

1 **Front-line glioblastoma chemotherapeutic temozolomide is toxic**
2 **to *Trypanosoma brucei* and potently enhances melarsoprol and**
3 **eflornithine**

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16 A B S T R A C T

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18 Sleeping sickness is an infectious disease that is caused by the protozoan parasite
19 *Trypanosoma brucei*. The second stage of the disease is characterised by the parasites
20 entering the brain. It is therefore important that sleeping sickness therapies are able to cross
21 the blood-brain barrier. At present, only three medications for chemotherapy of the second
22 stage of the disease are available. As these trypanocides have serious side effects and are
23 difficult to administer, new and safe anti-trypanosomal brain-penetrating drugs are needed.
24 For these reasons, the anti-glioblastoma drug temozolomide was tested *in vitro* for activity
25 against bloodstream forms of *T. brucei*. The concentration of the drug required to reduce the
26 growth rate of the parasites by 50% was 29.1 μM and to kill all trypanosomes was 125 μM .
27 Importantly, temozolomide did not affect the growth of human HL-60 cells up to a
28 concentration of 300 μM . Cell cycle analysis revealed that temozolomide induced DNA
29 damage and subsequent cell cycle arrest in trypanosomes exposed to the compound. As drug
30 combination regimes often achieve greater therapeutic efficacy than monotherapies, the
31 interactions of temozolomide with the trypanocides eflornithine and melarsoprol,
32 respectively, was determined. Both combinations were found to produce an additive effect. In
33 conclusion, these results together with well-established pharmacokinetic data provide the
34 basis for *in vivo* studies and potentially for clinical trials of temozolomide in the treatment of
35 *T. brucei* infections and a rationale for its use in combination therapy, particularly with
36 eflornithine or melarsoprol.

37

38 *Keywords:*

39 *Trypanosoma brucei*

40 Temozolomide

41 Trypanocides

42 Drug combination

43

44 **1. Introduction**

45

46 Human African trypanosomiasis (HAT), also known as sleeping sickness, is a tropical
47 neglected disease occurring in rural areas of sub-Saharan Africa (Steverding, 2008). The
48 disease is caused by the protozoan parasite *Trypanosoma brucei* and transmitted to humans
49 by the bite of infected tsetse flies (*Glossina* spp.). At first, the trypanosomes live and multiply
50 extracellularly in the blood and the lymphatic fluids of their human host (WHO, 2016). Then,
51 after months or years, the parasites invade the central nervous system. At this stage, the
52 typical symptoms are sleeping disorders and apathy, the characteristic signs that gave the
53 disease its name (WHO, 2017). Without chemotherapy, sleeping sickness is eventually fatal.
54 Unfortunately, only two drugs (melarsoprol and eflornithine) and one drug combination
55 (nifurtimox/eflornithine) are available for treatment of the neurological or second (late) stage
56 of the disease (Croft, 1997; Fairlamb, 2003; Steverding, 2010). All these treatments have
57 serious side effects and drawbacks. For example, melarsoprol can cause reactive
58 encephalopathy which is fatal in 3-10% of cases (WHO, 2017) while the treatment regime of
59 eflornithine is complex (iv infusion of 400 mg/kg/day 6 hourly) and requires hospital
60 admission (Kuzoe, 1993). Although two new drug candidates, fexinidazole and SCYX-7158,
61 are currently undergoing clinical trials (phase II/III and phase I, respectively; DNDi, 2017),
62 their licensing for treatment of sleeping sickness may still take years.

63 Another strategy to discover new therapies for tropical parasitic diseases is the testing of
64 existing, already approved drugs (Caffrey and Steverding, 2008). As the toxicological and
65 pharmacological properties of licensed drugs are established, a more rapid application of
66 existing drugs for treatment of a neglected tropical disease with limited clinical trials may be
67 possible. This has been shown in the case of the nifurtimox/eflornithine combination therapy
68 which development took only 8 years from two initial clinical trials in Uganda in 2001 and
69 2004 followed by a demonstration trial and a whole multicentric study in the Democratic
70 Republic of Congo until the drug combination was added to the WHO Essential Medicine
71 List for treatment of second stage *T. gambiense* sleeping sickness in April 2009 (Yun et al.,
72 2010).

