

Preclinical bioavailability–bioequivalence and toxico-kinetic profile of stable succinic acid cocrystal of temozolomide**

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Temozolomide (TMZ) is a frontline prodrug for the treatment of glioblastoma multiforme (brain cancer) approved by the US-FDA in 1999. A limitation with this otherwise potent and selective DNA alkylating agent is degradation of the prodrug to the inactive product 5-aminoimidazole-4-carboxamide (AIC) by incipient hydrolysis during storage. This transformation not only makes the drug less effective (due to hydrolysis of the active drug), but also causes discoloration from white to pink and tan brown colour (due to AIC contamination), which can make patients anxious and suspicious about the drug efficacy. We solved the stability issue of TMZ by preparing its succinic acid (SA) cocrystal and showed that TMZ-SA is stable for over 6 months in accelerated stability conditions of 40°C, 75% RH, as confirmed by PXRD. TMZ-SA retained its white colour for over 6 months compared to the tan-brown discoloration for TMZ in less than 1 month. Based on the enhanced stability and comparable dissolution profile of TMZ-SA, we now take forward TMZ-SA pharmaceutical cocrystal to stability

assessment by HPLC analysis and preclinical bioavailability and bioequivalence with the reference drug. We observed that there were no significant differences in the pharmacokinetic profile of the test cocrystal TMZ-SA compared to standard drug TMZ in Sprague Dawley rats. The bioavailability ratio was found to be in the range 102–109%. Pharmacokinetic parameters such as T_{max} , C_{max} , $T_{1/2}$, AUC and K_e are slightly superior for TMZ-SA and the toxico-kinetic profile is also better than TMZ. Most significantly, it has been shown that the active drug is released from the hydrogen-bonded cocrystal and was detected in the brain tissue of rats. Thus we report on a pharmaceutical cocrystal complying with the United States Food and Drug Administration guidance on pharmaceutical cocrystals. These results suggest that TMZ-SA cocrystal with improved physico-chemical properties is bioequivalent and has superior stability compared to the reference drug TMZ and a potential lead for an improved TMZ formulation.

Keywords: Bioavailability, bioequivalence, cocrystal, glioma, toxico-kinetic profile.

MALIGNANT glioma (glioblastoma multiforme and anaplastic astrocytoma) is an aggressive tumour which is difficult to treat and is associated with high morbidity and mortality¹. In adults, glioblastoma multiforme (WHO grade-IV) and anaplastic astrocytoma (WHO grade-III) are the most common types of malignant primary brain tumours with an annual incidence of around six cases per 100,000 and comprise approximately 70% of malignant brain tumours^{2,3}. Brain cancer treatment outcomes, even

with multiple therapies, including surgery, radiation and chemotherapy, are still dismal with median survival chances of less than one year¹. Various clinical trials between 1970 and 1990 suggested the use of radiotherapy, but the existing chemotherapies were of limited use. There is a small increase in survival between radiotherapy along with chemotherapy (46% at 1 year, 20% at 2 years) versus radiotherapy alone (40% and 15% respectively)⁴. Some well-known chemotherapeutic agents are heterocyclic drug molecules (Figure 1) such as mitozolomide, dacarbazine and temozolomide (TMZ)⁵. The biochemically active species in all these drugs is 5-(3-monomethyl-1-triazeno) imidazole-4-carboxamide (MTIC, Scheme 1). Mitozolomide was successful in rats, but produced toxic side effects in human phase I trials. Dacarbazine requires hepatic metabolism to generate the active intermediates species. For these reasons, both mitozolomide and dacarbazine have certain demerit whereas temozolomide

