Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials

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Abbreviations

BCNU: 1,3-bis(2-chloroethyl)-1-nitrosourea; NAD: nicotinamide adenine dinucleotide; 5HT₃: 5-hydroxytryptamine subset 3; CT: computerized tomography; MRI: magnetic resonance imaging; AUC: area under concentration × time curve; ¹⁸F-FDG: ¹⁸F-2-fluoro-2-deoxyglucose

Introduction

At present, there is no simple predictive test to identify an active anticancer agent in man; the development of compounds that finally reach the clinic commonly reflects a mixture of intelligence and guesswork, dogged persistence and a major element of luck. The discovery of temozolomide is the result of all the above but, crucially, was built on a symbiotic relationship between the laboratory sciences of chemistry and pharmacology, and an inquisitive clinical culture keen to explore the efficacy of a new drug in the only animal that really matters—the patient suffering from cancer.

The significant molecules which were stepping stones on the pathway to temozolomide are shown in Figure 1. Their common feature, spanning pyrazolo-1,2,4-triazines and aryldimethyltriazenes in the early 1960s to temozolomide of the mid 1990s, is the presence of arrays of nitrogen atoms
which confer unique chemical and biological properties (1). Between these two milestones, the antimelanoma drug dacarbazine (DTIC) entered clinical practice in the 1970s on the basis of its potent activity against a range of rodent tumours. However, DTIC requires metabolic activation in the liver to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) (2), and the modest performance of the drug in the clinic probably reflects poor distribution of the active metabolite MTIC in man.

In 1980, Robert Stone, a research student at Aston University sponsored by
(the then) May & Baker Ltd, synthesized the first examples of a new ring-system imidazo[5, 1-d]-1,2,3,5-tetrazine which, consistent with the molecular lineage of past products of the Aston group, possessed a bicyclic ring-system with multiple nitrogen atoms (any more might have rendered the construct dangerously unstable) (3). The lead compound mitozolomide (formerly azolastone) (see Figure 1) entered the clinic with high hopes in 1983, and a Phase I trial was completed by 1985. Unfortunately, despite its curative activity in many rodent tumour models (4), mitozolomide showed only limited clinical activity and elicited unpredictable and severe myelosuppression (5). At this stage, the programme, now lacking an industrial sponsor, might have foundered but the Cancer Research Campaign Phase I/II Subcommittee showed sufficient confidence to consider the clinical testing of the N-methyl congener of mitozolomide with a demonstrably different toxicological profile. Whereas mitozolomide cross-linked DNA (6), the methylating agent temozolomide is a monofunctional agent which has good tissue distribution and was shown to be schedule-dependent in terms of its antitumour activity (7). Additionally, temozolomide is a neat and robust molecule, stable at acid pH allowing it to be absorbed intact after oral administration (8), and has excellent bio-distribution, including penetration into the central nervous system (see later).

The emergence of temozolomide into the clinical spotlight is neither a triumph of rational drug design nor does it result from any outstanding biological insights. Rather, it is the product of a fruitful collaboration between chemists, pharmacologists, pharmacists and clinicians, all of whom served their apprenticeships well (1). Consistent financial and clinical support from the Cancer Research Campaign’s Scientific and Phase I/II Committees, respectively, underpinned the consolidation of a convincing scientific and clinical database which facilitated the licencing of temozolomide to Schering-Plough Corporation. Broader clinical testing of the drug is now being undertaken worldwide with a target of achieving marketing approval in the near future.

**Biological chemistry of temozolomide**

*Synthesis*

The publication by Ege and Gilbert (9) of the synthesis of azolo[5, 1-d]-1,2,3,5-tetrazinones by the reaction of diazoazoles with isocyanates provided the impetus to prepare the first of the imidazotetrazinone series of prodrugs (3). The original synthesis of temozolomide was from the reaction of diazo-IC with methyl isocyanate (Figure 2). This synthesis has the merit of complete ‘atom economy’ in that all atoms in the starting materials are incorporated into the product. Reaction times were long in solvents such as dichloromethane and ethyl acetate, but this was compensated for by the dual advantages of high yields and high purity of the product. The reaction time was improved by incorporating dimethyl sulphoxide (DMSO) in the reaction solvent. This route has proved highly versatile in giving access to a wide range of analogues (10, 11) from the many aminoimidazoles and isocyanates available commercially and in the literature. Despite the development of alternative syntheses of
temozolomide which do not require the use of methyl isocyanate or the potentially unstable diazo-IC (12, 13), the original synthesis remains the method of choice for the large-scale production of temozolomide.

The synthetic route has also accommodated modifications for the preparation of a variety of isotopically labelled forms of the drug (Figure 3). Temozolomide has been prepared with $^{15}$N (N-2, N-3) and $^{13}$C (CH$_3$) for nuclear magnetic resonance (NMR) studies (14, 15), and $^{13}$C (CH$_3$) for positron emission tomography (PET) imaging (16), and mitozolomide has been radiolabelled with $^{14}$C (C-6) for pharmacokinetic experiments (17).

**Chemical reactions**

The chemistry of imidazotetrazinones has been reviewed extensively by Threadgill (18). The reactivity in organic systems is dominated by retro-cyclo-addition to the isocyanate and diazo precursors, and the chemistry of their breakdown products (19). Potentially the most useful reaction of imidazotetrazinones is the easy conversion of the 8-carboxamide group to an 8-carboxylic acid (20), which provides a ready handle for the synthesis of a wide range of chimeric adducts.