73 An interesting class of drugs with anti-trypanosomal activity are anti-cancer agents as
74 has been shown for clinical approved topoisomerase and proteasome inhibitors (Deterding et
75 al., 2005; Steverding and Wang, 2009). However, in the case of sleeping sickness, drugs are
76 needed that are able to cross the blood-brain barrier in order to treat the neurological stage of
77 the disease. Here we pre-clinically evaluate the toxic effect of a glioblastoma
78 chemotherapeutic agent on trypanosomes. The compound temozolomide (Fig. 1) was
79 investigated for its trypanocidal activity alone and in combination with the anti-HAT drugs
80 eflornithine or melarsoprol against bloodstream forms of *T. brucei*.

81

82 **2. Materials and Methods**

83

84 *2.1. Drugs*

85

86 Temozolomide and eflornithine (DL- α -difluoromethylornithine) were purchased from
87 Sigma-Aldrich (Gillingham, UK) and Enzo Life Sciences Ltd. (Exeter, UK), respectively.
88 Melarsoprol was a gift from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany).

89

90 *2.2. Cell Culture*

91

92 Bloodstream forms of the *T. brucei* clone 427-221a (Hirumi et al., 1980) were grown in
93 Baltz medium (Baltz et al., 1985) while human myeloid leukaemia HL-60 cells (Collins et al.,
94 1977) were cultured in RPMI medium (Moore et al., 1967). Both media were supplemented
95 with 16.7% heat-inactivated foetal bovine serum. Trypanosomes and human cells were
96 maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

97

98 *2.3. Toxicity assay*

99

100 Toxicity assays were performed according to the method described by Merschjohann et
101 al. (2001) with some modifications. In brief, cells were seeded in 96-well plates in a final

102 volume of 200 μ l of appropriate medium containing various concentration of temozolomide
103 (twofold dilutions from 300 μ M to 2.34375 μ M) and 1 % DMSO. The initial cell densities
104 were 1×10^4 /ml for bloodstream-form trypanosomes and 1×10^5 /ml for human myeloid HL-
105 60 cells. After 24 h incubation, 20 μ l of 0.5 mM resazurin in PBL (sterile filtered) was added
106 and the cells were incubated for a further 48 h. The concentration of the resazurin stock
107 solution and the amount of the dye added were the same as those for the commercial Alamar
108 Blue assay (O'Brien et al., 2000). It should be also noted that the addition of resazurin does
109 not have any negative effect on the determination of MIC and GI₅₀ values. This was
110 previously shown when the resazurin assay was compared with direct cell counting
111 (Merschjohann et al., 2001). Subsequently, the absorbance was read on a microplate reader
112 using a test wavelength of 570 nm and a reference wavelength of 630 nm. The 50 % growth
113 inhibition (GI₅₀) value, i.e., the concentration of the drug necessary to reduce the growth rate
114 of cells by 50 % compared to the control, was determined by linear interpolation according to
115 the method described by Huber and Koella (1993). The minimum inhibitory concentration
116 (MIC) value, i.e., the concentration of the drug at which all cells were killed, was determined
117 microscopically.

118

119 2.4. Cell cycle analysis

120

121 Bloodstream forms of *T. brucei* (5×10^5 /ml) were incubated with 100 μ M temozolomide
122 or 0.5% DMSO for 21 h. After harvesting by centrifugation at $850 \times g$ and washing once with
123 PBS/1% glucose (PBSG), cells were re-suspended in 300 μ l PBSG and fixed with 700 μ l ice-
124 cold ethanol for 30 min. After diluting with 500 μ l PBSG, cells were centrifuged, washed
125 once with PBSG and re-suspended in the same buffer. The cells were stained with propidium
126 iodide staining solution (5 μ l/100 μ l cell suspension) and incubated with 200 μ g/ml RNase A.
127 After 1 h incubation at room temperature, cells were analysed on a CyFlow® Cube 6 flow
128 cytometer. Debris and cell fragments were excluded from analysis through gating on forward
129 and side scatter properties, and 50,000 cells were analysed.

