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PARP inhibitor cyanine dye conjugate with enhanced cytotoxic and antiproliferative activity in patient derived Glioblastoma cell lines.

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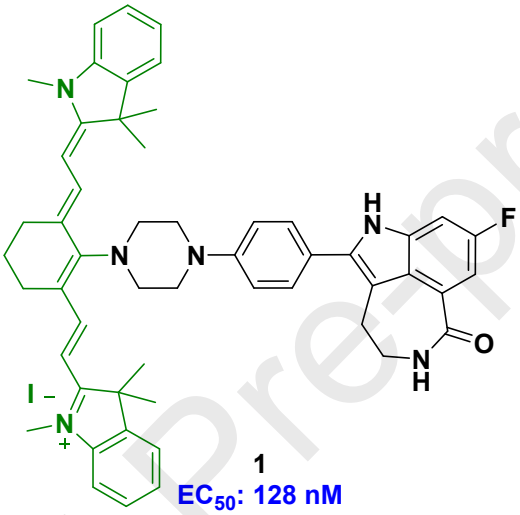
Abstract:

We describe the synthesis and *in vitro* activity of drug-dye conjugate **1,** which is a combination of the PARP inhibitor rucaparib and heptamethine cyanine dye IR-786. The drug-dye conjugate **1** was evaluated in three different patient-derived glioblastoma cell lines and showed strong cytotoxic activity with nanomolar potency (EC_{50}:128 nM), which was a 780 fold improvement over rucaparib itself. We also observe a synergistic effect of **1** with temozolomide (TMZ), the standard drug for treatment for glioblastoma even though these cell lines were resistant to TMZ treatment. We envisage such conjugates to be worth exploring for their utility in the treatment of various brain cancers.



1

EC_{50}: 128 nM

potent antiproliferative activity in three different patient derived glioblastoma cell lines

Key words:

Glioblastoma, temozolomide, PARP inhibitors, rucaparib, heptamethine cyanine dyes.

Cancer treatment has evolved since the discovery of nitrogen mustards in the 1940s to the current targeted therapies. The understanding of the biology of tumour initiation and progression has helped us to design drugs with improved therapeutic indexes. The challenge we face today in drug discovery is to translate the mechanistic revelation of cancer progression into meaningful drug design and delivery. This is particularly true for brain cancers where chemotherapy has not been able to have a significant impact.[1-2] The multifaceted challenges in designing central nervous system (CNS) drugs and the huge attrition rate has made it difficult to make any lasting progress in this area.[3] Among brain tumours, glioblastoma (GBM) is the most lethal, and it poses a challenge to known therapeutic intervention.[4] The median survival period of 15 to 20 months for patients suffering from GBM has not improved in the past decade. The DNA alkylating agent, temozolomide (TMZ) the mainstay in treating primary and recurrent GBM is ineffective in half of the patient population. This is because approximately 50–60% of GBM tumours have unmethylated O-6-methylguanine-DNA methyltransferase (MGMT), an enzyme which repairs the DNA damage caused by TMZ, thereby protecting cancer cells from cytotoxic alkylating agents.[5] Expression of the gene coding for this enzyme when methylated

