained in the cytoplasm of cancer cells (Fig. 8d–f). The same coronal section of a tumor-bearing brain in an animal injected with compound Probe 11 does not penetrate to any appreciable level the tumor cells in vivo, despite penetrating GL261 cells in vitro (Fig. 8g–i).

Discussion

The research of new targets for anti-cancer therapies and of markers for an efficient surgery and diagnosis still represents an ongoing challenge for several tumors and in particular for glioblastoma. With this paper, we describe new possible selective probes that are able to detect ALDH1A3 in GBM cancer cells.
Both fluorescent compounds showed biological interesting data, highlighting Probe 10 as a promising starting point for the development of ALDH1A3 probes. Indeed, this molecule can be considered as the first selective fluorescent probe for human ALDH1A3 ever reported and characterized in a specific cancer cell line. The biochemical characterization of Probe 10 demonstrates its selectivity toward ALDH1A3, and our in vitro experiments confirmed a strong signal not only in U87 cell line,
ALDH1A3⁺, but also in the patient-derived glioblastoma cell lines 3054 and 3060, compared with our negative controls where no significant emission was observed, meaning while, the in vitro imaging using the 4T1 cell line, ALDH1A⁻, but ALDHs positive, shows only unspecific signals. Moreover, fluorescence emission in ALDH1A3⁺ glioblastoma cell lines is only present in the cytosol, while the nucleus appeared unstained. Probe 10 is characterized by a benzaldehyde moiety on the lateral chain that is also present in DEAB. Based on this chemical feature, Probe 10 showed a marked binding preference for ALDH1As, with highly similar KD values. Our biochemical data confirmed that Probe 10 binds to the catalytic site of all three isoenzymes and it is metabolized in its corresponding acidic form by ALDH1A1 and ALDH1A2 but not by ALDH1A3. In the latter, Probe 10 was observed to behave as a strong competitive inhibitor with Ki value in the low micromolar range (0.880 µM). As confirmed by the in vitro imaging analysis, Probe 10 was detectable only in our positive controls U87MG, 3054 and 3060, whereas in the negative controls we were not able to detect any significant cytosolic emission. As a result, the Probe 10 displays the characteristics of an inhibitor of ALDH1A3 that most probably directly interact with the catalytic cysteine, Cys 314, while the other two isoenzymes metabolize the molecule in a faster way. We also performed a flow cytometry analysis that showed that Probe 10 is more selective than Probe 11 on the U87 ALDH1A3⁺ cells, even with a lower fluorescent intensity. As show in result section, we confirmed our data also in vivo on model of orthotopic transplantation of GL261 glioblastoma cells, positive at the ortholog of human ALDH1A3. Our results clearly show that Probe 10 is able to delimit the cancerous mass. Indeed, we obtained a significant signal only delimited on the tumor tissue in treated animals. This property could be exploited to improve precision in fluorescence-guided resection of malignant gliomas. This is a rapidly evolving technique that is currently based on the injection of fluorescent markers like fluorescein and 5-aminolevulinic acid that accumulate in the tumor by unspecific mechanisms. The finding of new markers, with the aim of designing more specific molecules for more effective therapies and faster diagnosis, is essential for the modern precision medicine and ALDH1A3 represents one of the most suitable candidates for this goal. Taken together, our results highlight that Probe 10 is a promising tool to selectively sort glioblastoma infiltrating cells, if compared with the probes already available on the market, such as ALDEFLOUR.

In conclusion, our results demonstrate that Probe 10 is the first ALDH1A3 selective fluorescent tool that is able to preferentially bind to the target enzyme, without inducing significant cytotoxic effects, both in vitro, in human Glioblastoma cells U87MG, 3054, and in vitro and in vivo murine GL261 cells outlining the tumor growing in the left striatum and invading the adjacent areas of the brain.

Materials and methods

General. NMR spectra were measured on Bruker Avance 400 MHz spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl3: δH = 7.21, δC = 77.0). MS spectra were acquired by Thermo Scientific Q-Exactive Plus (Supporting Information S1). Reactions were monitored by thin-layer chromatography on Merck 60 F254 (0.25 mm) plates, visualized by staining with 5% H2SO4 in EtOH and heating. Organic phases were dried with Na2SO4 before evaporation. Chemical reagents and solvents were purchased from Sigma-Aldrich, TCI Europe or Fluorochem and were used without further purification unless stated otherwise. Petroleum ether with boiling point of 40–60 °C was used. Silica gel 60 (70–230 mesh) was used for gravity column chromatography.