In aqueous systems, the reactivity of temozolomide is completely different and provides clues to account for the biological activity of the drug (21). When temozolomide is hydrolysed, the first product is the methyl triazene MTIC which ultimately transfers its methyl group to a nucleophile. The mechanism of
reaction of temozolomide in aqueous solution is shown in Figure 4. The rate-limiting step is the base-catalysed addition of water to C-4 to form a tetrahedral intermediate which collapses with breakdown of the tetrazinone ring followed by spontaneous decarboxylation. The further reaction of MTIC requires acid catalysis and is the fragmentation of the triazene to form the fugitive methyldiazonium ion. In deuterated buffer solutions, the methyldiazonium cation is in equilibrium with diazomethane which has been identified by the appearance of $^1$H-$^2$H couplings when the reaction was monitored by $^1$H NMR (21, 22). The final step is the reaction of methyldiazonium with a nucleophile; in solution, this may be water or components of the buffer, whereas biological (and clinical) activity depends on transfer of the methyl group to DNA (see later). Overall, the fates in vivo of all the atoms making up the temozolomide molecule have now been charted (Figure 5): N-2 and N-3 are lost as a molecule of dinitrogen; the C-4 carbon bearing an oxygen atom is converted to CO$_2$; and N-1 and all the imidazole atoms are excreted as a molecule of 5-aminoimidazole-4-carboxamide (AIC), a natural constituent of urine. Conversion of the carboxamide group of temozolomide to a carboxylic acid residue, monitored by using drug labelled with $^{14}$C in the C-6 position, is a very minor metabolic pathway (23). The temozolomide molecule can therefore be considered as a drug-delivery device exquisitely adapted to deliver a methylating fragment to DNA.

The pH dependence of MTIC production and degradation has been evaluated (22). The half-life of temozolomide in phosphate buffer at pH 7.4 and 37°C is
1.24h (7). Temozolomide is robustly stable under acid conditions but the rate of degradation increases rapidly on passing through neutral to basic pH. For MTIC, the converse is true; it is relatively stable at basic pH but degrades rapidly in acid. Indeed, MTIC can actually be generated preparatively from the reaction of temozolomide with 10% aqueous sodium carbonate (7). Thus there is only a small pH window close to physiological pH at which the whole process from prodrug activation to methyl group transfer can occur. This pH stability profile has a number of consequences for temozolomide as a drug; most importantly, the acid stability means that it survives the strong acid of the stomach so it can be administered orally in capsules. This is unusual since base-catalysed reactions usually have an acid-catalysed counterpart; a $^{15}$N NMR study has shown that in strong acid, the first site of protonation of temozolomide is at N-7 rather than O-4 which would be required to initiate an acid-catalysed ring opening (14). Differential tissue pH may also be one of several factors contributing to the activity of temozolomide against brain tumours. Brain tumours have a more alkaline pH than surrounding healthy tissue; a situation likely to favour activation of the prodrug in the tumour tissue (24).

**Reaction with DNA**

There has been much speculation about the direct interaction of intact temozolomide with DNA (22, 25, 26). Does a temozolomide-binding site exist and do specific DNA sequences influence the ring-opening reaction? The sequence selectivity of reaction of temozolomide with plasmid DNA has been determined by two methods. When DNA is exposed to alkylating agents, N-7 alkylation generates an alkali labile site. Piperidine cleavage of plasmid DNA following exposure to temozolomide was used to identify sites of guanine N-7 alkylation. A more sophisticated assay for DNA modification is the polymerase stop assay. This uses alkylated DNA as the template for polymerase chain reaction (PCR) extension of a primer and thus measures the ability of temozolomide alkylations to block the progress of the polymerase. The assay can identify sites of alkylation other than guanine N-7. There was notable agreement between the results of these two assays (27), with both showing a concentration of methylation at the middle guanines of runs of three or more.
consecutive guanines. This correlation also implies that the major lesion produced by this drug is N-7 methylation. This sequence selectivity is shared by a variety of low molecular weight alkylating agents of disparate chemical structure so, it could be argued, is more a property of DNA itself rather than of the drugs.

The alkylation of runs of guanines (G) can be rationalized in terms of electronic and steric properties of DNA (22). The GGG sequence has been calculated to be the most electron-rich (basic) micro-environment on DNA. Runs of guanines induce localized distortion of DNA away from an idealized B-form, resulting in a wider major groove and greater steric accessibility of functional groups in the base of the groove; for example, guanine N-7. Thus, electronic (basicity) and steric effects combine to enhance the nucleophilicity of guanine N-7 in runs of three or more guanines.

To probe the possible existence of a temozolomide-binding site on DNA, a number of footprinting experiments have been executed. Temozolomide (molecular weight 194) and MTIC (168) are too small for non-covalent footprints on DNA to be detected. Pre-treatment of DNA with unreactive temozolomide isosteres failed to protect DNA from subsequent exposure to temozolomide (27). Both of these results indicate that any association the intact drug may have with DNA is weak and that a stable complex is not formed.

Since the ring-opening of temozolomide is base-catalysed and GGG sequences on DNA have been shown to provide a basic micro-environment, it has been speculated that DNA sequences may influence the rate of ring-opening of temozolomide, and effectively catalyse the prodrug to drug conversion. However, incubation of the related drug mitozolomide in the presence of either GC or AT oligonucleotides and DNA components failed to demonstrate any factors other than pH which altered the rate of drug degradation (27). These experiments suggest, controversially in the light of PET scanning in patients with brain tumours (see later), that the only part of the temozolomide molecule that actually encounters DNA is the methyldiazonium cation. However, structure-activity relationships in temozolomide analogues with identical ring-opening chemistry (11) point to a crucial role for the substituent at C-8, which implies some non-covalent interactions at this locus with DNA which help to target the drug to GGG sequences. Studies to define precisely which molecular species from amongst the cascade of reactive moieties generated from temozolomide actually encounters and reacts with GGG sequences in DNA are continuing.