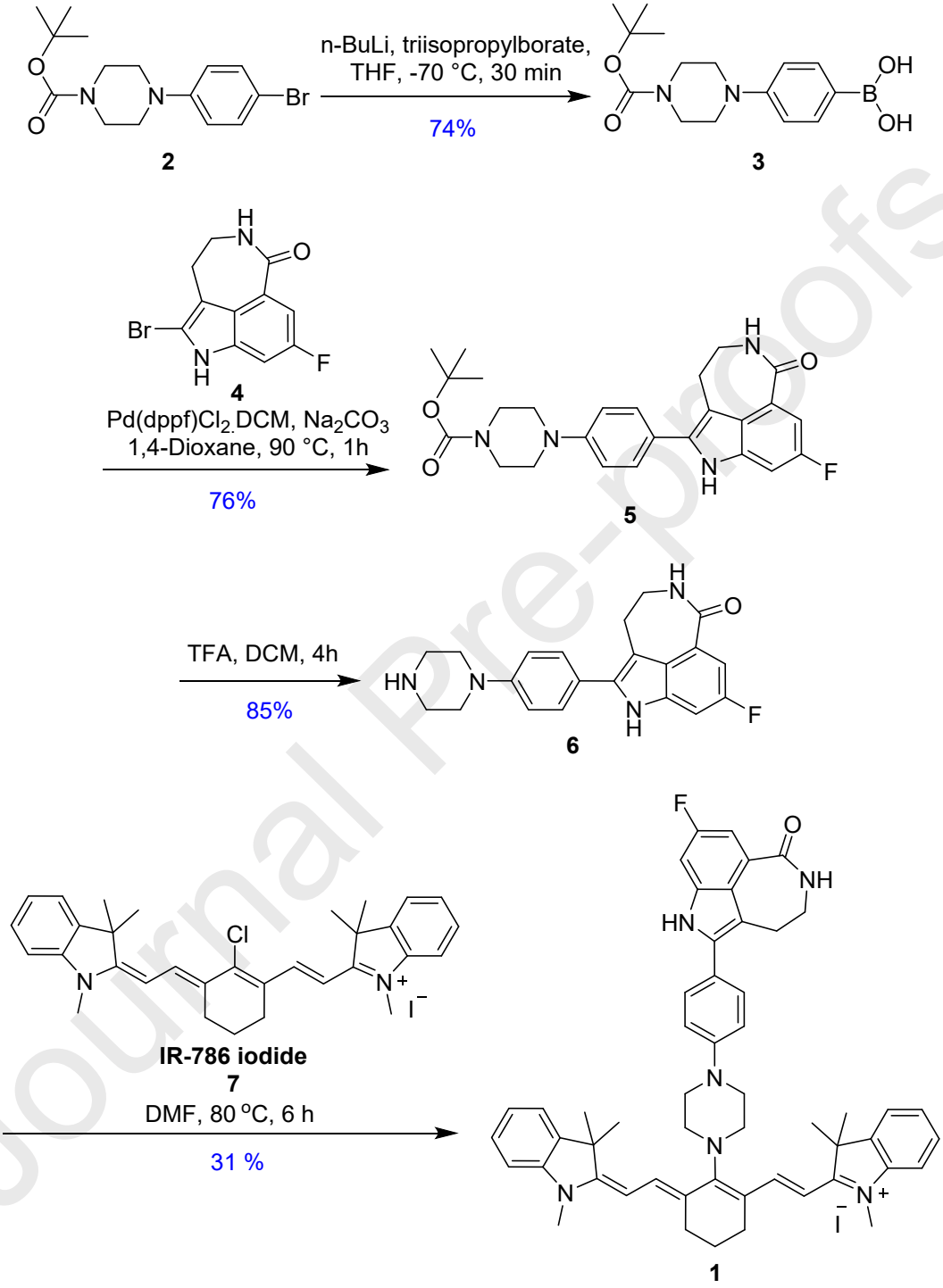
(epigenetic silencing) is suppressed resulting in reduced levels and hence activity of MGMT, which promotes the cytotoxic actions of TMZ. The recent advances in understanding the embryonic origin of brain tumours have shed light on the fact that mechanisms that regulate stem cell behaviour in the embryo could be sabotaged in a tumour microenvironment leading to increased proliferation and loss of cellular distinction.[6-7] The subtle difference in normal and malignant brain tissues makes it difficult for meaningful therapeutic intervention without invoking damage to surrounding normal brain tissues. In this scenario, a selective approach such as targeted drug delivery would be highly desirable. Although this has brought many benefits in treating peripheral cancers,[8-9] in the field of brain cancers, there is much to be achieved.