3-methoxy-4-propargyloxybenzaldehyde (3). Propargyl bromide (6.01 ml, 80% w/v in toluene, 52.580 mmol, 2 eq) was added to a suspension of vanillin (1, 4 g, 26.290 mmol, 1 eq) and potassium carbonate (5.09 g, 36.806 mmol, 2 eq) in acetone (80 ml). The suspension was heated to reflux for 12 h and the solvent was removed under reduced pressure. Water was added and the aqueous phase was extracted with EtOAc, washed with water, brine, and dried. The crude was purified by column chromatography (PE/EtOAc 9:1 as eluent) to give 3-methoxy-4-propargyloxybenzaldehyde (3, 2.58 g, 52%) as white crystalline solid. 1H NMR (400 MHz, CDCl3) δ (400 MHz, CDCl3) 8.87 (s, 1H), 7.47 (dd, J1 = 6.8, J2 = 1.4 Hz, 1H), 7.44 (d, J = 1.4 Hz, 1H), 7.14 (d, J = 6.8 Hz, 1H), 4.86 (d, J = 2.5 Hz, 2H), 3.95 (s, 3H), 2.56 (t, J = 2.5 Hz, 1H). 13C NMR (100 MHz, CDCl3) δ 200.9, 152.1, 150.0, 130.9, 126.3, 112.5, 77.4, 77.2, 56.6, 56.0; HRESIMS m/z [M + H]+ 191.0628 (calcld for C11H12O3, 191.0630).
(E)-6-(4-hydroxy-3-methoxyphenyl)hex-5-ene-2,4-dione (4). Boron oxide (14.6 g, 174.85 mmol, 5.3 eq) and acetylacetone (13.5 ml, 131.46 mmol, 4 eq) were dissolved in DMF (10 ml) stirred at 80 °C for 1 h. The mixture was cooled to 0 °C, then trimethyl borate (23.8 ml, 209.55 mmol, 6.38 eq) and vanillin (1.5 g, 32.86 mmol, 1 eq) were added. Butylamine (1.3 ml, 13.27 mmol, 0.4 eq) was then added dropwise. The obtained mixture was heated to 80 °C for 24 h, then cooled to room temperature and 5% AcOHaq (200 ml) was added. The suspension was then stirred for 2 h at room temperature and a yellow precipitate formed. The precipitate was filtered, washed with water (3 × 100 ml), and purified by chromatography over silica (PE/EtOAc 7:3) to afford (E)-6-(4-hydroxy-3-methoxyphenyl)hex-5-ene-2,4-dione (4, 3.22 g, 51%) as a yellow solid. 1H NMR (400 MHz, CDCl3) δ 7.53 (1H, d, J = 15.8 Hz, 1H), 7.09 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, 1H), 7.02 (d, J = 1.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.32 (d, J = 15.8 Hz, 1H), 5.91 (1H, bs, OH), 5.62 (s, 1H), 3.94 (s, 3H), 2.16 (s, 3H). 13C NMR (100 MHz, CDCl3) δ 197.0, 177.9, 147.7, 146.7, 140.0, 127.6, 120.2, 114.8, 110.5, 100.7, 55.9, 26.5; HRESIMS m/z [M + H]+ 235.0973 (calcd for C13H15O4, 235.0970).

1-(4-(Dimethylamino)phenyl)-5-hydroxyhexa-1,4-dien-3-one (5). Boron oxide (4.17 g, 46.933 mmol, 0.4 eq) and 4-dimethylaminobenzaldehyde (2.1 g, 6.705 mmol, 1 eq) were dissolved in EtOAc (50 ml) stirred at 80 °C for 1 h. The mixture was cooled to 0 °C, then trimethyl borate (761 ml, 6.705 mmol, 1 eq) and acetylacetone (6.88 ml, 67.047 mmol, 10 eq) were added. Butylamine (665 ml, 6.705 mmol, 1 eq) was then added dropwise. The obtained mixture was heated to 80 °C for 72 h, then cooled to room temperature. H2SO4 2 M (50 ml) was added, then the organic phase was washed with NaHCO3 s.s. (50 ml), water, brine and dried. The crude was purified by column chromatography (PE/EtOAc 9:1 as eluent) to afford 1-(4-(Dimethylamino)phenyl)-5-hydroxyhexa-1,4-dien-3-one (5, 291 mg, 19% yield) as a red powder. 1H NMR (400 MHz, CDCl3) δ 7.38 (s, 1H), 7.30 (d, J = 9.3 Hz, 2H), 6.64 (d, J = 9.3 Hz, 2H), 3.04 (s, 6H), 1.90 (d, J = 5.7 Hz, 6H); 13C NMR (100 MHz, CDCl3) δ 195.59, 179.56, 151.65, 140.98, 129.79, 122.79, 117.37, 111.92, 100.16, 40.11, 26.47; HRESIMS m/z [M + H]+ 232.1336 (calcd for C14H18NO2, 232.1338).

Fig. 7 In vitro experiments showing that ALDH1A3 is the most expressed isoform in GL261 murine glioma cells and both fluorescent substrates enter and label the same cells. a Results of quantitative rtPCR showing the levels of expression of ALDH1A1 (1a1), ALDH1A2 (1a2) and ALDH1A3 (1a3) in murine GL261 cells. ALDH1A3 is significantly more expressed than the other isoforms. b Living GL261 cells were analyzed through an ImageStreamX MarkII using two channels: brightfield (Ch 01) and fluorescence (Ch 02) after 1 h incubation in probe 10, cells were thoroughly washed in PBS and analyzed. Two different representative cells are shown. From above, row 1 brightfield images (Ch 01), row 2 fluorescent images (Ch 02). “In Focus Cells” were identified based on the “Gradient Root Mean Square (RMS) Contrast Feature” that captures in focus images of cells identified by high normalized pixel intensity gradient (RMS values) derived from Ch 01; then, a scatter plot of the “Aspect Ratio Feature” vs. brightfield “Area Feature” was used to identify single cells (singlets) from debris or cell clumps based on high aspect ratio and low area value. c Fluorescence intensity variations are shown in the histogram and dot plot (Area vs. Intensity). Scale bars: 5 µm are the same in all images.
AcOHaq (50 ml) was added. The suspension was then stirred for 2 h at room temperature and an orange solid. 1H NMR (400 MHz, CDCl3) δ 8.13 (d, J = 15.8 Hz, 1H), 7.57 (d, J = 15.8 Hz, 1H), 7.46 (d, J = 8.9 Hz, 2H), 7.08 (m, 3H), 6.69 (d, J = 8.6 Hz, 2H), 5.78 (s, 1H), 4.80 (d, J = 2.4 Hz, 2H), 3.92 (s, 3H), 3.03 (s, 6H), 2.53 (t, J = 2.3 Hz, 1H); 13C NMR (100 MHz, CDCl3) δ 185.08, 181.34, 151.78, 149.75, 149.45, 141.69, 139.25, 131.06, 130.05, 129.48, 128.32, 127.23, 124.40, 123.80, 122.70, 121.90, 118.92, 117.97, 113.81, 111.91, 110.33, 101.26, 78.12, 76.24, 56.63, 55.93, 40.13; HRESIMS m/z [M + H]+ 404.1859 (calcd for C22H24N2O6, 404.1862).

