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The synthesis of a novel Crizotinib heptamethine cyanine dye conjugate that potentiates the cytostatic and cytotoxic effects of Crizotinib in patient-derived glioblastoma cell lines.

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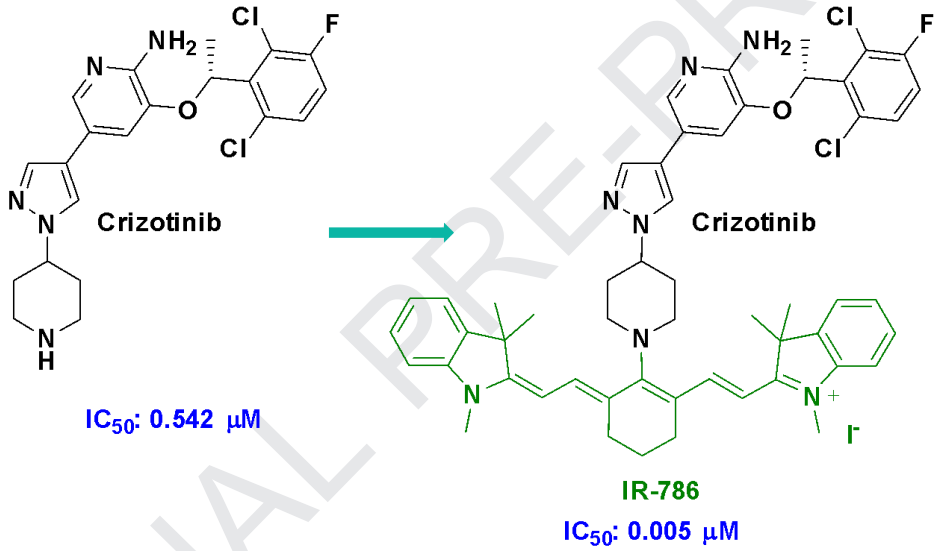
Abstract:

We describe the synthesis of drug-dye conjugate **1** between anaplastic lymphoma kinase inhibitor Crizotinib and heptamethine cyanine dye IR-786. The drug-dye conjugate **1** was evaluated in three different patient-derived glioblastoma cell lines and showed potent cytotoxic activity with nanomolar potency (EC_{50}: 50.9 nM). We also demonstrate evidence for antiproliferative activity of **1** with single digit nanomolar potency (IC_{50}: 4.7 nM). Furthermore, the cytotoxic effects conveyed a dramatic, 110-fold improvement over Crizotinib. This improvement was even more pronounced (492-fold) when **1** was combined with Temozolomide, the standard drug for treatment for glioblastoma. This work lays the foundation for future exploration of similar tyrosine kinase inhibitor drug-dye conjugates for the treatment of glioblastoma.



antiproliferative activity in patient derived glioblastoma cell lines

Key words:

Glioblastoma multiforme, Temozolomide, Anaplastic lymphoma kinase, Crizotinib, Heptamethine cyanine dyes.

Glioblastoma multiforme (GBM) is the most common primary brain cancer in adults and remains one of the most challenging cancers to treat.[1] This is largely due to the presence of treatment-resistance GBM stem cells and the heterogeneous nature of GBM pathology.[2-3] For these reasons, the median survival period for patients suffering from GBM is around 15 months.[4-5]

Temozolomide (TMZ) is the most commonly used chemotherapy agent along with radiotherapy after the initial tumour resection for the treatment of GBM.[6] TMZ is an imidazotetrazine class alkylating agent that is administered orally which can cross the blood-brain barrier (BBB) and accumulate in tumours. TMZ exerts its cytotoxicity by methylation of O_6 or N_7 position of guanine residues in DNA, which causes mispairing with thymine during DNA replication, eventually resulting in DNA damage. Almost half of the patient

population undergoing TMZ treatment do not respond to TMZ primarily due to the over-expression of O_6-methylguanine methyltransferase (MGMT), a DNA repair protein that removes O_6-methylguanine and other alkylated guanine adducts from DNA. Hence, MGMT promoter methylation status is an important prognostic factor for GBM, as it antagonises the cytotoxic effects of TMZ and other alkylating agents.[7-8]