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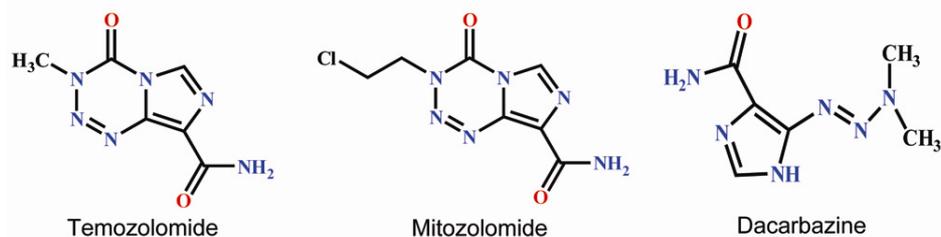
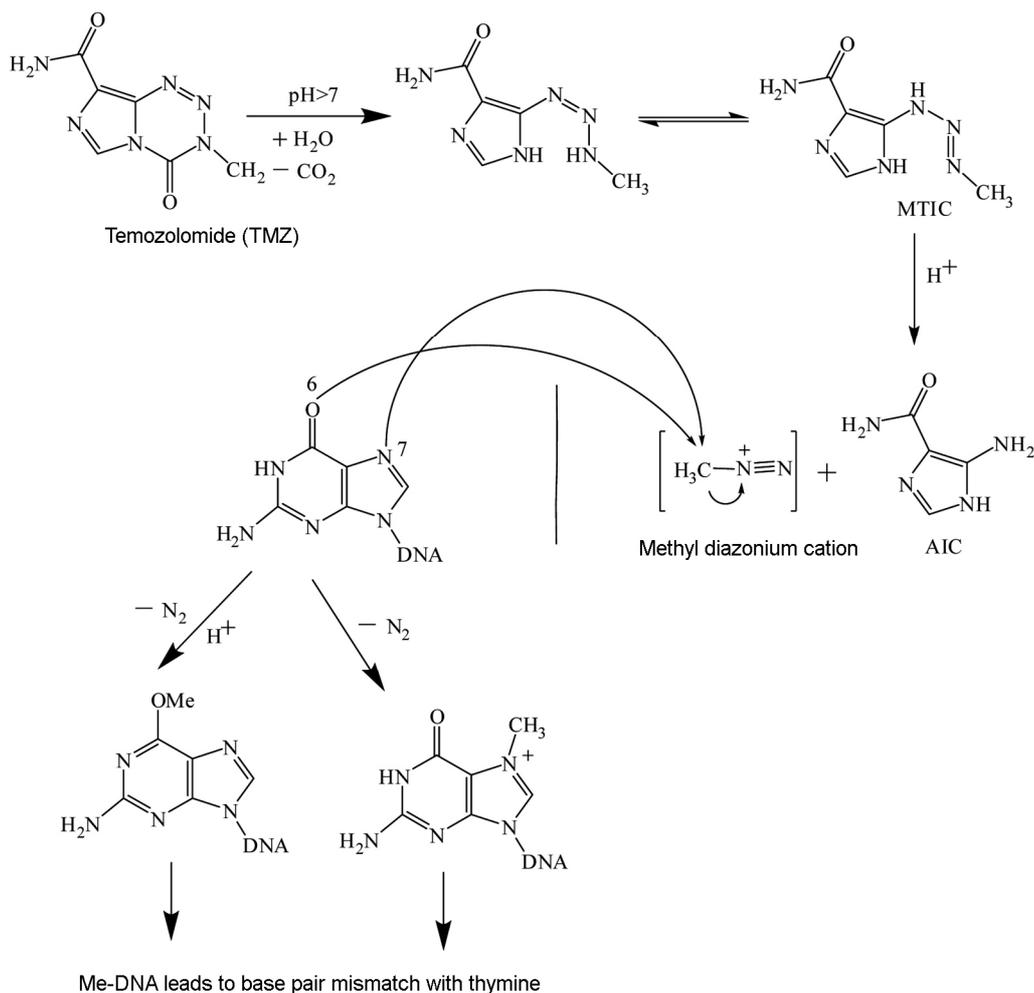


Figure 1. Structure of temozolomide, mitozolomide and dacarbazine.



Scheme 1. Schematic representation of DNA methylation by the prodrug temozolomide.

spontaneously hydrolyses at physiological pH to the active species MTIC with almost 100% bioavailability.