(1E,4Z,6E)-1-(4-(dimethylamino)phenyl)-5-hydroxy-7-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)hepta-1,4,6-trien-3-one (7). Boron oxide (17 mg, 0.223 mmol, 0.4 eq) and 5 (130 mg, 0.558 mmol, 1 eq) were dissolved in EtOAc (3 ml) stirred at 80 °C for 1 h. The mixture was cooled to 0 °C, then trimethylboron oxide (191 ml, 0.949 mmol, 1.67 eq) and 3 (106, 0.558 mmol, 1 eq) were added. Butylamine (9 ml, 0.095 mmol, 0.17 eq) was then added dropwise. The obtained mixture was heated to 80 °C for 24 h, then cooled to room temperature and 5% AcOHaq (50 ml) was added. The suspension was then stirred for 2 h at room temperature and an orange precipitate formed. The precipitate was filtered, washed with water (3 × 100 ml), and purified by chromatography over silica (PE/EtOAc 7:3 as eluent) to afford (1E,4Z,6E)-1-(4-(dimethylamino)phenyl)-5-hydroxy-7-(3-methoxy-4-(prop-2-yn-1-yloxy)phenyl)hepta-1,4,6-trien-3-one (7, 70 mg, 19% yield) as a red powder. 1H NMR (300 MHz, CDCl3) δ 8.13 (d, J = 15.8 Hz, 1H), 7.57 (d, J = 15.8 Hz, 1H), 7.46 (d, J = 8.9 Hz, 2H), 7.08 (m, 3H), 6.69 (d, J = 8.6 Hz, 2H), 5.78 (s, 1H), 4.80 (d, J = 2.4 Hz, 2H), 3.92 (s, 3H), 3.03 (s, 6H), 2.53 (t, J = 2.3 Hz, 1H); 13C NMR (100 MHz, CDCl3) δ 183.72, 182.82, 149.75, 148.65, 148.01, 146.90, 140.81, 140.11, 131.06, 130.05, 129.48, 128.32, 127.23, 124.40, 123.80, 122.70, 121.90, 118.92, 117.97, 113.81, 111.91, 110.33, 101.26, 78.12, 76.24, 56.63, 55.93, 40.13; HRESIMS m/z [M + H]+ 407.1496 (calcd for C22H24N2O6, 407.1495).

4-(2-Azidoethoxy)benzaldehyde (8). To a stirred solution of 4-hydroxybenzaldehyde (1 g, 8.188 mmol, 1 eq) in DMF (10 ml) K2CO3 (2.26 g, 16.376 mmol, 2 eq) and 2-bromoethanol (1.16 ml, 16.376 mmol, 2 eq) were added. The reaction was heated at 90 °C for 48 h, then cooled to room temperature. H2SO4 2 M (20 ml) was added, then the mixture was extracted with PE/EtOAc 7:3 (50 ml) and the organic phase was washed with water, brine and dried. The crude was purified by column chromatography (PE/EtOAc 5:5 as eluent) to afford 4-(2-Azidoethoxy)benzaldehyde (1.36 g, 100%) as a colorless oil. 1H NMR (400 MHz, CDCl3) δ 8.13 (d, J = 15.8 Hz, 1H), 7.57 (d, J = 15.8 Hz, 1H), 7.46 (d, J = 8.9 Hz, 2H), 7.08 (m, 3H), 6.69 (d, J = 8.6 Hz, 2H), 5.78 (s, 1H), 4.80 (d, J = 2.4 Hz, 2H), 3.92 (s, 3H), 3.03 (s, 6H), 2.53 (t, J = 2.3 Hz, 1H); 13C NMR (100 MHz, CDCl3) δ 183.72, 182.82, 149.75, 148.65, 148.01, 146.90, 140.81, 140.11, 131.06, 130.05, 129.48, 128.32, 127.23, 124.40, 123.80, 122.70, 121.90, 118.92, 117.97, 113.81, 111.91, 110.33, 101.26, 78.12, 76.24, 56.63, 55.93, 40.13; HRESIMS m/z [M + H]+ 407.1496 (calcd for C22H24N2O6, 407.1495).
Ethyl 2-azidoacetate (9). To a stirred solution of ethyl 2-bromooacetate (11, 1 ml, 9.017 mmol, 1 eq) in DMF (10 ml), sodium azide (798 mg, 12.382 mmol, 3 eq) and a catalytic amount of benzaldehyde (12) (110 mg, 1.282 mmol, 0.3 eq) were added. The reaction was heated at 85 °C for 12 h, then quenched with brine and extracted with PE. The organic phase was then washed with water, brine and dried. The crude was dissolved in DMF (10 ml), then sodium azide (798 mg, 12.382 mmol, 3 eq) and a catalytic amount of NaOH were added. The solution was stirred at room temperature for 24 h, then diluted with brine and extracted with EtOAc. The organic phase was dried and evaporated, then the crude was purified by chromatography over silica gel (PE:EtOAc:3:7 as solvent) to afford Probe (32 mg, 30%) as an orange solid.