130

131 2.5. Determination of drug interactions and isobologram construction

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133 The interactions between temozolomide and anti-HAT drugs were determined by a
134 modified isobolographic method (Fivelman et al., 2004). Based on GI_{50} values, the maximum
135 concentration of individual drugs was set at $5 \times GI_{50}$ ensuring that the GI_{50} was at the
136 midpoint of the serial dilution. The highest concentrations of solutions were prepared in
137 proportions of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 of temozolomide and anti-HAT drugs
138 (eflornithine and melarsoprol, respectively), which were then twofold serially diluted (6
139 dilution steps). The assay was set up in 96-well plates and wells without drugs served as
140 controls. All wells contained 1 % DMSO. The initial cell density was 1×10^4
141 trypanosomes/ml. The assay was evaluated and GI_{50} values calculated as described above.

142 The fractional inhibitory concentration (FIC) at the GI_{50} value was calculated as $FIC =$
143 $GI_{50(\text{combination})}/GI_{50(\text{alone})}$. The sum of FICs (ΣFIC) was computed as $\Sigma FIC = FIC_{(\text{drug A})} +$
144 $FIC_{(\text{drug B})}$. The mean sum of FICs ($x\Sigma FIC$) was averaged over the ΣFIC s. Isobolograms were
145 built by plotting the FIC of each drug ratio. The $x\Sigma FIC$ s were used to classify the interactions
146 according to Odds (2003). An $x\Sigma FIC$ of ≤ 0.5 , between 0.5 and 4, and of ≥ 4 indicates
147 synergy, indifference and antagonism, respectively.

148

149 3. Results and discussion

150

151 The trypanocidal and cytotoxic activity of the anti-glioblastoma drug temozolomide was
152 evaluated with bloodstream forms of *T. brucei* and human myeloid leukaemia HL-60 cells.
153 Temozolomide showed a concentration-dependent inhibitory effect on the growth of *T.*
154 *brucei* bloodstream forms with an MIC value of 125 μM and a GI_{50} value of 29.1 μM (Fig.
155 2). The trypanocidal activity of temozolomide was within the range of previously reported
156 cytotoxic activity of the drug against various glioblastoma cell lines, with published GI_{50}
157 values ranging between 10 μM to 250 μM (Gaspar et al., 2010; Perazzoli et al., 2015; Lee et
158 al., 2016). In addition, the GI_{50} value of temozolomide was similar to that of eflornithine
159 (23.9 μM determined under the same experimental conditions), one of the drugs used to treat

160 the second stage of sleeping sickness. Crucially important, temozolomide did not show any
161 cytotoxic effect against human HL-60 cells up to a concentration of 300 μ M (Fig. 2). HL-60
162 cells are usually used as reference as their sensitivity for approved trypanocides is well
163 established (Merschjohann et al., 2001; Steverding and Wang, 2009).