TMZ is an oral alkylating agent, a prodrug for MTIC, whose anticancer activity was first described in 1987 (ref. 6). TMZ spontaneously hydrolyses above pH 7 to MTIC, which breaks down to form the by-product 5-aminoimidazole-4-carboxamide (AIC) and the highly reactive methyl diazonium cation (CH_3N_2^+); the latter species is the nascent alkylating agent for DNA. The most nucleophilic centres for methylation of nucleotides are

O6 (5% of adducts) and N7 of guanine (70% of adducts), N1 and N3 of adenine, and N3 of cytosine (25% of adducts). Although O6 methylation is a minor site for DNA alkylation, it causes maximum cell apoptosis and death. The mechanism of activation of TMZ involves hydrolytic cleavage of the tetrazinone ring at neutral-to-basic pH conditions and release of the methylating agent (Scheme 1), which binds in the major groove of DNA^{7,8}. Temozolomide is an effective antitumour agent when a large population of cancer cells is actively replicating⁷.

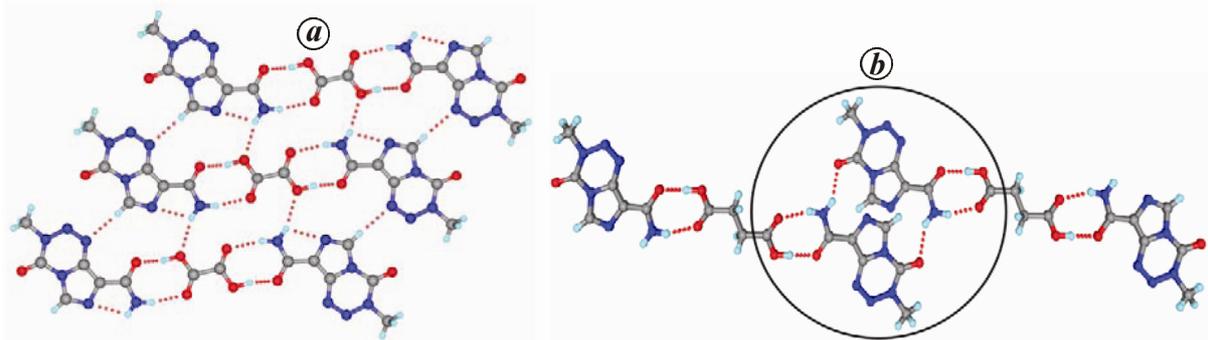


Figure 2. The amide–acid heterosynthon of N–H···O and O–H···O hydrogen bonds in the X-ray crystal structure of (a) TMZ–oxalic acid cocrystal (1 : 0.5), and (b) TMZ–succinic acid cocrystal (1 : 0.5). The dicarboxylic acid resides on the inversion centre special position in both crystal structures.

TMZ was approved as a first-line treatment for glioblastoma by the US-FDA and also followed by the European Medicines Agency (EMA). However there exists a stability problem associated with temozolomide as mentioned in the drug leaflet⁸. The product information leaflet on temodar (TMZ) in the *Physician Desk Reference 60th Edition*⁹ states the drug material is a light tan/light pink powder. The coloration is indicative of degradation to AIC during storage, thus making the prodrug less effective. A novel method to store the drug in desiccant bags was reported¹⁰, but a chemical approach to stabilize the formulation will make drug tableting more cost-effective, simpler and scalable.

Our research group recently showed that hydrolytic degradation of TMZ prodrug to AIC could be inhibited in combination with organic carboxylic acids, having pK_a in the range 2 to 5 as pH adjusters¹¹. Hence this idea was successfully demonstrated by formation of cocrystal utilizing robust hydrogen-bonding synthon between the CONH₂ group of TMZ and the COOH group of carboxylic acid cofomers via N–H···O and O–H···O $R_2^2(8)$ ring motif (Figure 2). Among the TMZ cocrystals with succinic acid (SA), oxalic acid (OA), salicylic acid, anthranilic acid, D,L-tartaric acid, etc. evaluated for structural homogeneity and solid form stability in accelerated ICH conditions, two pharmaceutical cocrystals of TMZ with oxalic acid and succinic acid appeared to be promising¹¹. Further, to check the cytotoxicity activity of TMZ-SA and TMZ-OA, these cocrystals were evaluated for sensitivity and cell-death activity in glioblastoma and cancer cell lines U373, U87 and LN18 (ref. 12). The IC₅₀ of TMZ and TMZ-SA in U87 is 1.24 mM. A study of TMZ stabilization in cucurbit [7]uril host cavity on primary GBM cell lines reported IC₅₀ values of 8.0 and 2.9 mM for TMZ and TMZ-Cucurbituril complex respectively¹². While the absolute drug concentrations for cell proliferation inhibition appear to be high being in millimolar range, the relative numbers are acceptable for stabilization of the prodrug. TMZ-OA resulted in the precipitation of oxalic acid in cell line plates at higher