General procedure for copper catalyzed 1,3-dipolar cycloaddition: synthesis of Probe as 10 example. To a stirred solution of 6 (50 mg, 0.123 mmol, 1 eq) in t-BuOH/H2O/CH2CN 2:1:1 (8 ml, 0.56 mmol, 2.5 eq) and a catalytic amount of CuSO4 and sodium ascorbate were added. The solution was stirred at room temperature for 24 h, then quenched with water and extracted with EtOAc. The organic phase was dried and evaporated, then the crude was purified by chromatography over silica gel (PE:EtOAc:3:7 as solvent) to afford 10 (32 mg, 30%) as an orange solid.

General procedure for diethylphosphate derivative: synthesis of probe as 13 example. To a stirred solution of 10 (50 mg, 0.251 mmol, 1 eq) in dry DCM (10 ml), TEA (105 ml, 0.733 mmol, 3 eq) and diethylchlorophosphate (54 ml, 0.377 mmol, 1.5 eq) were added. The reaction was stirred at room temperature for 24 h, then quenched with water and dried. The crude was purified by chromatography over silica (EtOAc as eluent) to afford Probe (14 mg, 23%) as orange solid. Probe 14 was obtained following the same protocol.

Expression and purification of the recombinant human aldehyde dehydrogenase 1A subfamily. A common experimental protocol has been developed with the aim of obtaining pure human ALDH1A1, 1A2 and 1A3 at a high yield, as already described[52]. Briefly, E. coli BL21 (DE3) were transformed with the full-length expression vector of each isoform and seeded onto 2XY agar plates containing 50 µg/ml ampicillin for ALDH1A1 and ALDH1A3 subtypes, and 50 µg/ml kanamycin for ALDH1A2. Petri plates were incubated for the overnight growth at 37 °C. The following day, colonies were scraped and used to inoculate 11 of 2XY liquid medium, which was previously added with 50 µg/ml ampicillin for ALDH1A1 and ALDH1A3, and 50 µg/ml kanamycin for ALDH1A2. Flasks were put under shaking at 37 °C and, once OD600 = 0.6–0.8 was reached, the temperature was shifted to 20 °C to induce the recombinant protein production. The induced strain was collected and centrifuged at 14,000 x g for 30 min. The harvested pellet was thawed and resuspended in lysis buffer (50 mM Na2HPO4, 300 mM NaCl, 1 mM β-mercaptoethanol, 20 mM imidazole, pH 7.5) with 1 µl per 80 ml of

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lysis buffer of benzonase nuclease (250 U/ml). E. coli BL21 (DE3) cells were disrupted using a French Press system, three times at 1.5 Kbar, adding 100 µl per 40 ml of lysis buffer M. After a Protease inhibitor cocktail from SIGMA. To obtain the clarified cell lysate, the cell debris was removed by centrifugation at 18,000 rpm for 50 min. The recombintant proteins were purified by a His-tag affinity chromatography followed by size-exclusion chromatography, using an AKTA FPLC system at 4 °C. To better evaluate the purity and homogeneousity of the protein after each purification step, the elution fractions were analyzed by SDS-PAGE. The final protein concentration was determined through the Bradford protein assay. In the first purification step, the collected supernatant was loaded on a Qiagen Ni-NTA Superflow 5 ml cartridge that was previously equilibrated with 10 column volumes of lysis buffer. The Ni-NTA cartridge was washed with 15 column volumes of 50 mM Na2HPO4, 300 mM NaCl, 1 mM β-mercaptoethanol, 50 mM imidazole, pH 7.5, until the absorbance at 280 nm returned to the baseline. The recombintant hALDH1A was eluted with 50 mM Na2HPO4, 300 mM NaCl, 1 mM β-mercaptoethanol, 250 mM imidazole, pH 8, by applying a linear gradient in 10 column volumes. Eluted fractions were pooled and concentrated to 5 ml with Merck Millipore Amicon Ultra-15 30 kDa and loaded on a HiLoad 16/600 Superdex 200 pg column on AKTA FPLC system. Elution buffer contained 20 mM Tris HCl pH 8.0, 150 mM KCl, 1 mM β-mercaptoethanol, and a flow rate of 1 ml/min was applied. By means of this procedure, 20 mg of pure and active human ALDH1A1, ALDH1A2 and ALDH1A3 were obtained, stocked at −80 °C and later used for biochemical analysis.