Among recently emerging targeted therapies, the poly ADP ribose polymerase (PARP) inhibitors have shown promise in treating cancer such as ovarian[10-11] and breast cancer[12] with many undergoing clinical trials for various other cancers.[13-16] These inhibitors work by inhibiting the DNA damage repair function of PARP enzymes, which can ultimately result in cell death. These inhibitors are particularly effective in cancer patients with tumours having mutations in homologous recombination repair enzymes BRCA1, BRCA2, and PALB2,[17] which increases the accumulation of unrepaired DNA double-strand breaks that lead to apoptosis.[18-19] There are marketed therapies currently which take advantage of genomic instability in cancer to achieve selectivity in targeting cancer cells over normal cells.[20] This has brought about an increased efficacy in treatment but off-target toxicity still persists. PARP inhibitors (PARPi) can radiosensitize glioblastoma cancer stem cells (GSCs) which makes them a useful combination to use with alkylating agents such as temozolomide.[21-22] Although this is very encouraging, their ability to cross the blood-brain barrier (BBB) to achieve effective therapeutic doses is very limited.[23-26] Literature reports certain PARP inhibitors being efflux pump substrates which are partly responsible for development of drug resistance over the period of treatment.[27-29] Besides, it is reported that the PARP inhibitor Veliparib radiosensitizes both normoxic and hypoxic cell lines which could invoke systemic toxicity.[30-31] Therefore improving the tumour specificity of PARPi will expand their utility beyond the treatment of breast and ovarian cancer in oncology.

A class of near infrared-emitting dyes called heptamethine cyanine dyes (HMCDs) is reported to possess BBB crossing properties and selectivity towards tumour compared to normal tissue.[32] These dyes evade efflux pumps[33-34] and utilize tumour hypoxia and organic anion-transporting polypeptides (OATPs) overexpressed in cancer cells to achieve their specificity.[35] There are literature examples of cytotoxic drug-dye conjugates showing efficacy in treating brain cancers in mouse models.[36-37] The drug-dye conjugate incorporating crizotinib, an anaplastic lymphoma kinase (ALK) inhibitor showed 100-fold enhanced cytotoxicity over the parent kinase inhibitor.[38] This improvement was even more pronounced (492-fold) when the above conjugate was combined with TMZ, the standard drug for treatment for GBM. Encouraged by these results, we have applied this strategy to improve the efficacy of PARP inhibitors in the treatment of GBM. Herein, we conjugated HMCD, IR-786 **7** with modified PARPi **6**, an analogue of rucaparib. Rucaparib is a PARPi which is reported to be an efflux pump substrate and therefore not amenable to cross the BBB.[24] To the best of our knowledge, there are no reported studies exploring HMCD-modified rucaparib conjugate for the treatment of GBM. Such conjugates may provide novel ways of treating brain cancers.

The synthesis of conjugate **1** is described in scheme 1. Lithium halide exchange with bromide **2** afforded boronic acid **3** which subsequently underwent Suzuki-Miyaura cross coupling reaction with commercially available bromide **4** to obtain intermediate **5**. Removal of the *tert*-butyloxycarbonyl protecting group using trifluoroacetic acid afforded rucaparib analogue **6**. **6** was then conjugated to HMCD IR-786 by facile displacement of chloro group to obtain

conjugate **1**. This modification was affected after careful consideration of published crystal structure of rucaparib with PARP and Tankyrase protein so as not to perturb the interaction of rucaparib with the protein hot spots.[39-40] Both rucaparib and analogue **6** showed similar modest activity in patient-derived glioblastoma cell line (Figure 1D).



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

The drug-dye conjugate **1**, IR-786 **7** and rucaparib were screened against three patient-derived cell lines obtained from Auckland City Hospital (Sup. Table S1). As shown in Table 1,

and Figure 1, the drug-dye conjugate **1** was more potent at reducing cell numbers when compared to rucaparib (EC_{50} of 128 nM vs >100,000 nM, respectively after 48 h). Treatment with rucaparib was unable to result in enough cell death to determine an EC_{50} within the range of concentrations used.