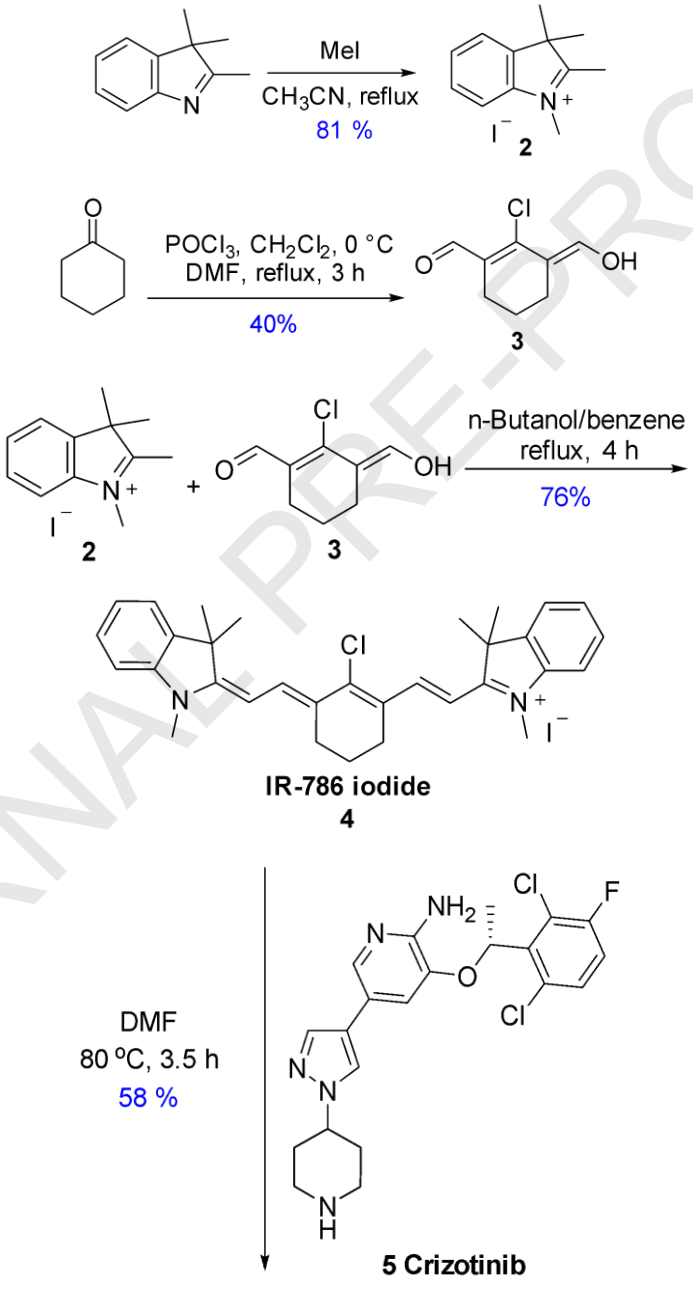
Recent literature report that heptamethine cyanine dyes (HMCDs) possess BBB crossing properties and exhibit selectivity towards tumours compared to normal tissue.[9] These beneficial properties are conferred by tumour hypoxia and organic anion-transporting polypeptides (OTAPs) - both of which are common in GBM.[10] However, the low efficacy of HMCDs towards brain cancer cells mean these compounds are not useful stand-alone agents for the treatment of cancers. This drawback might be overcome by conjugating HMCD with more effective anti-cancer drugs. Such conjugates might also show more favourable pharmacokinetics by more easily crossing the BBB. Indeed, there are previous studies showing that such drug-dye conjugates have good efficacy in treating brain cancers in mouse models.[11] The structural features that are essential for the uptake of these HMCDs into the tumour tissue may include the presence of ionisable groups and/or presence of chloro-cylcohexenyl moiety as the uptake is mediated by reaction with cysteine residues in human serum albumin.[12] The reactivity of chloro-cylcohexenyl moiety has been exploited previously for site specific protein labelling[13] and also for development of nanotheranostics for tumour targeting.[14]