Absorbance, emission and excitation wavelengths evaluation. Fluorescent compounds were analyzed on a Tecan Spark to evaluate the correct parameters to further set the biochemical characterizations. The absorbance was evaluated in a range from 300 to 700 nm, with a wavelength step size of 5 nm. Based on the absorbance values, we settled a 3D fluorescence emission scan to evaluate the excitation and emission peaks. The analysis was conducted using the same buffer mix, as already described. The excitation range was set between 390 and 450 nm for both molecules, with a step size of 5 nm. The emission range was set between 485 and 700 nm for both molecules, with a step size of 5 nm. All these characterizations were performed on a Tecan Spark using Greiner Bio-one 96-UV-Transparent Microplates and the tests were carried out using a total volume of 100 µl for each well.

Chemical stability and cross reactions with biomolecules assay. Based on the values obtained by the 3D analysis, a wide series of biomolecules directly used in the biochemical experiment, both in vitro and in vivo, and listed in Table 1, were tested in complex with a fixed concentration of 10 µM of both probes, to measure any possible cross reaction signal.

Kₐ evaluation of the probes in complex with human ALDH1A isoforms. To evaluate the Kₐ constant between the two probes and the isoenzymes ALDH1A1, ALDH1A2 and ALDH1A3 we performed a fluorescence emission assay. We used a Tecan Spark with Greiner Bio-one 96-UV-Transparent Microplates. A single reaction was performed in a total volume of 100 µl per well containing 20 mM Tris HCl pH 8.0, 1 mM β-mercaptoethanol, 150 mM KCl, 500 µM NAD⁺ and 10 µM fluorescent probe with a 5% DMSO final concentration, in the presence of different ALDH1As concentrations, from 100 µM to 1.1719 µM. Each reaction mix was preincubated for 10 min at 25 °C before the analysis. The catalytic activity was measured by monitoring the absorbance at 340 nm (εNADH = 6.220 M⁻¹ cm⁻¹) for 30 min at 25 °C and the inhibitory parameters were calculated by processing the raw data on GraphPad.

Kₐ, IC₅₀ and Kᵢ evaluation of probes on the ALDH1A isoforms. Initially, the inhibitory activities of our fluorescent probes were screened at a fixed concentration of 50 µM using a Tecan Sunrise 96 Multple Plate Reader with Greiner Bio-one 96-UV-Transparent Microplates. The analysis was performed in triplicate in a total volume of 100 µl per well containing 20 mM Tris HCl pH 8.0, 1 mM β-mercaptoethanol, 150 mM KCl, 500 µM NAD⁺, 1.41 µM DMSO, 2.8 µM hALDH1A and Probe 10 was tested as substrate at different concentrations, from 300 µM to 3.125 µM. Each reaction mix was preincubated for 10 min at 25 °C before the analysis. The catalytic activity was measured by monitoring the absorbance at 340 nm (εNADH = 6220 M⁻¹ cm⁻¹) for 12 h at 25 °C and the inhibitory parameters were calculated by processing the raw data on GraphPad.

Table 1. List of a several biomolecules directly used in the biochemical experiment, both in vitro and in vivo.

| Chemical species | Assay concentration |
|------------------|---------------------|
| CONTROL (only SEC buffer) | See material and methods |
| Tris HCl pH = 8.0 | 20 mM |
| Tris HCl pH = 8.5 | 20 mM |
| Hesper Na pH = 7.0 | 20 mM |
| Hesper Na pH = 7.5 | 20 mM |
| H₂O₂ | 100 µM |
| FBS | 0.1% v/v |
| Trypsine | 0.05 mg/ml |
| NaOH | 100 µM |
| FeCl₂ | 100 µM |
| β-mercaptoethanol | 1 mM |
| Cysteine | 100 µM |
| Glycine | 100 µM |
| Glutamate | 100 µM |
| Lysine | 100 µM |
| Tryptophane | 100 µM |
| Tyrosine | 100 µM |
| NaCl | 150 mM |
| KCl | 150 mM |
| MgCl₂ | 2.5 mM |
| ZnSO₄ | 100 µM |
| NAD⁺ | 500 µM |
| DMSO | 15% |
| Probe 10/11 diluted in SEC buffer | Probe 10/11 = 10 µM |
| Probe 10/11 diluted in SEC buffer + ALDH1A1 | ALDH1A1 = 100 µM |
| Probe 10/11 diluted in SEC buffer + ALDH1A2 | ALDH1A2 = 100 µM |
| Probe 10/11 diluted in SEC buffer + ALDH1A3 | ALDH1A3 = 100 µM |