**Pre-clinical evaluation and optimization of temozolomide activity**

*Cytotoxicity of temozolomide in vitro*

The antitumour activity of temozolomide is largely attributed to the methylation of DNA which, as described previously, is dependent upon formation of a reactive methyldiazonium cation. This species produces methyl adducts at the accessible nucleophilic atoms in DNA by an $S_N2$ reaction (22, 25). At least 70% of total DNA methylation by temozolomide occurs at $N'$-guanine, while 9.2 and
5% of adducts are formed at N^-adenine and O^-guanine, respectively (28). Since DNA-adduct formation is dependent upon the net difference between the rates of alkylation and repair, recent experimental initiatives have investigated the inhibition of DNA repair as a possible adjuvant to temozolomide treatment.

The cytotoxicity of temozolomide is affected by three DNA-repair activities in particular:

1) O^-alkylguanine-DNA alkyltransferase. Much evidence suggests that the cytotoxicity of temozolomide can be correlated with the formation of O^-methylguanine (29–31), despite the fact that this lesion accounts for only a small percentage of the total DNA adducts formed. Adducts produced at the O^-position of guanine are, however, considered particularly mutagenic (32) and cytotoxic (33).

Methyl adducts at O^-guanine in DNA are repaired by the cytoprotective DNA repair protein, O^-alkylguanine-DNA alkyltransferase (AGT, EC 2.1.1.63), which transfers the methyl group to an internal cysteine acceptor residue (34). This reaction is stoichiometric and results in an irreversible inactivation of AGT, requiring de novo protein synthesis to restore repair activity. Depletion of AGT by pre-treatment with a substrate analogue has therefore been investigated as a useful therapeutic strategy to circumvent AGT-mediated resistance to temozolomide (35). Although free O^-methylguanine was initially employed for this purpose (36), O^-benzylguanine (O^-BG) has subsequently been used since it is a much better substrate for AGT; a benzyl group being more readily displaced than a methyl in a bimolecular displacement reaction (37). Pre-treatment of Mer^+ human tumour cell lines (i.e. those with an AGT activity of >15 fmol/mg protein) with O^-BG in vitro can increase the cytotoxicity of temozolomide by up to 3.5-fold (30, 31). Since temozolomide activity is known to be schedule dependent, repeat dosing of temozolomide±O^-BG pre-treatment (at 24-h intervals for 5 days) has also been examined, and the potentiation of cytotoxicity (defined as the ratio between the temozolomide IC_{50} without and with inhibitor treatment) was found to increase linearly with each subsequent dosing (Figure 6) (31). Continuous 1 μM O^-BG treatment was found to be more efficacious than a 1 h intermittent 100 μM O^-BG pre-treatment on this schedule, emphasizing that the regeneration of ACT activity following inhibitor treatment may have a profound effect on temozolomide cytotoxicity. These results suggest that dosing of an AGT inhibitor and temozolomide may be clinically useful in the treatment of tumours with high AGT activity.

Pre-treatment of tumour cells with temozolomide has also been used as a method for depleting AGT activity, in order to circumvent AGT-mediated resistance to a chloroethylnitrosourea (CENU) (38, 39). However, appreciable enhancement of CENU cytotoxicity is only evident when a cytotoxic concentration of temozolomide is used. O^-alkylguanine-DNA alkyltransferase depletion with a non-toxic O^-alkylguanine is therefore likely to be of more benefit in vivo (39).

2) DNA-mismatch repair. O^-methylguanine produced in DNA by temozolomide is thought to be cytotoxic as a consequence of its processing by a DNA-mismatch repair pathway which normally corrects GT mismatch replication
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Figure 6. Potentiation of temozolomide cytotoxicity in Mawi (human colorectal carcinoma cell line) by repeat dosing with O\(^6\)-benzylguanine (O\(^6\)-BG) Mawi cells were seeded in 96-well plates 24 h before receiving between one and five treatments (one every 24 h) of temozolomide for 3 h, with/without 100 \(\mu\)M O\(^6\)-BG for 1 h (○) or 1 \(\mu\)M O\(^6\)-BG for 24 h (■) (commencing 1 h before alkylating treatment). Cytotoxicity was determined on Day 8 using the colorimetric sulphorhodamine-B assay for protein. Potentiation was defined as the relative increase in cytotoxicity afforded by a particular O\(^6\)-BG treatment (determined by a comparison of IC\(_{50}\) values). Each data point represents the mean potentiation ± S.E. from three independent experiments. Adapted from Wedge et al. (31) with permission.

errors. This pathway involves mismatch binding by a heterodimer consisting of hMSH2 (40) and GTBP/p160 proteins (41, 42), and DNA incision following recruitment of an additional heterodimer consisting of hPMS2 and hMLH1 proteins (43). A section of DNA is then removed between the incision and the mismatch, and replaced by resynthesis and ligation. When this pathway is targeted to the strand directly opposite O\(^6\)-methylguanine, its futile attempt to find a complementary base results in continued excision/insertion which produces long-lived nicks in the DNA (44). The resulting interruptions in the
daughter strands are inhibitory to replication in the subsequent S-phase (45, 46), and may account for two cell divisions being required before the emergence of temozolomide cytotoxicity (35).

Since the cytotoxicity of temozolomide is dependent upon a functional DNA-mismatch repair pathway, resistance may be conferred by a mutation in any one of the genes encoding for a protein involved in mismatch recognition/incision. These mutations commonly occur as germline mutations in hereditary non-polyposis colorectal cancer (47), and in a number of sporadic colon tumours (48). Such abnormalities result in a temozolomide ‘tolerant’ phenotype which is unaffected by AGT activity. Treatment of mismatch-repair-deficient tumour cells with O6-BG cannot, therefore, restore sensitivity to temozolomide (49, 50).