concentration (1 mM), whereas TMZ-SA is superior as a stable drug cocrystal and inhibited glioblastoma cell growth comparable to that of TMZ. For these reasons, as well as the comparable dissolution rate and excellent colour stability of TMZ-SA (Figure 3), we continued studies on the bioavailability and bioequivalence (BABE) of TMZ-SA cocrystal, which is the subject matter of this article.

Experimental section

Bioavailability investigations of TMZ-SA in comparison to TMZ in Sprague Dawley (SD) rats were carried out along with stability studies in ICH conditions.

Materials

Temozolomide was purchased from Giovell Healthcare (New Delhi) and used directly for experiments. The preparation and characterization of TMZ-SA cocrystal were done according to Babu *et al.*¹¹. Succinic acid, theophylline and various solvents were purchased from Sigma-Aldrich and Merck (Hyderabad).

Stability studies

Stability studies on TMZ and TMZ-SA cocrystal were carried out in a stability chamber by keeping the samples (about 2 g each) in an open petri dish at accelerated ICH conditions of 40°C and 75% RH and 30°C and 65% RH (ref. 11). Quantification of the decomposition products was analysed by HPLC. Pure TMZ (10 mg) was dissolved in 10 ml volumetric flask with HPLC mobile phase solution. Then 20 µl of the final resulting solution was analysed by HPLC. The absorbance intensity of TMZ peak at 254 nm (UV–Vis) with 5.0 min retention time (HPLC) and retention time at 2.5 min pertains to the inactive species AIC. The samples were analysed at regular