LC-HRMS analysis of ALDH1As isoenzyme activity in the presence of Probe 10 or Probe 11. These analyses were carried out by using UPLC-HRMS procedure with Vanquish UPLC system coupled to a Thermo Scientific Q-Exactive Plus, operating in negative electrospray ionization mode. The chromatographic separation was carried out using a Phenomenex Synergi μ 4 μm Polar-RP 80 Å (150 × 2 mm) equipped with a Phenomenex Polar-RP (4 mm × 2 mm) security guard column. The mobile phase consisted of: A: acetonitrile 0.1% formic acid and B: 0.1% formic acid. We used a linear elution gradient, starting from 80% of A to 5% A in 5 min, 3 min at 5% A, an equilibration of 4 min for a total runtime of 10.00 min, at 300 µl/min flow rate. The autosampler temperature was set at 15 °C, the injection volume was 5 µl and column was maintained at 40 °C. The heated electrospray ionization source was used, with a spray voltage of 3200 V, with a capillary temperature of 300 °C, a heater temperature of 350 °C, a sheat gas flow of 45 arbitrary units (AU), an auxiliary gas flow of 10 AU and a sweep gas flow of 0 AU. During the Parallel Reaction Monitoring acquisition in negative ion mode, the instrument operated at 17,500 resolution in presence of the inclusion list (m/z = 596.20384 and m/z = 612.18975 for Probe 10 and Probe 10-COOH respectively; m/z = 534.18819 and m/z = 596.155888 for Probe 11 and Probe 11-COOH respectively) with an automatic gain control target of 2 × 10⁵ charges and a maximum injection time of 100 ms. Before injecting the samples, the blanks in the list were analyzed and every biological sample was analyzed in duplicate. The biological samples analyzed were as follow: (1) Enzymatic assay Buffer; (2) Enzymatic assay Buffer + NAD⁺; (3) Enzymatic assay Buffer + Probe 10 or Probe 11; (4) Enzymatic assay Buffer + ALDH1A1; (5) Enzymatic assay Buffer + ALDH1A2; (6) Enzymatic assay Buffer + ALDH1A3; (7) Enzymatic assay Buffer + ALDH1A1 + Probe 10 or Probe 11; (8) Enzymatic assay Buffer + ALDH1A1 + Probe 10 or Probe 11; (9) Enzymatic assay Buffer + ALDH1A3 + Probe 10 or Probe 11. All samples were diluted 1:1 with ACN, then 1:100 in ACN-Water 1:1 prior the LC-HRMS analysis.
Cell culture. U87MG human glioblastoma, and 4T1 murine mammary carcinoma cell lines were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich). The HEK293T human embryonic kidney cell line and GL261 high grade glioma cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich). All these cell lines with the exception of GL261 were purchased from ATCC. Media were supplemented with 10% fetal bovine serum (Gibco), 2 mg/ml glutamine, 10 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). Normal human fetal astrocytes were kindly provided by Eleonora Aronica. All these cell lines with the exception of GL261 were purchased from ATCC. Media were supplemented with 10% Fetal bovine serum (Gibco) and 1% Penicillin-streptomycin solution (Gibco). Cells were cultured in Dulbecco's modified eagle medium (DMEM) + F10 medium. Cells were maintained in a controlled atmosphere of 5% CO2 with humidity at 37°C. Cells were detached by trypsin treatment and visualized without fixation with 0.5 ml of PBS buffer two times, then a 10 μM solution of probes was added to each well for 2h. For nuclei staining, 1 μg/ml of DAPI was added the last 15 min of treatment. The cover slips were washed and removed with PBS buffer, and fixed with 3.7% formaldehyde solution for 10 min. The cover slips were washed with 1 ml of PBS, then slides were prepared using Mounting Media (Merck Life Science). Fluorescence images were acquired using a Leica THUNDER Imager 3D Live Cell (Leica Microsystems, Wetzlar, Germany) microscope equipped with an U 40x/1.3 objective using the LAS X software.

Flow cytometry. U87MG, 3054, 3060, HEK293T, hASTRO and 4T1 cells were resuspended in PBS to a final concentration of 106 cells/ml. Each sample was then resuspended in probe 10 and probe 11 solutions (1 μM in ALDEFLUOR assay buffer, STEMCELL). Triplicate samples were prepared for each dye. Cells were pretreated with DEAB for 15 min and then incubated with the probes for 30 min at room temperature, with rocking to prevent cell clumping and ensure an even dye distribution. At the end of the incubation period, cells were harvested by centrifugation at 1000 rpm for 5 min at 4°C. The probes were removed and washed with PBS buffer, and fixed with 1% formaldehyde solution for 10 min. The cover slips were washed with 1 ml of PBS, then slides were prepared using Mounting Media (Merck Life Science). Data were further analyzed by Ideas software (Annims, version 2.0). The following parameters: 10,000 images per sample, 488 nm laser (25 and 100 mW) to excite the probes, 785 nm laser used to provide a side scatter signal and measurement of SpeedBeads (Annims, LumineX Corporation, Austin, TX, USA), 830 nm laser used for internal bead calibration of core flow speed and focus, x60 objective, in low-speed flow. Data were further analyzed by Ideas software (Annims, version 6.1).

Cell viability. In total, 10 x 10^5 U87MG human glioblastoma, patient-derived glioma cells 3054 and 3060, HEK293T human embryonic kidney, 4T1 murine mammary carcinoma, human fetal astrocytes cell lines were plated in their respective medium and treated for 72 h with our respective medium and treated for 72 h with our respective medium and treated for 72 h with our respective medium and treated for 72 h with our respective medium. At the end of the incubation period, cells were harvested by centrifugation at 1000 rpm for 5 min at 4°C. The probes were removed and washed with PBS buffer, and fixed with 1% formaldehyde solution for 10 min. The cover slips were washed with 1 ml of PBS, then slides were prepared using Mounting Media (Merck Life Science). Data were further analyzed by Ideas software (Annims, version 6.1).

Animal experiments. We used in vitro and in vivo cells from a mouse high grade glioma cell line, GL261. Briefly, ten to three-month-old female C57BL mice were stereotactically implanted under deep general anesthesia (isoflurane supplemented with nitrous oxide) with 1 x 10^5 GL261 glioblastoma cells. The tumors were allowed to grow for 3 weeks, at which time mice were inoculated with the probes and treated with probe 10 (18 μg) or probe 11 (5 μg). All drugs were administered intraperitoneally 6 days after tumor implantation and the animals were euthanized under deep anesthesia 6 h after the i.p. injection of the probes. Briefly, they were transected with probe 10 (18 μg) or probe 11 (5 μg). All drugs were administered intraperitoneally 6 days after tumor implantation and the animals were euthanized under deep anesthesia. After tumor implantation and cell injection, the animals were euthanized under deep anesthesia 6 h after the i.p. injection of the probes. Briefly, they were transected with probe 10 (18 μg) or probe 11 (5 μg).