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. Data represents mean ± SEM (n=3).

	Compound (EC_{50} nM)	Compound with TMZ (EC_{50} nM)
IR-786 (7)	1735 ± 249	390 ± 45
1	128 ± 30	56 ± 6
Rucaparib	>100,000	>100,000

The reported mode of uptake for HMCDs are through the activated hypoxia-inducible factor 1α-organic anion transporting polypeptide (OATP) axis. Therefore, we investigated whether **1** maintained the same mode of cellular uptake. We utilized a known OATP pump blocker sodium taurocholic acid (250 μM) and the HIF1 α-OATP axis activator, 3,4-DHB (1 mM). Figure 3 clearly illustrates the increase in uptake of **1** with a 24 h 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 the conjugate **1** has a high reliance on the OATPs for their uptake into the tumour cells.

Additionally, Figure 1 highlights that the three GBM patient cell lines were largely unresponsive to TMZ. However, co-treatment of **1** with 100 μM of TMZ increased the cytotoxic potency of **1** to 56 nM, which was two-fold higher than treatment with **1** alone (Figure 1). This response is suggestive of synergism of conjugate **1** with TMZ. Additionally, the co-administration of TMZ with **7** had an appreciable increase in potency in relation to treatment with **7** alone. In contrast, co-administration of rucaparib with TMZ did not produce a change in potency for either compound.

Despite the lack of cytotoxic activity exhibited by rucaparib on GBM cells, there was an apparent antiproliferative effect (IC_{50} of 53 μM). However, by conjugating rucaparib to our dye (drug-dye conjugate **1)**, it significantly increased the cytotoxic activity on the GBM cells (EC_{50}:128 nM). Furthermore, conjugate **1** significantly increased the antiproliferative activity on the GBM cells when compared to rucaparib alone (IC_{50}: 21 nM vs 53 μM; Table 2, $p < 0.001$). No cytotoxic activity was observed at this concentration. In terms of proliferation, despite treatment with up to 100 μM, TMZ failed to exert a 50% reduction in proliferating cells across all three primary GBM patient-derived cell lines. Additionally, co-treatment of rucaparib with 100 μM of TMZ resulted in a 24-fold increase in the potency of rucaparib in antiproliferative activity (2262 nM) (Figure 3). However, drug-dye conjugate **1** did not show any evidence of synergism with TMZ in terms of the proliferation of GBM cells (IC_{50} of 21nM to 20 nM with TMZ; Table 2).

The ability of tumour specific accumulation of a class of near infra-red emitting dyes, HMCD and their application in conjugating various drug-like molecules to enhance potency and reduce off-target toxicity as reported in the literature[36-37, 41-42] prompted us to try a similar strategy to conjugate a PARPi to HMCD IR-786 **7**. This effort resulted in drug-dye conjugate **1** with nanomolar potency in three different patient-derived GBM cell lines. The observed potency is at least 780 fold higher than rucaparib or the modified rucaparib **6**. The potency of compound **1** was positively influenced by the addition of TMZ (two-fold improvement compared to **1** alone), although TMZ itself did not have any effect in reducing cell number, due

to the inherited resistance of these cell lines to TMZ treatment. IR-786 **7** itself was 13.5 fold and 7 fold less potent than **1** alone or in combination with TMZ. Such dramatic improvements in potency exhibited by **1** could be due to altered transport mechanism of such compounds since rucaparib itself is reported to be an efflux pump substrate[43] contributing to its diminished activity. Conjugate **1** appears to rescue this activity by targeting an alternate pathway of transport, mainly the OATPs, which is activated by hypoxic tumour conditions. Our experiments demonstrate that this mode of transport was indeed present, and is consistent with similar compounds reported in the literature.[44-46] Although other mechanisms cannot be ruled out, the activity of conjugate **1** gives a good rationale for exploring OATPs in oncology to overcome drug resistance, which is a formidable barrier to overcome in CNS tumours.