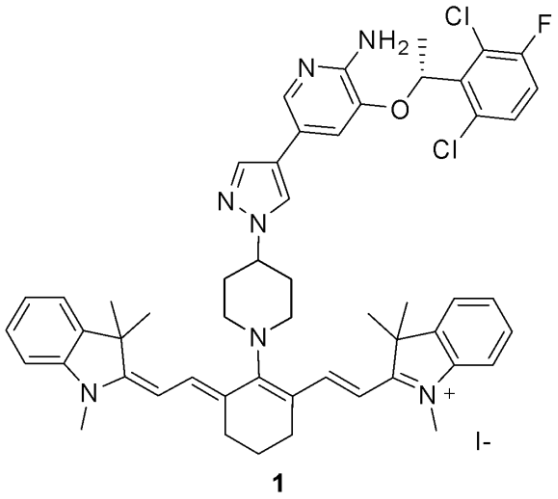
Mutations in receptor tyrosine kinase pathways (RTK) are responsible for tumour growth in over 80% of GBM patients.[15] Despite these prevalent mutations in RTKs, clinical studies have not been able to demonstrate efficacy in treating GBMs using FDA approved tyrosine kinase inhibitors (TKI).[16-17] This anomaly is largely due to their inability to cross the BBB and the apparent lack of tumour specificity.[18]

Anaplastic lymphoma kinase (ALK) is a druggable tyrosine kinase receptor that is over-expressed in tumours, and represents a potential therapeutic target for the treatment of GBM.[19] Normal ALK receptor is implicated in the genesis and progress of GBM with evidence suggesting the involvement of autocrine and paracrine growth loops with the ALK ligands, pleiotrophin and midkine.[20] Crizotinib is one of the first ALK inhibitors approved for the treatment of non-small cell lung cancer (NSCLC), showing favourable efficacy in patients with advanced ALK-positive non-small cell lung cancer (NSCLC).[21] Crizotinib also inhibits Met and ROS1 pathways, thereby serving as a multi-kinase inhibitor. This is an advantage in treating GBM, as multiple aberrant RTK signalling pathways are involved in GBM progression.[22-23]

We, therefore, envisaged to design and synthesize HMCD-TKI conjugates to study their effects on various patient-derived GBM cell lines. As shown in scheme 1, we designed Crizotinib-HMCD conjugate **1** via facile nucleophilic

displacement of the chloride group in HMCD **4**. The HMCD **4** itself was assembled using a previously reported procedure in good yields.[24] Piperidine amine from Crizotinib was chosen as the point of attachment to **4** as this region was shown to be exposed to the solvent region in the crystal structure of ALK co-crystallized with Crizotinib.[25] We hypothesized that a chemical modification at this region would have minimal impact on the interaction of Crizotinib with key residues in the ATP binding pocket of ALK, therefore, maintaining the potency of the drug-dye conjugate.





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Scheme 1. Synthesis of Crizotinib-IR786 drug-dye conjugate **1**.

Drug-dye conjugate **1,** dye **4,** and Crizotinib **5** were screened against three patient-derived GBM cell lines obtained from Auckland City Hospital (Supporting Information Table S1). As shown in Table 1, and Figures 1 and 2, the drug-dye conjugate **1** was significantly more potent at reducing cell numbers when compared to Crizotinib (EC_{50} of 50.9 nM vs 5595 nM respectively; $P<0.0001$). The reported mode of uptake for HMCDs are through the activated hypoxia-inducible factor 1α-organic anion transporting polypeptide (OATP) axis.[26] Therefore, we investigated whether our Crizotinib-HMCD dye conjugate maintained the same mode of cellular uptake. We utilized a known OATP pump blocker sodium taurocholic acid[27] (250 μM) and the HIF1α-OATP axis activator, 3,4-DHB (1 mM).[28] Figure 1 clearly illustrates the increase in drug-dye conjugate **1** uptake with a 24 hour pre-incubation of 3,4-DHB, and the dramatic decrease in uptake with a 30 min pre-incubation with the blocker, sodium taurocholic acid. These results suggest our conjugate also has a high reliance on the OATPs for their uptake into the tumor cells.