References
1. Louis, D. N. et al. The 2021 WHO classification of tumors of the central nervous system: a summary. Neuropathology. 3, 1231–1251 (2021).
2. Phillips, H. S. et al. Molecular subclasses of high-grade glioma predict survival, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 9, 157–173 (2006).
3. Verhaak, R. G. W. et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17, 98–110 (2010).
4. Dunn, G. P. et al. Emerging insights into the molecular and cellular basis of glioblastoma. Genes Dev. 26, 756–784 (2012).
5. Batlle, E. & Clevers, H. Cancer stem cells revisited. Nat. Med. 23, 1124–1134 (2017).
6. Chen, X., Liao, R., Li, D. & Sun, J. Induced cancer stem cells generated by radiochemotherapy and their therapeutic implications. OncoTARGET 8, 17301–17312 (2017).
7. Fedele, M., Cerchia, L., Pegoraro, S., Sgarra, R. & Manni-Fellitieti, G. Proneural-mesenchymal transition: phenotypic plasticity to acquire multi-therapies resistance in glioblastoma. IJMS 20, 2746 (2019).
8. Behan, J., Finocchiario, G. & Hanna, G. The landscape of the mesenchymal signature in brain tumors. Brain 142, 866–869 (2016).
9. Singh, S. K. et al. Identification of human brain tumour initiating cells. Nature 432, 396–401 (2004).
10. Carro, M. S. et al. The transcriptional network for mesenchymal transformation of brain tumours. Nature 463, 318–325 (2010).
11. Mao, P. et al. Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc. Natl Acad. Sci. USA 110, 8644–8649 (2013).
12. Stupp, R. et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N. Engl. J. Med. 352, 987–996 (2005).
13. Perry, J. R. et al. Short-course radiation plus temozolomide in elderly patients with glioblastoma. N. Engl. J. Med. 376, 1027–1037 (2017).
14. Zhang, W. et al. ALDH1A3: a marker of mesenchymal phenotype in gliomas associated with cell invasion. PLoS ONE 10, e0142856 (2015).
15. Ferrari, D. M., Gelardi, E. L., Garavaglia, S., Muggiano, R. & Rizzi, M. Targeting NAD-dependent dehydrogenases in drug discovery against infectious diseases and cancer. Biochem. Soc. Trans. 48, 693–707 (2020).
16. Koppaka, V. et al. Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. Pharmac. Rev. 64, 520–539 (2012).
17. Marchitti, S. A., Brocker, C., Stagos, D. & Vasiliiou, V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin. Drug Metab. Toxicol. 4, 697–720 (2008).
18. Moretti, A. et al. Crystal structure of human aldehyde dehydrogenase 1A3 associated with bupropion inhibition. Proc. Natl Acad. Sci. USA 103, 10166–10171 (2006).
19. Duan, T.-J., Cai, J., Guo, Y.-F., Bian, X.-W. & Yu, S.-C. ALDH1A3, a metabolic target for cancer diagnosis and therapy. Int. J. Cancer 139, 965–975 (2016).
20. Tomita, H., Tanaka, K., Tanaka, T. & Hara, A. Aldehyde dehydrogenase 1A1 in stem cells and cancer. Oncotarget 7, 11018–11032 (2016).
21. Nwani, N. et al. A novel ALDH1A1 inhibitor targets cells with stem cell characteristics in ovarian cancer. Cancers 11, 502 (2019).
22. Kharkar, P. S. Cancer stem cell (CSC) inhibitors in oncology—a promise for a better therapeutic outcome: state of the art and future perspectives. J. Med. Chem. 63, 15279–15307 (2020).
23. Geraldi, E. L. M. et al. A selective competitive inhibitor of aldehyde dehydrogenase 1A3 hinders cancer cell growth, invasiveness and stemness in vitro. Cancers 13, 356 (2021).
24. Cheng, P. et al. FOXD1-ALDH1A1 signaling is a determinant for a self-renewal and tumorigenesis of mesenchymal glioma stem cells. Cancer Res. 76, 7219–7230 (2016).
25. Li, J. et al. A specific inhibitor of ALDH1A3 regulates retinoic acid biosynthesis in glioma stem cells. Commun. Biol. 4, 1–16 (2021).
26. Clark, D. W. & Palle, K. Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets. Ann. Transl. Med. 4, 518 (2016).
27. Januchowski, R., Wojtowicz, K. & Zabel, M. The role of aldehyde dehydrogenase (ALDH) in cancer drug resistance. Biomed. Pharmacother. 67, 609–680 (2013).
28. Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. Nat. Rev. Cancer 13, 714–726 (2013).
29. Januchowski, R. et al. Inhibition of ALDH1A1 activity decreases expression of drug transporters and reduces chemotherapy resistance in ovarian cancer cell lines. Int. J. Biochem. Cell Biol. 78, 248–259 (2016).
30. Quattrini, L. et al. Imidazo[1,2-α]pyridine derivatives as aldehyde dehydrogenase inhibitors: novel chemotypes to target glioblastoma stem cells. J. Med. Chem. 63, 4603–4616 (2020).