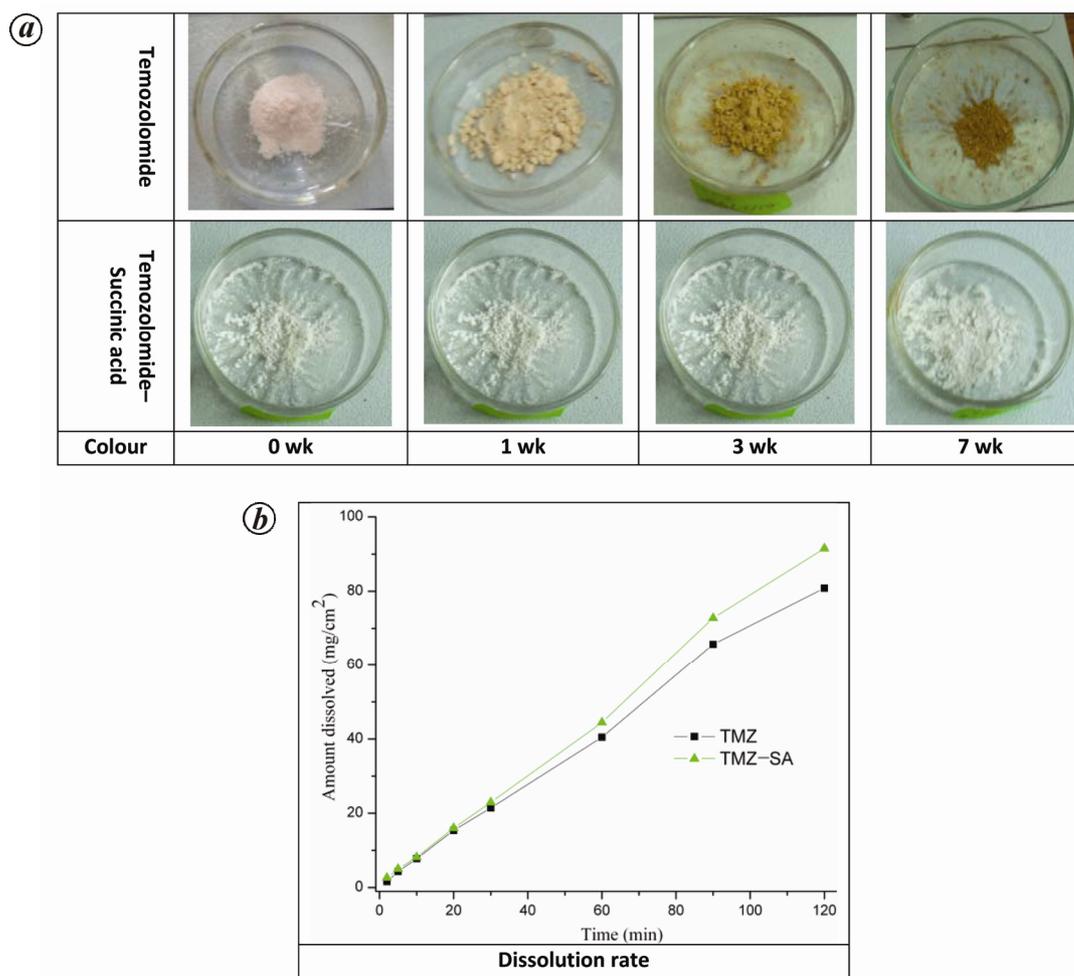


Figure 3. *a*, Colour stability of TMZ-SA compared to dark tan discoloration of temozolomide. *b*, Comparable dissolution rate in pH 7 buffer medium of test cocrystal with that of the reference drug. There was no change in the colour appearance of TMZ-SA up to 28 weeks (6 months), while the pure drug deteriorated in appearance.

intervals of 30 days up to 6 months. The intensity of pure TMZ at 254 nm around 5 min RT decreased whereas AIC at 2.5 min RT increased over time, thus indicating hydrolysis of TMZ to AIC.

Test species

A total of 14 SD rats (7M + 7F) of 6–8 weeks age weighing 200–250 g of either sex were obtained from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (ICMR), Hyderabad, and were selected after careful initial screening for any external signs of disease or injury. They were housed in individual cages in the conventional animal facilities of NCLAS, a registered facility with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) (154/1999), Government of India. The environmental conditions were kept at $21^{\circ} \pm 2^{\circ}\text{C}$, with 10–15 air changes per hour and relative humidity of 50–55% with a 12 h light/dark cycle. Animals were quarantined for 4

days and all of the free access to sterile formulated feed pellets and filtered, potable clean water.

In vivo pharmacokinetic study

A crossover design study was carried out on SD rats. They were selected as a model to generate the kinetic study, as their metabolism and pharmacokinetic studies have greater relevance when conducted in both sexes of young adult animals of the same species and strain used for other toxicity tests with the test substance¹³. The study was undertaken in 6–8-week-old SD rats (7M + 7F) weighing 200–250 g. Also, extra 20% rats were taken so that a minimum of 5M + 5F data will be available at the end of the study, even if there are any pre-terminal deaths in the experimental phase. The rats were acclimatized for 7 days; the test compound was administered orally at a dose of 35 mg/kg for TMZ-SA cocrystal and 27 mg/kg for TMZ (adjusted to same active drug based on cocrystal stoichiometry; TMZ M.W. 194, TMZ-SA_{0.5} M.W. 253).

Blood samples were collected at the start of the experiment and at $T = 30, 60, 90, 120, 180, 240, 480, 360$ min, 720 min (12 h) and 1440 min (one day) after administration of the test compound. Blood plasma was separated and processed for estimation of TMZ concentration. The samples collected at 0, 60 and 720 min were subjected to hematology and serum test for clinical chemistry profile as a part of the toxicokinetics investigation. Being a crossover design study, the animals were kept for wash-out period of 8 days and then exposed with standard drug TMZ.