The prognosis of GBM patients remains dismal despite the aggressive treatment regimen. The PARP family of enzymes has a pleiotropic role in DNA repair, and therefore, has attracted attention for the development of inhibitors as monotherapies and a chemo-radiation sensitizer. However, the use of PARP inhibitors like rucaparib in the treatment of GBM has been challenged by poor brain penetration and tumor heterogeneity which is a hallmark of most brain cancers.[25, 47] Although this might be the case, these classes of drugs are extremely valuable for the treatment of CNS tumors due to the pleiotropic role of PARP enzymes in DNA repair. With increasing evidence of unsuitability in targeting cancer stem cells in brain cancer due to the transient nature of the biomarkers expressed by them, and compounded by tumour plasticity[48-49], a shift in therapeutic approach is warranted. The drug-dye conjugate **1** serves as an example of the beneficial effects of combining PARP inhibitors like rucaparib with HMCDs to afford a significantly more potent compound with good efficacy in human glioblastoma cell lines. Despite rucaparib being relatively inactive across the human glioblastoma cell lines, conjugation to IR-786 **7**, afforded a cytotoxic compound with nanomolar potency. The co-incubation of **1** with TMZ provided evidence for synergism by reducing the EC_{50} value by two-fold. This highlights the potential of **1** as both a monotherapy and a chemotherapy sensitizer. The radiation-sensitisation effects of these compounds and their mechanisms of action 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.

