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

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A B S T R A C T

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

Keywords:

Trypanosoma brucei

Temozolomide

Trypanocides

Drug combination

1. Introduction

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

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

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

2. Materials and Methods

2.1. Drugs

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

2.2. Cell Culture

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

2.3. Toxicity assay

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

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

2.4. Cell cycle analysis

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

2.5. Determination of drug interactions and isobologram construction

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

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

3. Results and discussion

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

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

To investigate the anti-trypanosomal mechanism of action of temozolomide, the DNA content of bloodstream form trypanosomes exposed to the drug was analysed by flow cytometry. After 21 h exposure to 100 μM temozolomide, a 2.8-fold decrease, and a 6.4-fold and a 1.7-fold increase in the proportion of cells with normal (2C), lower (<2C) and double (4C) DNA content were observed, respectively (Fig. 3). This finding indicates that trypanosomes exposed to the drug are unable to finish cell division after completing DNA synthesis (cell arrest at the G_2-M boundary) followed by fragmentation of DNA with subsequent cell death. The trypanocidal mode of action of temozolomide resembles the cytotoxic mechanism of the drug reported previously for human glioblastoma cells (Hirose et al., 2001). The cytotoxic activity of temozolomide is based on its alkylating activity of guanine and adenine (Denny et al., 1994). Although accounting for only 5% of the total adducts formed, O^6-methylguanine plays the critical role in the antitumor activity of drug. Even though the methyl group in O^6-methylguanine can be removed by O^6-methylguanine-DNA methyl transferase (MGMT), cells with low levels of this DNA repair enzyme are sensitive to the cytotoxic action of temozolomide (Dolan et al., 1991). In the case of *T. brucei*, it seems that this protozoan does not have this enzyme as no MGMT gene was found when searching the TriTrypDB database. Non-removal of the methyl group at position O^6 of guanine will lead to mispairing of the base with thymine during the next DNA replication. This triggers the DNA mismatch repair (MMR) system that does not repair O^6-methylated guanine residues but tries to correct the nucleotide on the newly synthesised strand (Jiricny, 2006). As the MMR system cannot find the correct partner, it will reinsert thymidines during the repair process. A futile cycle of repetitive non-productive repairs is the result leading to the accumulation of persistent nicks in the DNA during the subsequent cell cycle. These nicks ultimately inhibit initiation of replication inducing blockage of the cell cycle at the G_2-M boundary. Subsequently, the DNA will fragment and the cell dies. The observed effect of

temozolomide on the DNA content of trypanosomes exposed to the drug (increased G_2-M peak and increased sub-G_1 peak), is consistent with the cytotoxic mechanism mediated through base modification recognised by the MMR machinery as describe above.

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

Temozolomide is an oral alkylating prodrug that is 100% bioavailable and able to cross the blood-brain barrier (Agarwala and Kirkwood, 2000). The prodrug is stable at the acidic pH of the stomach but spontaneously converts to the active metabolite 5-(3-dimethyl-1-triazenyl) imidazole-4-carboxamide when in contact with the slightly basic pH of the blood and tissues (Newlands et al., 1997). The concentrations of the temozolomide in the brain and cerebrospinal fluid (CSF) are about 30% of plasma levels (Agarwala and Kirkwood, 2000). These pharmacological properties make the drug an interesting agent for treating the neurological stage of sleeping sickness. The mean peak CSF concentration of temozolomide in Rhesus monkeys receiving the drug as a 1 h intravenous infusion was 26±4 μM at 2.5 h (Patel et al., 2003). Although this concentration is close to the GI_{50} value determined for bloodstream forms of *T. brucei in vitro*, it is arguable whether it would be high enough to

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

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

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

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

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

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

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Table 1

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

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

[a] TMZ, temozolomide.

[b] DFMO, eflornithine (DL-α-difluoromethylornithine).

[c] CI, confidence interval.

[d] ΣFIC, sum of FIC values.

Table 2

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

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

[a] TMZ, temozolomide.

[b] ME, melarsoprol.

[c] CI, confidence interval.

[d] ΣFIC, sum of FIC values.