(3) Base excision repair and poly(ADP-ribose) polymerase. Methyl adducts produced at N⁶-guanine and N⁰-adenine by temozolomide could also hinder DNA-replication, as enzymatic or spontaneous depurination will ultimately result in DNA strand breakage (51, 52). Evidence that these lesions may not be crucial to temozolomide cytotoxicity, stems from the observation that the expression of N⁶-methylguanine-DNA and N⁰-methyladenine-DNA glycosylases (which excise the respective methylated purine base) does not correlate with temozolomide resistance (35, 53, 54). Nevertheless, it may be possible to potentiate the cytotoxic contribution of these adducts via the inhibition of poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30); an enzyme activated by temozolomide treatment (55). It is suggested that PARP has a role in DNA repair since it binds tightly to DNA-strand breaks, possibly to prevent spurious transcription or recombination, but is released following auto-poly(ADP-ribosyl)ation, thereby allowing DNA-repair enzymes access to the lesion (56). Inhibitors of the NAD⁺ binding domain of PARP prevent the synthesis of ADP-ribose polymers (57), and significantly potentiate the cytotoxicity of temozolomide (49, 58). However, PARP inhibitors do not always produce a proportional effect on temozolomide cytotoxicity and DNA-strand breakage (58), which suggests that the enhancement observed could be related to the inhibition of ADP-(ribosyl)ation of acceptor proteins, such as those involved in the regulation of apoptosis or cell cycle progression in response to DNA damage (59, 60). Whatever the mechanism of enhancement, the combination of PARP inhibitor (3-aminobenzamide) with an AGT inhibitor (O⁶-BG) has been found to potentiate the cytotoxicity of temozolomide (49), and may have clinical application.

In addition to being inhibitory to replication, DNA methylation by temozolomide may also perturb other DNA-dependent processes, such as the regulation of gene expression. A series of studies by Tisdale (61–63) indicated that temozolomide could induce differentiation in the K562 human erythroleukaemia cell line. This phenomenon was attributed to a drug-induced hypomethylation of DNA, which occurs because the enzymatic methylation of cytosine in DNA is prevented by the presence of adjacent O⁶-methylguanine residues (64), although it is uncertain as to whether these adducts contribute significantly towards cytotoxicity/antitumour activity. It has also been suggested that formation of a carbamoylating isocyanate from temozolomide, may result
Figure 7. Temozolomide biodistribution. Female Balb/C mice were implanted with PC66 plasmocytoma, and temozolomide was administered i.p. at 20 mg/kg. Temozolomide was extracted from acidified plasma and tissue homogenates by ethylacetate using ethazolastone as an internal standard, then measured by high-performance liquid chromatography (8).

in the inhibition of certain enzyme activities, such as the inhibition of cellular esterase activity observed in EMT6 mouse mammary tumour cells (66).

Antitumour activity of temozolomide in vivo

Early pre-clinical experimentation with temozolomide indicated that it possessed good antitumour activity when administered intraperitoneally (i.p.) against both haematological (L1210 and P388 leukaemias) and solid (e.g. M5076 sarcoma, ADJ/PC6A plasmacytoma, B16 melanoma, Lewis lung carcinoma) murine tumour models (7). This activity was found to be highly schedule dependent, with multiple administration being more effective than a single bolus dose. Like mitozolomide, temozolomide demonstrated good tissue distribution, including penetration across the blood-brain barrier (67) (Antoniw and Newlands, unpubl. obs., Figure 7), and was found to have a bioavailability of
0.98 when administered orally. Antitumour activity was therefore maintained when temozolomide was administered orally to L1210-bearing mice (7).

The plasma pharmacokinetics of temozolomide following oral administration to mice were characterized by a rapid absorption phase (the peak plasma concentration of temozolomide being achieved within 30 min of administration), and mono-exponential elimination (with an elimination half-life of 1.29 h) (7). The predominant route of temozolomide elimination is by renal excretion and occurs largely as intact drug, although an unidentified acidic urinary metabolite is also produced (23). Incubation studies with liver microsomal preparations in vitro suggest that there is no appreciable hepatic metabolism of temozolomide in mice (23). These observations were found to correlate with the subsequent findings of Phase I clinical studies, which indicated that rapid absorption of temozolomide also occurred in man (the peak plasma concentration occurred within 0.7 h of oral dosing), and drug elimination could be best described by a one-compartment model with a half-life of 1.81 h (8). However, in addition to the unresolved metabolite, patient urine was also found to contain the carboxylic acid metabolite of temozolomide, 3-methyl-3,4-dihydro-4-oxoimidazo[5,1-d]tetrazine-8-carboxylic acid, which has equivalent cytotoxicity to temozolomide and is thought to be generated extrahepatically (23).

Temozolomide antitumour activity has been demonstrated in a panel of human melanoma, ovarian and colon tumour xenografts (68). Recent experiments have also indicated excellent antitumour activity against a number of subcutaneous and intracerebral human brain tumour xenografts (69, 70). Of particular interest is the finding that when temozolomide is administered daily for 5 days, it exhibits activity against Mer+ central nervous system xenografts resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (70), which is possibly the single most effective compound for the clinical treatment of these malignancies (71). Strong evidence of synergy between temozolomide and BCNU has also been demonstrated in these xenograft models, and the sequence of administration has been found to influence host toxicity; administration of temozolomide 2 h after BCNU being more tolerable and effective than if administered 2 h before (69).