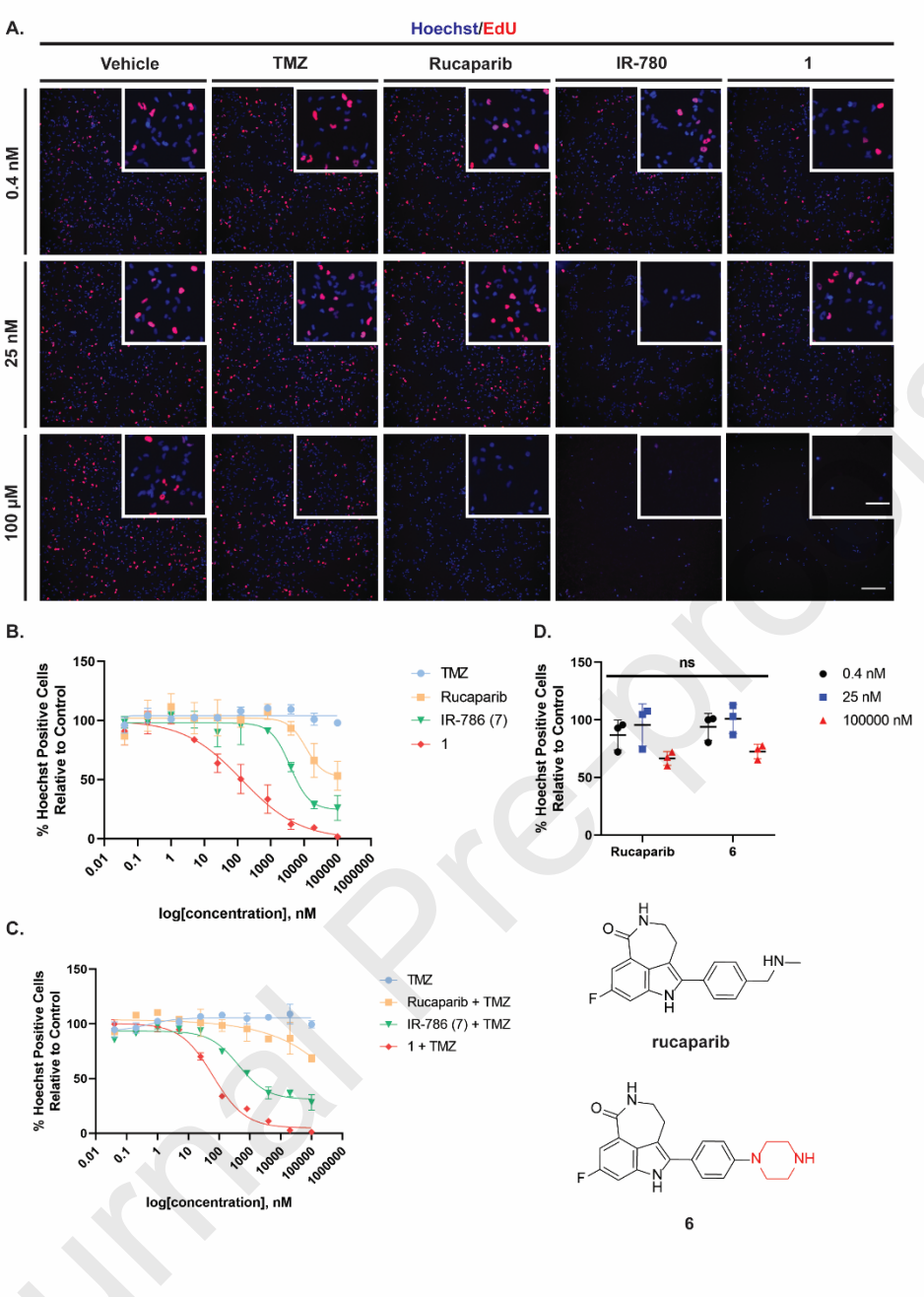


Figure 1. Viability and proliferative activity of GBM cells as indicated by total cell count after 48 h (**A-B**) incubation with conjugate (**1**), rucaparib, IR-786 (**7**) and TMZ, and co-incubation with 100 µM TMZ (**C**). The EC_{50} was determined using Hoechst counts to represent the total number of cells (**B** -**C**). The activity of rucaparib compared to **6** is seen in **D.** A non-linear curve was fitted using Graphpad Prism using the concentration of each compound versus the total number of Hoechst-positive cells normalised to DMSO (three parameters). Data represents mean ± SEM ns = P > 0.05 to rucaparib (one-way ANOVA with Dunnett's post-test). Data displayed is from three independent GBM cases (T115, T84 and T141)

Table 2. IC_{50} of the proliferation of the GBM cells as indicated by the proportion of EdU-positive cells which is normalised to the vehicle DMSO after 48 h. Data represents mean ± SEM. * = P < 0.05, *** = P > 0.005 relative to rucaparib.

	Compound (IC_{50} nM)	Compound with TMZ (IC_{50} nM)
IR-786 (7)	357 ± 39 ***	120 ± 30 *
1	21 ± 4 ***	20 ± 7 *
Rucaparib	53443 ± 473	2262 ± 488