Figure legends

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

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

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

Fig. 4. Isobolograms showing the *in vitro* interactions between temozolomide and eflornithine (A) and between temozolomide and melarsoprol (B) against bloodstream forms of *T. brucei*. Assays were performed by a fixed-ratio method based on GI_{50} values, with the combinations being tested at constant ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. Results shown are from three independent experiments (see Tables 1 and 2). The dashed line is the 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 xΣFIC values shown are the mean sums of the
401 FICs for the interactions tested.
402

Fig. 1

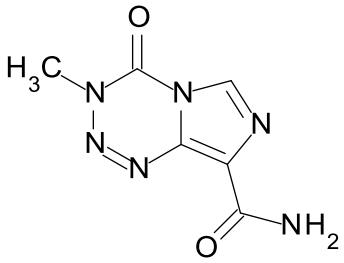
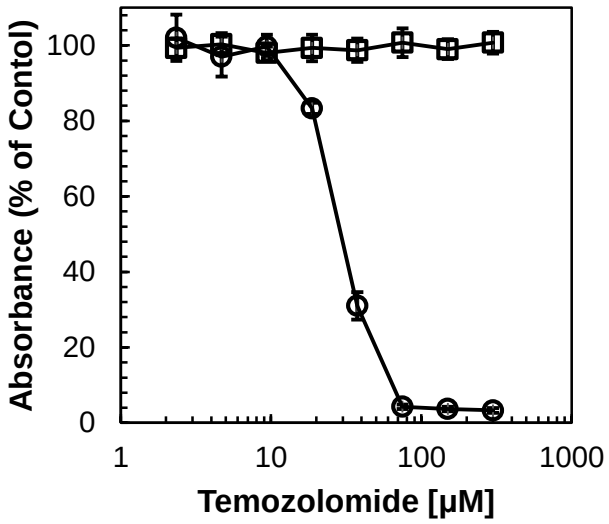
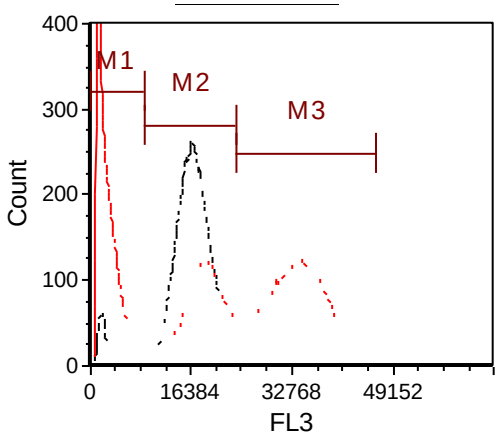


Fig. 2


Absorbance (% of Contol)
100
80
60
40
20
0
1
10
100
1000
Temozolomide [μM]

412 **Fig. 3**

413



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417 **Fig. 4**
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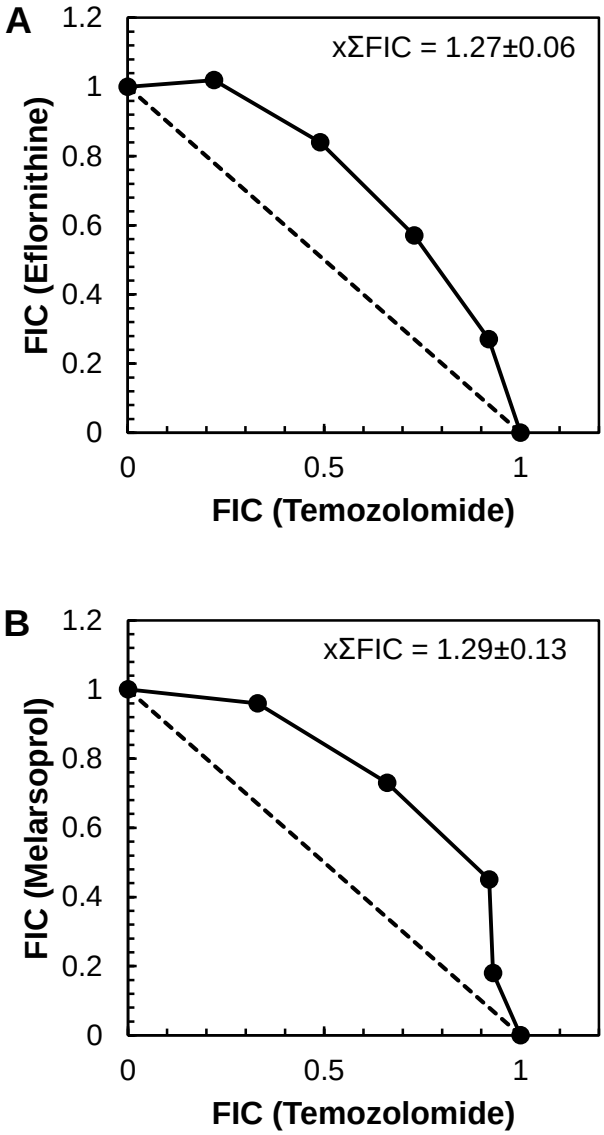

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