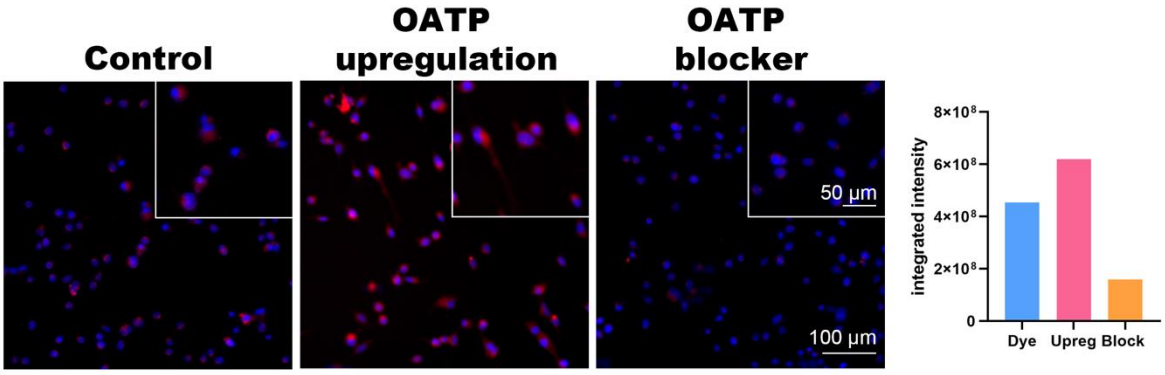

Figure 1. Fluorescence microscope images illustrating the uptake of the drug-dye conjugate **1** (red) into patient-derived GBM cells (nuclei; blue). **1** (1 μM) was added to the cells for 1 h in all conditions before imaging. Pre-incubation with 1 mM of 3,4-DHB greatly increased **1**'s uptake as shown by

the increased far-red fluorescence signal. Conversely, blocking the OATP pump with a pre-incubation with sodium taurocholic acid greatly attenuated this fluorescence signal. The bar graph is a quantification of the average cell integrated fluorescence intensity of the far-red signal.

Additionally, Figure 2 highlights that the three GBM patient cell lines were largely unresponsive to **TMZ**. However, co-treatment of **1** with an equal concentration of **TMZ** increased the cytotoxic activity of **1** to 15.3 nM, which was three-fold higher than the **1** alone ($P<0.01$; Table 1, Figure 2 and supplementary information Figure S1). This response points towards a synergism of conjugate **1** with **TMZ.** In contrast, the co-administration of **TMZ** with Crizotinib **5**, did not produce a significant change in potency ($P>0.05$). Additionally, the co-administration of **TMZ** with **4** had an appreciable increase in potency ($P<0.05$) in relation to Crizotinib.

Drug-dye conjugate **1** also exhibited antiproliferative activity on GBM cells at subordinate concentrations to the induction of cell death (Figure 3 and 4, supplementary information Figure S2). At half the EC_{50} (25 nM), treatment with **1** resulted in a significant reduction in proliferative cells measured by an EdU cell proliferation assay ($P<0.001$). In terms of proliferation, the treatment with **TMZ** at 100 μM failed to exert a 50% reduction in proliferating cells in all three primary GBM patient-derived cell lines.

Despite the variability in the average response of each primary GBM patient cell-line to treatment with Crizotinib **5**, **4** and **1**, there is a dramatic, and significant effect on cell death and proliferation (Tables 1 and 2, Figure 2 and 3, supplementary information Figure S3, S4, S5). The consistent potency of **1** across three heterogeneous patient-derived GBM cell lines, in comparison with **TMZ** treatment, serves as an example for the beneficial effects of combining TKIs such as Crizotinib with a dye to improve therapeutic effects in GBM cells.