164 To investigate the anti-trypanosomal mechanism of action of temozolomide, the DNA
165 content of bloodstream form trypanosomes exposed to the drug was analysed by flow
166 cytometry. After 21 h exposure to 100 μ M temozolomide, a 2.8-fold decrease, and a 6.4-fold
167 and a 1.7-fold increase in the proportion of cells with normal (2C), lower (<2C) and double
168 (4C) DNA content were observed, respectively (Fig. 3). This finding indicates that
169 trypanosomes exposed to the drug are unable to finish cell division after completing DNA
170 synthesis (cell arrest at the G₂-M boundary) followed by fragmentation of DNA with
171 subsequent cell death. The trypanocidal mode of action of temozolomide resembles the
172 cytotoxic mechanism of the drug reported previously for human glioblastoma cells (Hirose et
173 al., 2001). The cytotoxic activity of temozolomide is based on its alkylating activity of
174 guanine and adenine (Denny et al., 1994). Although accounting for only 5% of the total
175 adducts formed, O⁶-methylguanine plays the critical role in the antitumor activity of drug.
176 Even though the methyl group in O⁶-methylguanine can be removed by O⁶-methylguanine-
177 DNA methyl transferase (MGMT), cells with low levels of this DNA repair enzyme are
178 sensitive to the cytotoxic action of temozolomide (Dolan et al., 1991). In the case of *T.*
179 *brucei*, it seems that this protozoan does not have this enzyme as no MGMT gene was found
180 when searching the TriTrypDB database. Non-removal of the methyl group at position O⁶ of
181 guanine will lead to mispairing of the base with thymine during the next DNA replication.
182 This triggers the DNA mismatch repair (MMR) system that does not repair O⁶-methylated
183 guanine residues but tries to correct the nucleotide on the newly synthesised strand (Jiricny,
184 2006). As the MMR system cannot find the correct partner, it will reinsert thymidines during
185 the repair process. A futile cycle of repetitive non-productive repairs is the result leading to
186 the accumulation of persistent nicks in the DNA during the subsequent cell cycle. These
187 nicks ultimately inhibit initiation of replication inducing blockage of the cell cycle at the G₂-
188 M boundary. Subsequently, the DNA will fragment and the cell dies. The observed effect of

189 temozolomide on the DNA content of trypanosomes exposed to the drug (increased G₂-M
190 peak and increased sub-G₁ peak), is consistent with the cytotoxic mechanism mediated
191 through base modification recognised by the MMR machinery as describe above.

192 Combination therapy has been shown to be a valuable option for the treatment of
193 parasitic infections. For instance, the first-line recommended treatments of malaria and
194 sleeping sickness are, according the World Health Organization, dual-drug therapies (WHO,
195 2016, 2017). Therefore, the interaction of temozolomide with the two drugs used in second
196 stage treatment of sleeping sickness, eflornithine and melarsoprol, was evaluated with the
197 fixed-ratio isobologram method (Fivelman et al., 2004). The experimental design allowed the
198 determination of FIC values for each combination (Tables 1 and 2). Both
199 temozolomide/eflornithine and temozolomide/melarsoprol combinations had Σ FIC values
200 ranging between 1.1 and 1.4. The $x\Sigma$ FIC of both combinations was very similar with
201 calculated values of 1.27 ± 0.06 (temozolomide/eflornithine) and 1.29 ± 0.13
202 (temozolomide/melarsoprol) which were not significantly different ($p = 0.737$, student's t-
203 test). As these $x\Sigma$ FIC values are within the range of 0.5 to 4, the interaction between
204 temozolomide and the two anti-trypanosomal drugs is indifferent (additive) (Odds, 2003).
205 The indifferent interaction was also confirmed by plotting the individual FIC values in
206 isobolograms (Fig. 4), where all points were close to the additivity line.

207 Temozolomide is an oral alkylating prodrug that is 100% bioavailable and able to cross
208 the blood-brain barrier (Agarwala and Kirkwood, 2000). The prodrug is stable at the acidic
209 pH of the stomach but spontaneously converts to the active metabolite 5-(3-dimethyl-1-
210 triazenyl) imidazole-4-carboxamide when in contact with the slightly basic pH of the blood
211 and tissues (Newlands et al., 1997). The concentrations of the temozolomide in the brain and
212 cerebrospinal fluid (CSF) are about 30% of plasma levels (Agarwala and Kirkwood, 2000).
213 These pharmacological properties make the drug an interesting agent for treating the
214 neurological stage of sleeping sickness. The mean peak CSF concentration of temozolomide
215 in Rhesus monkeys receiving the drug as a 1 h intravenous infusion was $26\pm 4 \mu\text{M}$ at 2.5 h
216 (Patel et al., 2003). Although this concentration is close to the GI₅₀ value determined for
217 bloodstream forms of *T. brucei in vitro*, it is arguable whether it would be high enough to

218 significantly affect the growth of the CSF forms of the parasites. However, in contrast to
219 bloodstream forms, CSF forms are more fragile and lyse quickly (Pentreath et al., 1992)
220 which could indicate that they may be also more susceptible to drugs. It remains to be shown
221 in an animal model for the second stage of the disease whether temozolomide can indeed
222 eliminate trypanosomes from the brain.