Three studies have examined single-dose administration of a combination of AGT inhibitor and temozolomide in human brain tumour xenograft models. Temozolomide antitumour activity was found to be unaffected by 30 mg/kg O6-BG (i.p., 1 h pre-treatment) in a glioblastoma xenograft with relatively little AGT activity (<12 fmol/mg protein) (69), but moderately enhanced in a medulloblastoma xenograft with an AGT activity of between 64 and 124 fmol/mg protein (69). In contrast, a recent study described significant enhancement of temozolomide activity in a glioblastoma model by administration of 40 mg/kg O6-BG (i.p., 1 h pre-treatment), resulting in a tumour growth delay equivalent to that produced by a 3.5-fold greater dose of temozolomide alone (72). Surprisingly, the AGT activity of this xenograft was extremely low (<6 fmol/mg protein) and may implicate the involvement of a pharmacokinetic interaction between temozolomide and O6-BG.

A comparative study of both single and multiple dosing of temozolomide with O6-BG has also been performed in a human malignant melanoma xenograft with an AGT activity of 95 fmol/mg protein (73). O6-BG pre-treatment (35 mg/
kg) did not significantly affect the tumour growth delay induced by treatment with a single dose of 200 mg/kg temozolomide, while daily $O^6$-BG pre-treatment (Days 1–5) considerably enhanced the antitumour activity of the same total dose of temozolomide divided into five equal fractions (40 mg/kg) and administered on five consecutive days (Figure 8). This effect is undoubtedly related to a progressive and persistant depletion of AGT activity which enables a greater net retention of $O^6$-methylguanine adducts in DNA. The study also found that a combination of $O^6$-BG and temozolomide produced a greater antitumour effect than an equitoxic dose of temozolomide alone on a repeat
dosing (i.e. daily \(\times 5\)) schedule, indicating that DNA-repair inhibition may afford an increase in the therapeutic index of temozolomide. However, these results may be partly compromised by interspecies variation in the sensitivity of AGT to inactivation with O\(\beta\)-BG; murine tissues being approximately 5–10-fold more resistant to such inactivation than human tissues (74, 75).

No reports have yet documented the effect of combining a PARP inhibitor with temozolomide in vivo. This has been hindered by the requirement for more soluble and potent inhibitors than the benzamides, which have only been synthesized recently (58).

Additional in vivo pre-clinical experimentation has addressed the possibility of combining temozolomide with the DNA topoisomerase I inhibitor topotecan (9-dimethylaminomethyl-10-hydroxycamptothecin). These two compounds demonstrate synergistic antitumour activity against the human SF-295 glioma xenograft, with no appreciable increase in toxicities (76). Temozolomide has also been shown to possess antimitastatic activity, reducing the metastatic potential of Lewis lung carcinoma in mice (77). This effect is attributed to the inhibition of protein kinase C by temozolomide, which prevents phosphorylation of the tumour cell adhesion factor-6 integrin; an important event in the metastatic progression of these tumour cells (77).

Relevance of pre-clinical studies

Pre-clinical studies have undoubtedly made a significant contribution to the clinical development of temozolomide in identifying its schedule-dependent activity, its ability to penetrate central nervous system tissues, and its excellent oral bioavailability. An examination of how DNA-repair processes may compromise temozolomide cytotoxicity has now introduced the possibility of using DNA-repair inhibitors as a therapeutic adjuvant. Whether these approaches will circumvent clinical resistance to temozolomide and extend its usage to the treatment of a wider range of malignancies remains to be seen. The success of such therapy requires an increase in the therapeutic index of temozolomide (i.e. the activity/toxicity ratio) and will therefore be dependent upon the relative augmentation of toxicity in tumour compared with normal tissues. Preferential inhibition of PARP activity in tumour tissue is at least conceivable, given that low PARP expression has been suggested to be a contributory factor to the development of genetic instability and malignant progression (78). Conversely, the degree of sensitization to a DNA-alkylating agent following treatment with an AGT inhibitor has been shown to be proportional to AGT activity (79, 80), and so greater potentiation of antitumour activity would be anticipated in high AGT-expressing tumours, rather than tissues with low AGT activity, such as human bone marrow (81). Nevertheless, enhanced temozolomide cytotoxicity has been demonstrated in vitro following treatment of human primary bone marrow granulocyte/macrophage precursor cells with O\(\beta\)-BG, which suggests that AGT depletion will exacerbate temozolomide-related myelosuppression (82). In addition, AGT depletion may also increase the carcinogenicity/mutagenicity of temozolomide, and thereby increase the possibility of inducing a secondary malignancy (83). However, these risks are easily outweighed by the potential to extend patient survival.
(particularly those with high-grade glioma), suggesting that the combination of an AGT inhibitor and temozolomide should be examined clinically.

Further pre-clinical experimentation with temozolomide should involve an evaluation of the interaction with other antitumour agents known to perturb DNA metabolism, in an attempt to identify possible synergistic combinations. The effect of combining X-irradiation with temozolomide treatment is also being evaluated, since the combination of chemotherapy with loco-regional radiotherapy is clearly an attractive proposition for the treatment of glioma (84).

Clinical development of temozolomide

Phase I trial of temozolomide

Temozolomide is the second of a series of imidazotetrazine derivatives developed by Stevens et al. (3) to enter the clinic. The lead compound mitozolomide completed its Phase I trial in 1985 (5) and several Phase II trials of mitozolomide were performed in small cell carcinoma of the lung and malignant melanoma. The further development of mitozolomide was precluded owing to the severe and unpredictable myelosuppression that occurred (85–91).

Since the imidazotetrazine derivatives exhibited such broad-spectrum antitumour activity in murine tumours and human tumour xenografts, temozolomide was selected for further clinical development as it had much lower toxicity in the pre-clinical screen (7).

In the pre-clinical toxicology, the formulation of temozolomide was dissolved in DMSO, and a toxic dose of temozolomide could not be obtained because, at 420 mg/m², the quantity of DMSO itself became toxic. A further important difference between mitozolomide and temozolomide was that temozolomide activity was highly schedule dependent in the pre-clinical screen (7).