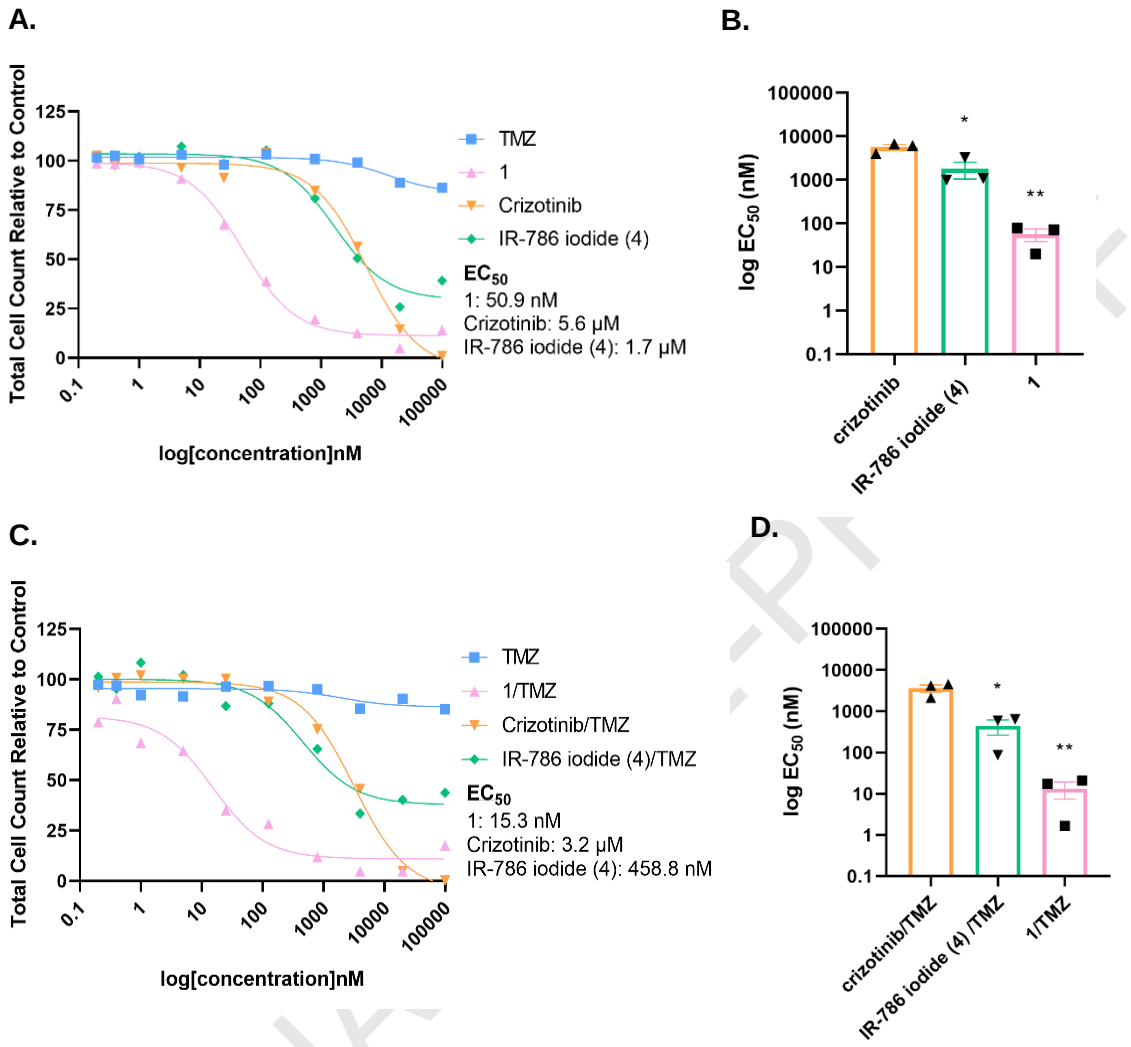

Figure 2. Viability of GBM cells as indicated by total cell count after 48 hours incubation with **1**, Crizotinib, **4** and **TMZ** (A). Co-incubation with **TMZ** (100 µM) demonstrated synergism, despite being unresponsive to TMZ treatment alone (IC_{50} 1/TMZ: 15.3 nM) (C). The average EC_{50} and the inter-patient variability to each compound is highlighted in B and D. TMZ did not reach a 50% reduction in cell number in any of the three primary GBM patient-derived cell lines.