31. Quattrini, L. et al. Progress in the field of aldehyde dehydrogenase inhibitors: novel Imidazo[1,2-α]pyridines against the 1A family. ACS Med. Chem. Lett. 11, 963–970 (2020).
32. Shahcheraghi, S. H. et al. Therapeutic potential of curcumin in the treatment of glioblastoma multiforme. Curr. Pharm. Des. 25, 333–342 (2019).
33. Purkayastha, S. et al. Curcumin blocks brain tumor formation. Brain Res. 1266, 130–138 (2009).
34. Gersey, Z. C. et al. Curcumin decreases malignant characteristics of glioblastoma stem cells via induction of reactive oxygen species. BMC Cancer 17, 99 (2017).
35. Nelson, K. M. et al. The essential medicinal chemistry of curcumin: miniperspective. J. Med. Chem. 60, 1620–1637 (2017).
36. Park, K.-S. et al. A curcumin-based molecular probe for near-infrared fluorescence imaging of tau fibrils in Alzheimer’s disease. Org. Biomol. Chem. 13, 11194–11199 (2015).
37. Wang, X. et al. Curcumin exerts its tumor suppressive function via inhibition of NEDD4 oncoprotein in glioma cancer cells. Int. J. Oncol. 51, 467–477 (2017).
38. Kato, N. et al. Boron difluoride curcuminoid fluorophores with enhanced two-photon excited fluorescence emission and versatile living-cell imaging properties. Chem. Eur. J. 22, 5219–5232 (2016).
39. Hishim, M., Matsumoto, T. & Arai, H. Diagnosis and treatment of early-stage glioblastoma. Asian J. Neurosurg. 14, 589–592 (2019).
40. Caprioglio, D. et al. Triazole-curcuminoids: a new class of derivatives for ‘tuning’ curcumin bioactivities. Bioorg. Medicinal Chem. 24, 140–152 (2016).
41. Koeberle, A. et al. SAR studies on curcumin’s pro-inflammatory targets: discovery of prenylated pyrazolocurcinoids as potent and selective novel inhibitors of 5-lipoxygenase. J. Med. Chem. 57, 5638–5648 (2014).
42. Caldarelli, A., Penucchini, E., Caprioglio, D., Genazzani, A. R. & Minassi, A. Synthesis and tubulin-binding properties of non-symmetrical click C5-curcuminoids. Bioorg. Med. Chem. 21, 5510–5517 (2013).
43. Minassi, A., Sánchez-Duffhues, G., Collado, J. A., Muñoz, E. & Appendino, G. Dissecting the pharmacophore of curcumin. Which structural element is critical for which action? J. Nat. Prod. 76, 1105–1112 (2013).
44. Pabon, H. J. A synthesis of curcumin and related compounds. Recl. Trav. Chim. Pays-Bas 83, 379–386 (1964).
45. Cai, L., Innis, R. B. & Pike, V. W. Radioligand development for PET imaging of beta-amyloid (Abeta)–current status. Curr. Med. Chem. 14, 19–52 (2007).
46. Agalave, S. G., Majum, S. R. & Pore, V. S. Click chemistry: 1,2,3-triazoles as pharmacophores. Chem. Asian J. 6, 2069–2718 (2011).
47. Anand, P., Kunnunakkara, A. B., Newman, R. A. & Aggarwal, B. B. Bioavailability of curcumin: problems and promises. Mol. Pharm. 4, 807–818 (2007).
48. Van Nong, H. et al. Fabrication and vibration characterization of curcumin extracted from turmeric (Curcuma longa) rhizomes of the northern Vietnam. SpringerPlus 5, 1147 (2016).
49. Morgan, C. A., Parajuli, B., Buchman, C. D., Dria, K. & Hurley, T. D. N,N-diethylaminobenzaldehyde (DEAB) as a substrate and mechanism-based inhibitor for human ALDH isoenzymes. Chem. Biol. Interact. 234, 18–28 (2015).
50. Diez Valle, R., Hadjipanayis, C. G. & Stummer, W. Established and emerging uses of 5-ALA in the brain: an overview. J. Neurooncol. 141, 487–494 (2019).
51. Neira, J. A. et al. Aggressive resection at the infiltrative margins of glioblastoma facilitated by intraoperative fluorescence guidance. J. Neurosurg. 127, 111–122 (2017).
52. Katsevman, G. A., Turner, R. C., Urhie, O., Voelker, J. L. & Bhatia, S. Utility of sodium fluoresein for achieving resection targets in glioblastoma: increased gross- or near-total resections and prolonged survival. J. Neurosurg. 132, 914–920 (2019).
53. SigmaPlot Extract Graphs and Data Analysis (StatSoft v12.3)SigmaPlot (RRID:SCR_003210). URL: http://www.sigmaplot.com/products/sigmaplot/.
54. Xie, Y. et al. The human glioblastoma cell culture resource: validated cell models representing all molecular subtypes. EBioMedicine 2, 1351–1363 (2015).
55. Azzalin, A. et al. A new pathway promotes adaptation of human glioblastoma cells to glucose starvation. Cells 9, 1249 (2020).
56. Statzm, T. et al. Detailed characterization of the mouse glioma 261 tumour model for experimental glioblastoma therapy. Cancer Sci. 97, 546–553 (2006).