223 Rather employing temozolomide as monotherapy, it would be probably more promising
224 to use the drug in combination with eflornithine or melarsoprol. Although the combination of
225 temozolomide with the anti-trypanosomal drugs did not result in the desirable synergistic
226 interaction, an additive (indifferent) effect is also of value as it would permit decreased
227 dosage while maintaining efficacy (Chou, 2006). In this context it is interesting to note that
228 the recommended nifurtimox/eflornithine combination therapy as first-line treatment of the
229 second stage of sleeping sickness failed to show synergistic anti-trypanosomal activity *in*
230 *vitro* (Vincent et al., 2012). Further investigations are requires to establish the *in vivo* efficacy
231 of temozolomide in combination with eflornithine or melarsoprol.

232 As for many anti-cancer drugs, temozolomide causes side effects but is usually well
233 tolerated. The most common adverse events are nausea, vomiting, headache, fatigue and
234 constipation with severity levels generally being mild to moderate (Agarwala and Kirkwood,
235 2000). Thus, the side effects of temozolomide are similar or less severe than those of the anti-
236 HAT drugs currently used for treatment of second stage sleeping sickness (Abdi et al., 1995).

237 The relative low anti-trypanosomal activity of temozolomide should not discourage from
238 carrying out further research into this compound. For example, nifurtimox is also not very
239 trypanocidal (GI_{50} for bloodstream form trypanosomes was determined to be between 4 to 5.6
240 μ M (Enanga et al., 2003; Vincent et al., 2012)), and yet the nitroheterocyclic drug has
241 become in combination with eflornithine the first-line treatment of late stage sleeping
242 sickness. In addition, temozolomide has been shown to be active in patients with high-grade
243 gliomas (Agarwala and Kirkwood, 2000), despite the drug has a similar low activity against
244 glioblastoma cells *in vitro* as against trypanosomes. In this context it should also be noted that
245 *in vitro* studies not necessarily reflect the situation *in vivo*. For instance, the host's immune
246 response may contribute to the effect of drugs and pharmacokinetic factors may lead to a

247 different exposure of pathogens to drugs. Importantly, alkylating agents have been previously
248 shown to eliminate trypanosomes from the bloodstream of mice within 48-72 h (Penketh et
249 al., 1990).

250 In summary, we have shown that the glioblastoma chemotherapeutic agent
251 temozolomide is toxic to bloodstream forms of *T. brucei* and potently enhances the activity of
252 the existing drugs eflornithine and melarsoprol. As temozolomide is a clinically approved
253 oral chemotherapy with well-established pharmacokinetic data and if the agent is
254 demonstrated to be effective against trypanosomes *in vivo*, a more rapid application of the
255 drug for treatment of second stage sleeping sickness with less extensive clinical trials might
256 be possible, particularly in combination with eflornithine or melarsoprol.

257

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259

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262

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356

357 **Table 1**358 GI_{50} and FIC values of temozolomide-eflornithine combination against bloodstream forms of *T. brucei*.

Combination ratio (%)		$GI_{50} \pm SD$ (95% CI ^c)		FIC		
TMZ ^a	DFMO ^b	TMZ (μ M)	DFMO (μ M)	TMZ	DFMO	Σ FIC ^d
100	0	27.1 \pm 0.8 (25.5-28.7)				
80	20	24.8 \pm 0.4 (24.0-25.6)	5.2 \pm 0.1 (5.0-5.4)	0.92	0.27	1.19
60	40	19.9 \pm 0.8 (18.3-21.5)	11.1 \pm 0.5 (10.1-12.0)	0.73	0.57	1.30
40	60	13.2 \pm 0.8 (11.7-14.7)	16.5 \pm 0.9 (14.7-18.3)	0.49	0.84	1.33
20	80	6.0 \pm 0.8 (4.4-7.5)	19.9 \pm 2.6 (14.9-24.9)	0.22	1.02	1.24
0	100	19.6 \pm 2.6 (14.5-24.7)				

359 ^a TMZ, temozolomide.360 ^b DFMO, eflornithine (DL- α -difluoromethylornithine).361 ^c CI, confidence interval.362 ^d Σ FIC, sum of FIC values.