Table 1. EC_{50} of the viability of the GBM cells as indicated by total cell count which is normalised to the vehicle DMSO after 48 h. Potency reflects a fold change relative to Crizotinib alone. ns = $p > 0.05$, **=$p<0.01$, to Crizotinib (n=3).

	Compound (EC_{50} nM)	**Potency**	**Compound with TMZ (EC_{50} nM)**	**Potency**
Crizotinib	5600±460	1	3200±1000	1.7 ns
4 (IR-786 iodide)	1680±110	3.3 *	460±80	12.2 *
1	50±20	109.9**	15±6	365.7**

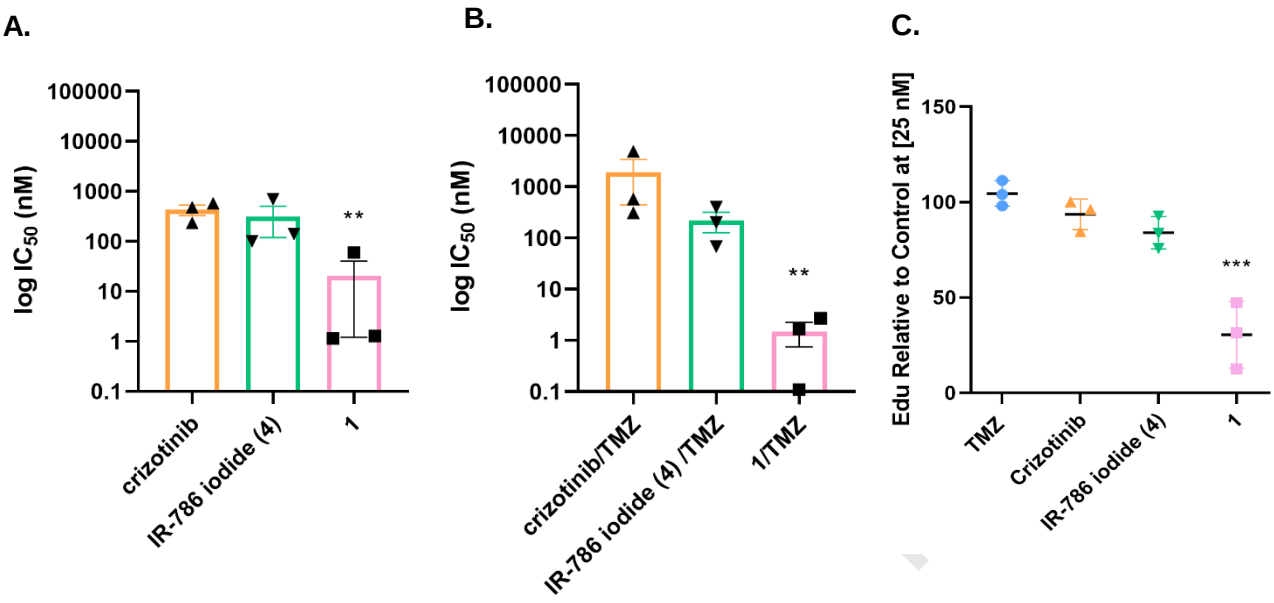

Figure 3. Graphs showing the individual and the average IC_{50} (A-B) values for **Crizotinib**, **4 and 1** of the three primary GBM patient cell lines. Comparatively, B highlights the synergism of TMZ co-incubated with the above compounds, on their respective and IC_{50}. C demonstrates that at a concentration below the EC_{50} **1** has a significant effect on proliferation. *=P<0.05 ** = P<0.01 ***= P<0.001 relative to Crizotinib. TMZ did not reach 50% reduction in proliferation in all of the primary GBM patient-derived cell lines, hence was not included in the above analysis.

Table 2. IC_{50} of the proliferation of the GBM cells, using the EdU cell proliferation assay which is normalised to DMSO after 48 h. Potency reflects a fold change relative to Crizotinib. ns = p > 0.05, * = p<0.05, ** = p < 0.001 to Crizotinib (n=3).