The Phase I trial of temozolomide was conducted under the auspices of the Cancer Research Campaign Phase I/II Subcommittee. The starting dose of temozolomide was 50 mg/m², initially given intravenously as a 1-h infusion (8). The pharmacology of temozolomide was examined following both intravenous and oral administration. Good oral bio-availability was confirmed at a dose of 200 mg/m². All subsequent clinical studies have been with temozolomide given orally. The doses of temozolomide were escalated from 50 mg/m² up to 1200 mg/m², when leukopenia and thrombocytopenia became dose limiting on the single-dose schedule. The increase in area under curve (AUC) of temozolomide was linear with dose. No activity was seen in the 51 patients who received temozolomide as a single dose with a 1-h infusion.

In view of the schedule dependency in the pre-clinical screen, temozolomide was given five times daily at doses of 750, 1000 and 1200 mg/m² in 42 patients. Again, myelosuppression was dose limiting at 1200 mg/m². A well-tolerated schedule for clinical use was identified giving temozolomide 150 mg/m² orally for five consecutive days (total dose 750 mg/m² for the first course), and if no myelosuppression was detected on Day 22 of the 4-week cycle, subsequent courses were given at 200 mg/m² for 5 days (total dose 1 g/m² on a 4-week
cycle). Approximately one in 20 patients’ bone marrow is sensitive to temozolomide, and these patients need to continue at the starting dose of 750 mg/m² for subsequent courses. The toxicity of this schedule includes nausea and vomiting which can usually be controlled by 5HT₁-antagonists such as ondansetron and granisetron. The myelosuppression is predictable with the nadir on Day 22. Little cumulative toxicity has been seen using this schedule, and patients have tolerated this 4-weekly cycle of chemotherapy for up to 3 years. In contrast to the lack of clinical activity seen with the single-dose schedule, activity in the Phase I trial on the 5-day schedule was seen in four (two complete responses, two partial responses; 17%) of 23 clinic patients with melanoma, and one patient with mycosis fungoides (complete response lasting 7 months). Two patients with recurrent high-grade gliomas also had evidence of clinical activity. Subsequent clinical studies so far have concentrated on confirming the activity in melanoma and high-grade gliomas.

**Phase II trials of temozolomide**

The Cancer Research Campaign sponsored a multi-centre Phase II trial of temozolomide in metastatic melanoma (92). Patient eligibility for this study involved the usual criteria of advanced malignant melanoma with measurable or assessable disease which had progressed prior to entering the study. The schedule used was as defined in the Phase I trial with the first course being 750 mg/m² split over 5 days, and if no myelosuppression occurred on Day 22, subsequent courses were given at 1000 mg/m² split over 5 days on a 4-day week cycle. Sixty patients were entered in this study, 55 of these were eligible for toxicity and 49 were eligible for response. All responses were reviewed independently; a complete response was seen in three patients (all with lung metastases) and a partial response was seen in nine patients (21% CR + PR rate) (Table 1). The toxicities encountered were mainly haematological, and the schedule was well tolerated in this multi-centre study. The median survival for all patients was 5.5 months (range 0.5–29.5), and for the responders, the median survival duration was 14.5 months with four patients still alive at the time of writing this review (92). This study confirmed that temozolomide had significant activity in patients with metastatic melanoma, was well tolerated and had comparable activity to other agents that have been used in this disease. Clearly,

<table>
<thead>
<tr>
<th>Number (%)</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Complete response</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Partial response</td>
<td>9 (16)</td>
</tr>
<tr>
<td>No change</td>
<td>8 (14)</td>
</tr>
<tr>
<td>Not assessible (early death)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>29 (52)</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
</tr>
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</table>
combinations of temozolomide with other cytotoxic agents need to be assessed in future studies in patients with metastatic melanoma.

The Cancer Research Campaign also sponsored a Phase II trial of temozolomide in low-grade non-Hodgkin's lymphoma (93). This study was limited to 18 patients, all of whom had received prior chemotherapy. The number of prior chemotherapy schedules that patients had previously received ranged from one to seven, with a median of three. Only one of these 18 patients responded to temozolomide, and the conclusion of this study was that temozolomide had little activity in previously treated, low-grade non-Hodgkin's lymphomas. In view of the complete response identified in the Phase I trial in one patient with mycosis fungoides, further trials with different sub-groups of lymphomas are probably warranted with temozolomide. Consideration should be given to repeating a similar trial to the one described above in patients with low-grade non-Hodgkin's lymphoma who have not received prior chemotherapy.

The activity of temozolomide in primary brain tumours has been of particular interest in view of the very limited activity of other cytotoxic agents in this group of diseases. The present authors concentrated their studies mainly on patients with high-grade (Grades III and IV) astrocytomas. The present authors reported the original cohort of 28 patients in 1993 (94) using the same schedule identified in the Phase I trial with course one at a dose of 750 mg/m² given orally, split over 5 days (150 mg/m²/day), and if no myelosuppression was seen on Day 22, the dose was escalated to 1000 mg/m² split over 5 days (200 mg/m²/day), given on a 4-week cycle. Given the difficulty of defining endpoints on computerized tomography (CT) and magnetic resonance imaging (MRI) scans, these results were reported as showing a major improvement on CT scan in five of 10 patients with astrocytomas recurrent after radiotherapy, and a reduction in CT lesions in four of seven patients with newly diagnosed astrocytomas given temozolomide for two courses prior to irradiation. In addition, one child who had recurrent medulloblastoma with bone metastases also had a clinical response in terms of relief of bone pain, and one patient with recurrent ependymoma after surgery and radiotherapy had a major tumour reduction and continued on temozolomide for a total of 24 courses, and has now been off all treatment without recurrent tumour for 2 years.