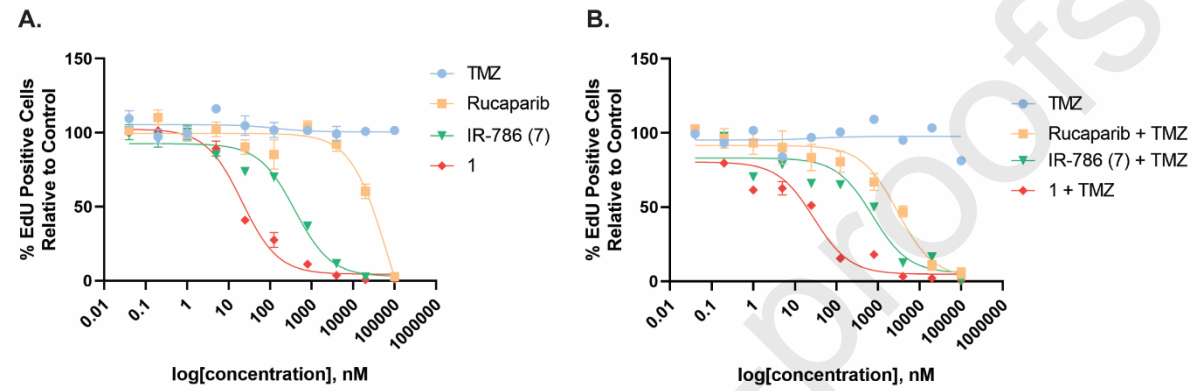


Figure 2. Proliferative activity of GBM cells as indicated by an EdU proliferation assay after 48 h (A) incubation with conjugate (**1**), rucaparib, IR-786 (**7**) and TMZ, and co-incubation with 100 μM TMZ (B). The IC_{50} was determined using the percentage of EdU-positive cells as a measure of proliferation. A non-linear curve was fitted using Graphpad Prism using the concentration of each compound versus the percentage of EdU-positive cells normalised to DMSO (three parameters). Data represents mean ± SEM. Data displayed is from three independent GBM cases.

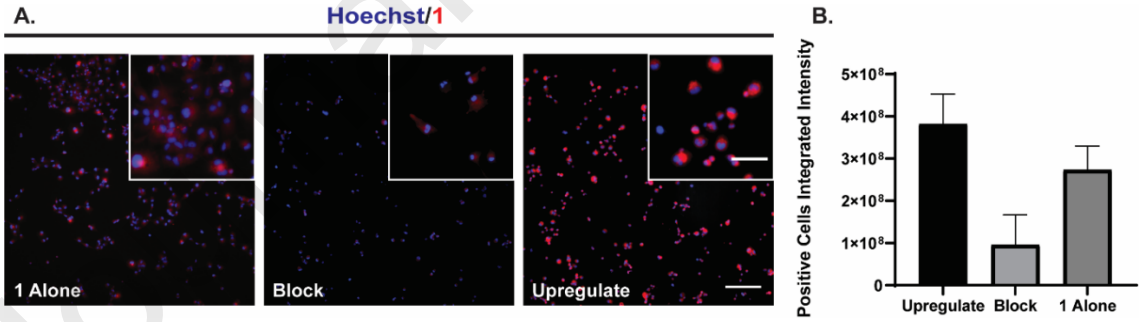


Figure 3. Fluorescence microscope images illustrating the uptake of the drug-dye conjugate **1** (red) into patient-derived GBM cells. **1** (1 μM) was added to the cells for 1 h in all conditions before imaging. Pre-incubation with 1 mM of 3,4-DHB greatly increased **1**'s uptakes as shown by the increased far-red fluorescence signal. Conversely, blocking the OATP pump with pre-incubation with sodium taurocholic acid greatly attenuated this fluorescence signal. The bar-graph is a quantification of the average cell integrated intensity of the far-red signal . Data represents mean ± SEM (n=3).

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Supplementary material:

Detailed synthetic procedures, characterization of compounds, biological assays can be found in the online version at

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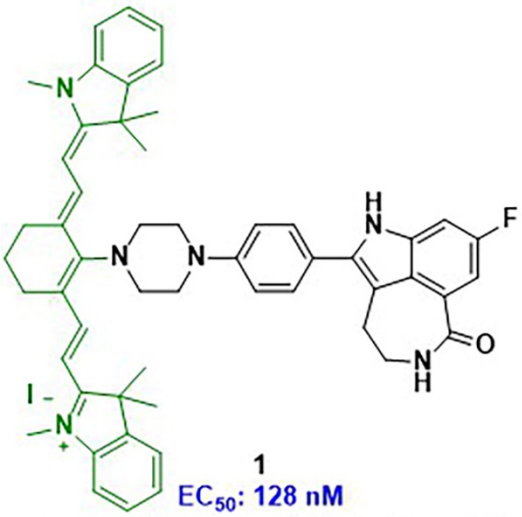

1

EC_{50}: 128 nM

potent antiproliferative activity in three different patient derived glioblastoma cell lines