	Compound (IC_{50} nM)	**Potency**	**Compound with TMZ (IC_{50} nM)**	**Potency**
Crizotinib	540±160	1	480±140	1.13 ns
4 (IR-786 iodide)	280±70	1.9 ns	120±140	4.7 ns
1	4.7±3.3	115.0**	1.0±0.6	492.5 **

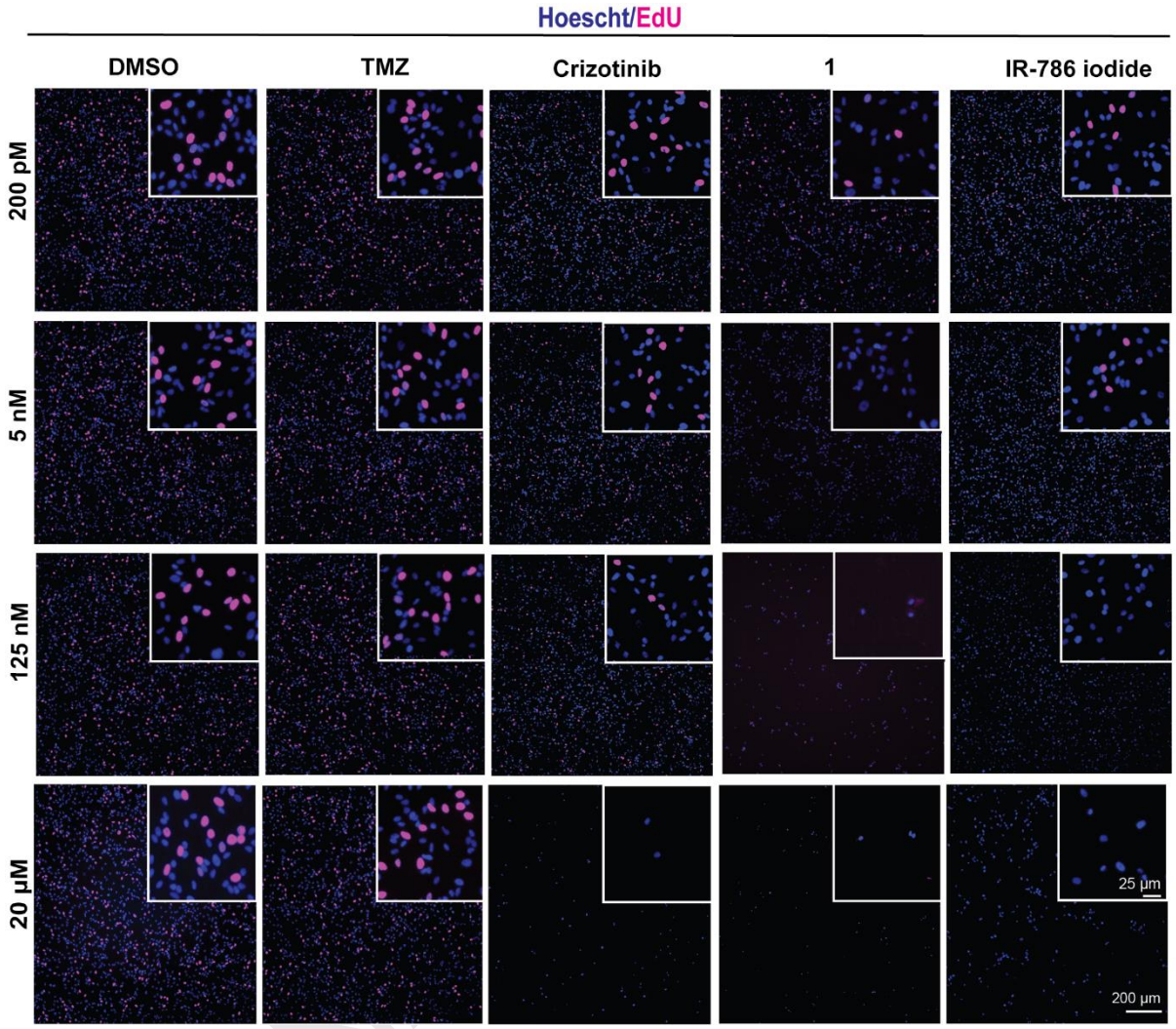

Figure 4. Representative images of the cell reduction and cytostatic effects following incubation with **DMSO, TMZ,** Crizotinib**, 1** and **4** on primary GBM patient-derived cell line T141 at the 48 h time point. Blue represents the cell nuclei counterstained with Hoechst, and the Red represents the EdU incorporated cells. Scale: 200µm, inset: 25µm.

GBM remains a formidable challenge to treat due to tumour heterogeneity with a high chance of recurrence. The efficacy of current drugs targeting GBM is far from ideal and therefore, warrants the need to explore other classes of drugs. Tyrosine kinase inhibitors (TKIs) have had a major impact on the treatment of cancers and other diseases such as rheumatoid arthritis, glaucoma, Crohn disease, and ulcerative colitis, as evidenced by the 48 small molecule kinase inhibitors so far approved by the FDA for clinical use.[29] Although TKIs have had minimal impact in treating brain cancers mainly due to poor brain exposure, they could serve as a starting point for synthetic modifications to improve their efficacy. The drug-dye conjugate **1** serves as an example for the beneficial