The Charing Cross Hospital experience using temozolomide in patients with astrocytoma has recently been updated (95). From the original Phase I trial through to 1995, 75 consecutive patients with astrocytomas were treated. These patients form two groups; 48 patients with recurrent disease following radiotherapy, and 27 patients treated after their initial surgery and prior to their cranial irradiation. The mean age of these patients is 46.6 years, and 48 (64%) were male and 27 (36%) were female. The grade of the tumours were Grade II, one (1%); III, 14 (19%); IV, 58 (78%); and mixed III/IV; two (3%) patients. The prior treatment these patients had received included radiotherapy in 42 (56%) patients; radiotherapy and chemotherapy in five (7%) patients; and no treatment other than surgery in 28 (37%) patients. Temozolomide was given in the same schedules throughout as defined in the recommendation from the Phase I trial (8). The clinical response was assessed using the Medical Research Council (MRC) scale of neurological status (see Table 2), and CT or MRI scans were reported to have shown minor or major improvements. Since the endpoints
Table 2. Assessment of response by both computerized tomography (CT) and magnetic resonance imaging (MRI) scanning and Medical Research Council (MRC) grade*

<table>
<thead>
<tr>
<th></th>
<th>Patients treated prior to radiotherapy (%)</th>
<th>Patients with recurrent disease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive disease</td>
<td>2 (7)</td>
<td>14 (29)</td>
</tr>
<tr>
<td>No charge</td>
<td>13 (48)</td>
<td>18 (38)</td>
</tr>
<tr>
<td>Objective response</td>
<td>8 (29)</td>
<td>12 (25)</td>
</tr>
<tr>
<td>Not assessable</td>
<td>4 (15)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Early deaths</td>
<td>—</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

*MRC scale of neurological status: 0, no neurological deficit; 1, function adequate for useful work; 2, moderate functional impairment; 3, neurological deficit causing major functional impairment; and 4, no useful function.

†Assessed at maximum neurological and CT or MRI scan improvement, i.e. usually 2 or 5 months after starting temozolomide.

are indistinct in the majority of patients, using the normal criteria of partial and complete response is rarely possible except by over-interpreting the data on the scans. The combination of clinical improvement and a clearcut reduction in tumour mass on CT or MRI scanning has been classified as an objective response. In the authors' experience, there is a close correlation in virtually all patients between clinical and neurological assessment of the patient and subsequent changes in the scan. In this series, there was only one patient who showed a reduction in tumour mass effect on the CT scan who did not get clinical benefit. Using this criterion of objective response, in the 27 patients with primary disease treated with two courses of temozolomide prior to radiotherapy, eight (29%) patients fulfilled the criteria for an objective response. In the 48 patients who were treated with temozolomide after recurrence following their radiotherapy, 12 (25%) patients fulfilled the criteria for an objective response. This gave an overall objective response rate of 20 (27%) of 75 patients (Table 1).

The conclusions of the present study were: (1) temozolomide clearly has activity against high-grade gliomas both prior to radiotherapy and on relapse after radiotherapy; (2) the responses induced with this 5-day schedule of administration of temozolomide are usually of short duration and have relatively little impact on overall survival; and (3) studies evaluating chemotherapy in primary brain tumours should include a 'quality-of-life assessment and performance data' in addition to CT scanning or MRI scanning assessment. Although in this study, temozolomide had little effect on overall survival, patients who had an objective response clearly benefitted in terms of mental and physical performance during the period of response.

A number of ongoing Phase II trials sponsored by Schering-Plough Corporation, who have taken over the development of temozolomide, are currently accruing patients and, also, a number of additional Phase II trials are in the planning stage. Trials in high-grade gliomas are currently accruing patients rapidly, as are the trials for malignant melanoma. Future trials that are planned include nasopharyngeal carcinoma, renal cell carcinoma, pancreatic carcinoma and sarcomas. All these studies are planned with the schedule of
administering temozolomide orally for five consecutive days on a 4-week cycle giving a total dose of 1000 mg/m² per course.

Additional Phase I trials

Temozolomide given continuously over 6 or 7 weeks orally. At the end of the Phase I trial (8), a group of patients were treated with temozolomide given continuously to identify whether or not this was potentially superior to the 5-day administration of temozolomide, since temozolomide is such a schedule-dependent drug. Hints of activity giving temozolomide continuously were seen in patients relapsing after the 5-day schedule. A Phase I trial has recently been completed with the starting dose of temozolomide given at 50 mg/m²/day orally for 6 and 7 weeks. Doses were escalated to 75 mg/m²/day, then 100 mg/m²/day. At 100 mg/m²/day, Grade IV myelosuppression was identified and the dose was subsequently reduced to 85 mg/m²/day. Again, myelosuppression was identified at this dose. Ten patients have now completed administration of temozolomide at 75 mg/m²/day for 6 or 7 weeks and, following a break, a second course was administered to those patients with stable disease or evidence of tumour response. The recommended dose for further studies using continuous administration of temozolomide is currently 75 mg/m²/day (96). At this dose, more temozolomide can be administered per unit time than on the 5-day schedule. Temozolomide given at 75 mg/m²/day continuously is the equivalent of 2.1 g/m²/4 weeks which is twice the dose that can be administered when temozolomide is given on the Days 1-5 schedule. The pharmacology also confirms that when temozolomide is given continuously, the AUC per 4 weeks is 2.3 (1.6–3.8)-fold [mean (range)] greater on the continuous schedule than on the 5-day schedule. Activity in this continuous schedule was seen both in melanoma and, in particular, in patients with astrocytomas. However, the data suggest that temozolomide was even more active when given in the continuous schedule than when given in the 5-day schedule in patients with astrocytomas (Figure 9). This data needs to be confirmed in future studies.