363

364 **Table 2**365 GI_{50} and FIC values of temozolomide-melarsoprol combination against bloodstream forms of *T. brucei*.

Combination ratio (%)		$GI_{50} \pm SD$ (95% CI ^c)		FIC		
TMZ ^a	ME ^b	TMZ (μ M)	ME (nM)	TMZ	ME	Σ FIC ^d
100	0	27.7 \pm 0.5 (26.7-28.4)				
80	20	25.8 \pm 0.7 (24.5-27.1)	0.9 \pm 0.0 (0.8-0.9)	0.93	0.18	1.11
60	40	25.6 \pm 0.8 (24.0-27.2)	2.3 \pm 0.1 (2.1-2.4)	0.92	0.45	1.37
40	60	18.4 \pm 0.4 (17.7-19.1)	3.7 \pm 0.1 (3.5-3.8)	0.66	0.73	1.39
20	80	9.2 \pm 0.3 (8.5-9.9)	4.9 \pm 0.2 (4.5-5.3)	0.33	0.96	1.29
0	100		5.1 \pm 0.0 (5.1-5.2)			

366 ^a TMZ, temozolomide.367 ^b ME, melarsoprol.368 ^c CI, confidence interval.369 ^d Σ FIC, sum of FIC values.

370

371 **Figure legends**

372

373 **Fig. 1.** Chemical structure of temozolomide (4-methyl-5-oxo-2,3,4,6,8-
374 pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide). The PubChem Compound Identifier
375 (CID) of the compound is 5394.

376

377 **Fig. 2.** Effect of temozolomide on the growth of bloodstream forms of *T. brucei* and human
378 myeloid leukaemia HL-60 cells. Trypanosomes (circles) and HL-60 cells (squares) were
379 incubated with varying concentrations of temozolomide. After 72 h of culture, cell viability
380 and proliferation was determined with the colorimetric dye resazurin. Mean values \pm SD of
381 three experiments are shown.

382

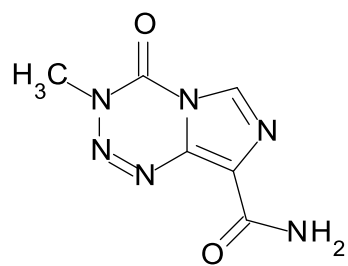
383 **Fig. 3.** Cell cycle distribution of bloodstream forms of *T. brucei* exposed to temozolomide.
384 Trypanosomes were treated with 100 μ M temozolomide (red line) or with 0.5% DMSO
385 (black line, control). After 21 h incubation, the cells were stained with propidium iodide and
386 the DNA content analysed by flow cytometry. The gates M1, M2 and M3 represent cell
387 populations with less than the normal DNA content (<2C), with normal DNA content (2C)
388 and with double DNA content (4C). The respective gated populations comprised M1 = 4.9%,
389 M2 = 71.0% and M3 = 23.5% of cells for the control parasites and M1= 31.4%, M2 = 25.6%
390 and M3 = 41.1% of cells for the temozolomide-treated trypanosomes. It should be noted that
391 the population distribution of control cells is consistent to previously observations for
392 bloodstream forms of *T. brucei* (Kabani et al., 2010).

393

394 **Fig. 4.** Isobolograms showing the *in vitro* interactions between temozolomide and
395 eflornithine (A) and between temozolomide and melarsoprol (B) against bloodstream forms
396 of *T. brucei*. Assays were performed by a fixed-ratio method based on GI₅₀ values, with the
397 combinations being tested at constant ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. Results shown
398 are from three independent experiments (see Tables 1 and 2). The dashed line is the
399 theoretical line that produced a sum of the FICs of 1 at all ratios tested and represents an