effect of combining a TKI such as Crizotinib and a dye to afford a potent compound with good efficacy in human glioblastoma cell lines. At the time of submitting this manuscript, there has been only one reported TKI-HMCD dye conjugate reported in the literature showing micro molar potency in HepG2 live cancer cell line.[30] To the best of our knowledge, this is the first TKI-HMCD dye conjugate showing nanomolar inhibition in primary patient-derived glioblastoma cell lines. Furthermore, the three GBM patient cell-lines were largely unresponsive to TMZ treatment with an IC_{50} that was greater than 100 μM. However, co-incubation of **1** with TMZ provided evidence for synergism, reducing the EC_{50} for tumour cell number by three-fold. The mechanism of action of these conjugates and their biological targets are currently being investigated. Future work is directed towards improving physiochemical properties of similar conjugates to explore their utility in animal models of glioblastoma and in human patients.

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Supplementary material:

Detailed synthetic procedures, characterization of compounds, cell images can be found in the online version at

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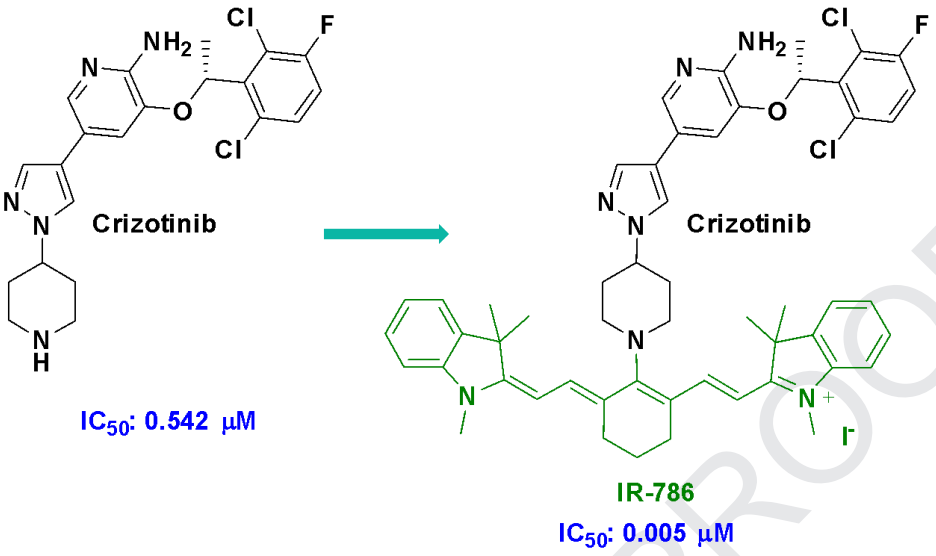
Graphical abstract:



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