Current plans with the continuous temozolomide schedule are to develop it in combination with cranial irradiation administered over 7 weeks. A series of pilot trials are planned assessing response by: (1) quality-of-life inventories (97): (2) ¹⁸F-fluoro-2-deoxyglucose (¹⁸F-FDG) scanning (98); and (3) precisely registered MRI scans (99,100).

Temozolomide given twice a day orally. Temozolomide is known to deplete the DNA-repair protein O⁶-alkylguanine-DNA alkyltransferase. A Phase I trial giving a dose of 200 mg/m² of temozolomide followed by 9 12-hourly doses of 50, 75 and 100 mg/m² over a period of 5 days has been conducted by Gerson et al. in 1996 (101). This trial confirms that on this schedule, temozolomide rapidly depletes alkyl-transferase and it may be that a twice-daily schedule may be superior to the single dose/day and should be studied further.

Paediatric Phase I trial. A Phase I trial in paediatric patients has been completed (102). In patients who had received prior nitrosourea therapy, the dose-limiting toxicity was myelosuppression at 600 mg/m² (split over 5 days on a 4-week
Magnetic resonance imaging scans of a 33-year-old female presenting 5 years previously with right frontal low-grade astrocytoma. Treated initially by surgery then radiotherapy 3 years later. Upper scans at start of temozolomide (dose 75 mg/m²/day for 7 weeks) when patient was in a wheelchair, had a left hemiparesis and could not complete a sentence. Lower scans after two courses of temozolomide of 7 weeks each (with an interval of 6 weeks between courses). Patient is now fully mobile with fluent speech and continues on the 5-day/4-week schedule of temozolomide.
cycle). In patients who had not received prior nitrosourea therapy, the dose-limiting toxicity was, like the adult Phase I trial, at 1200 mg/m² (split over 5 days). Activity was seen in patients with both high-grade supratentorial astrocytoma and brain stem gliomas. Clearly, further evaluation of temozolomide is indicated in paediatric tumours and, in particular, in primary brain tumours.

**Positron emission tomography.** Positron emission tomography scanning is becoming an integral part in the early clinical development of new anticancer agents. Positron emission tomography has been used to assess changes in tumour metabolism using ¹⁸F-FDG. ¹⁸F-FDG follows the same metabolic pathway as that of glucose; it is phosphorylated to ¹⁸F-fluoro-deoxyglucose-6-phosphate (²⁻⁸F-FDG-6-P) and is trapped in this form at a rate proportional to glucose utilization.

During the clinical development of temozolomide, paired ¹⁸F-FDG-PET scans were performed in patients prior to, and 7–14 days after, a 5-day course of oral temozolomide. A reduction in ¹⁸F-FDG uptake of -30, -30 and -29% was seen in patients who subsequently responded clinically and radiologically. In contrast, patients with progressive disease had increased glucose utilization rates (¹⁸F-FDG uptake of +25, +25 and +20%). In those patients with stable disease, the change in ¹⁸F-FDG uptake was -2 and -11% (103). Thus, ¹⁸F-FDG uptake has the potential of being an early surrogate marker and, perhaps, a predictor of response (103).

It is planned to serially perform ¹⁸F-FDG-PET studies in cohorts of patients receiving treatment with radiotherapy alone, temozolomide administered over 7 weeks and the combination of temozolomide and radiotherapy. Thus, the PET studies will parallel the trial providing an early surrogate assessment of response and also, possibly, survival.

Positron emission tomography can also provide quantitative normal tissue and tumour pharmacokinetic data in man. Temozolomide has been radiolabelled with a positron emitting ¹¹C in the methyl group (16), and tracer amounts (50 µg) injected into patients with dynamic PET scan studies being performed over 90 min (Figure 10). By sampling normal tissue and tumour regions, time-activity curves of mean radiotracer concentration against mid-frame time can be generated. Using tracer-PET studies, it has been demonstrated that the kinetic behaviour of all extra-cranial organs sampled (including liver) is virtually identical, and that the tumour area under the curve for ¹¹C-temozolomide is significantly higher statistically (p<0.001 using both parametric and non-parametric tests) than in normal brain tissue (10 patients) (104).

**Future prospects**

Temozolomide is a well-tolerated orally bio-available DNA-methylating agent. It has significant activity in metastatic malignant melanoma, in patients with primary brain tumours and, in particular, in patients with high-grade astrocytomas. The spectrum of activity of temozolomide in human malignancy needs to be evaluated in further Phase II trials initially using the 5-day schedule repeated on a 4-week cycle that was identified in the Phase I trial. Given the
preliminary results obtained by continuous administration, other schedules of administration should also be explored. The combination of temozolomide with other cytotoxic drugs needs to be evaluated. The potential efficacy of temozolomide and radiotherapy combinations needs to be fully evaluated and is an important area for future research, particularly in primary brain tumours. The ability of tumour cells to repair the DNA methylation induced by temozolomide (by pathways such as alkyl-transferase) may be circumvented using DNA-repair inhibitors. In particular, an alkyl-transferase inhibitor, O²-BG, which has already been tested in the clinic (105, 106), should be evaluated in combination with temozolomide. The potentiation of temozolomide's activity with this and other DNA-repair inhibitors is an exciting future area to explore experimentally and in the clinic.

Acknowledgements

The work reported in this review by the authors was supported by the Cancer Research Campaign, U.K., and by Schering-Plough Research Institute, Kenilworth, NJ, U.S.A. The authors would like to thank Dr R. C. Moschel for the supply of O²-BG and Dr G. P. Margison for the tritiated DNA substrate for AGT assay. The authors would also like to thank Dr J. Catino (Schering-Plough Research Institute) for supplying temozolomide, and Dr P. Karran (Imperial
Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts, U.K.) for informative discussions concerning mismatch binding mutations.

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