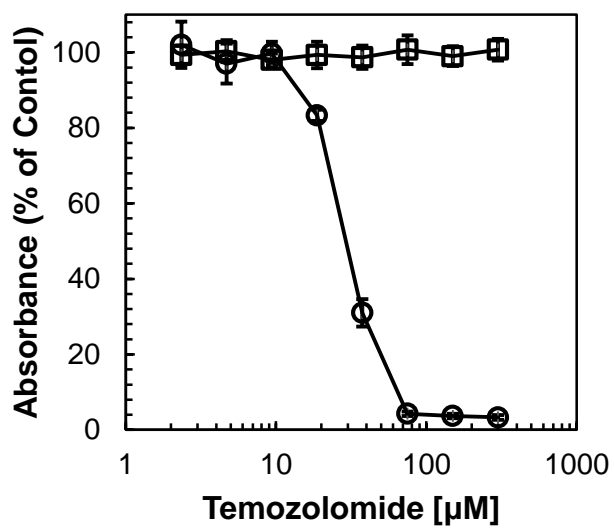
400 additive effect of the two compounds. The $\bar{x}\Sigma FIC$ values shown are the mean sums of the
401 FICs for the interactions tested.
402

403 **Fig. 1**
404



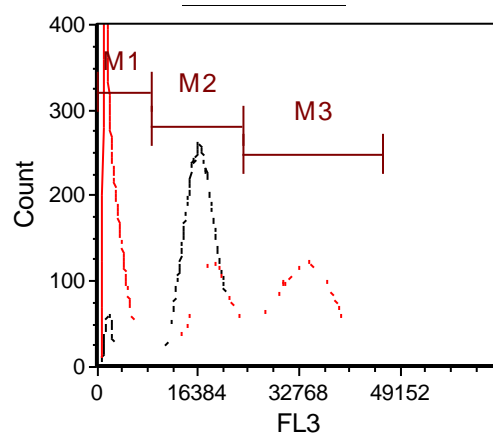
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408 **Fig. 2**



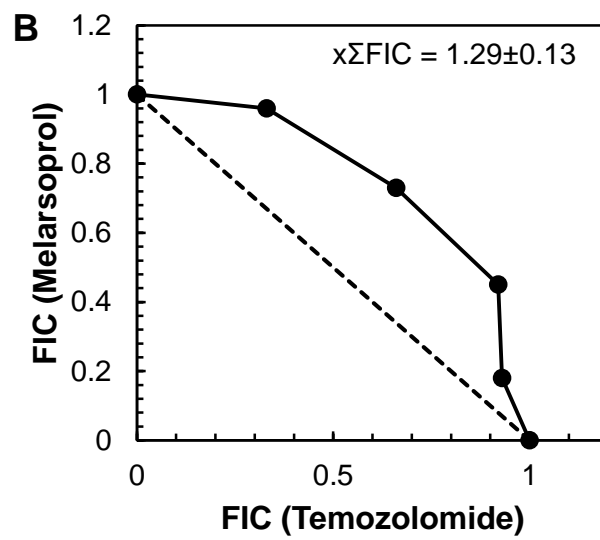
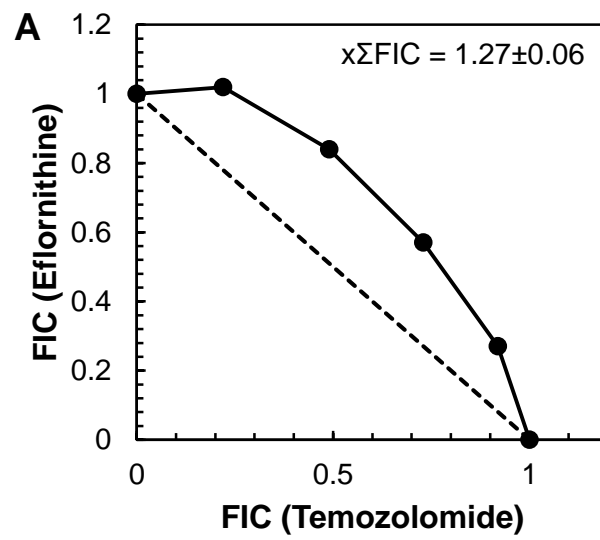
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412 **Fig. 3**
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417 **Fig. 4**
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