Sample processing for TMZ and TMZ-SA cocrystal

TMZ and its cocrystal TMZ-SA were extracted from the plasma using liquid-liquid extraction procedure. The plasma samples were allowed to thaw in a water bath at room temperature and 0.25 ml aliquots were pipetted into separate 16×125 mm screw-cap glass culture tubes. All samples were acidified by the addition of 50 μ l of 1 N HCl and 5 ml ethyl acetate was added to each tube. The contents were mixed in a shaker for 10 min. The samples were centrifuged at 3000 g for 5 min at 4°C. The organic layer was evaporated to dryness at 37°C under N₂. The residue was then reconstituted in 600 μ l of mobile phase (20 : 80 methanol: 0.5% glacial acetic acid)¹⁴ and then analysed by HPLC. The equation for each calibration curve was derived by linear regression using the peak area of TMZ and TMZ-SA cocrystal relative to the internal standard of standard calibration curve. The concentration of active species in plasma samples was determined using the slope and intercept values of the calibration curve equation.

Drug transport to the brain tissue

Blood-brain barrier studies were performed in Sprague Dawley rats of 6–8-week-old and weighing 200–250 g. The brain samples were collected immediately after 2 h of drug administration on the last day of the recovery period (15th day) of sub-chronic study. The brain tissue was isolated, rinsed with water and immediately frozen on the surface of liquid nitrogen and stored at –80°C until analysis. For analytical determination, brain tissues were selected to avoid additional blood presence; thus only serum-free tumour tissues were taken. TMZ and its cocrystal were extracted from the brain tissue using liquid-liquid extraction for HPLC analysis¹⁵.

Quantitative analysis

The quantification of prodrug TMZ and TMZ-SA cocrystal was determined by a validated HPLC method. All the samples were analysed by HPLC using a Shimadzu pump

(LC-20AD), diode array (SPD-M20A) detector, degasser (DGU-20A3) and reverse phase enable column C18G (250 \times 4.6 mm, 5 μ m particle size) analytical column. The chromatographic conditions were: UV 330 nm for TMZ and TMZ-SA cocrystal, and at 276 nm for theophylline (internal standard), injection volume: 20 μ l, retention time of TMZ and TMZ-SA is about 5 min, and theophylline (IS) is 12 min, HPLC isocratic flow rate is 1.1 ml min⁻¹ (ref. 16).

Clinical hematology

Blood samples from the rat were drawn at 0 and 60 min time intervals after test compound exposure from the orbital plexus. The samples were collected into tubes containing EDTA K2 using heparinized microhaematocrit tubes and analysed in an automated blood cell counter. The hematological profile includes WBC, RBC, Hb/HGB, HCT, MCV, MCH, MCHC, PLT, neutrophils, lymphocytes, monocytes, eosinophils and basophils, which were monitored using ADVIA (model no. 120), Mumbai.

Clinical chemistry

Blood samples from the orbital plexus were drawn after test compound exposure at 0, 60 and 720 min. The samples were collected in vacutainer tubes (BD, USA) and centrifuged at 3000 rpm for 10 min to separate the plasma for clinical chemistry analyses for toxico-kinetic study. The clinical chemistry includes renal function test (creatinine and urea), and liver function test (ALT, AST, ALP and TBILI) which were analysed using ACE ALERA Clinical Chemistry Analyzer (model no. 6090477).

Statistical analysis

The standard curve was calculated by linear relationship between concentration and area with regression factor $R^2 = 0.999$. Area under the curve (AUC) for serum concentration versus time plots was calculated by the linear trapezoidal rule. The maximum plasma concentration C_{\max} and time T_{\max} required to reach C_{\max} were obtained from the plasma concentration curve. Statistical analysis was carried out on SPSS 15.0 Windows version, where matched paired *t*-test comparison and repeated measures of ANOVA were applied for different time points.

Results and discussion

Stability studies

Stability studies were carried out under accelerated ICH (International Conference on Harmonization) conditions

Table 1. Stability data of TMZ and TMZ-SA at 40°C and 75% RH for up to 6 months. The concentration of TMZ by HPLC analysis (% relative to at $T = 0$) is mentioned in Figure 4 a

	First month	Second month	Third month	Fourth month	Fifth month	Sixth month
TMZ	94.7 ± 1.10	58.1 ± 1.76	30.2 ± 0.32	30.1 ± 0.28	30.1 ± 0.01	30.1 ± 0.01
TMZ-SA	99.9 ± 0.04	99.8 ± 0.09	99.6 ± 0.09	99.6 ± 0.19	99.5 ± 0.21	99.4 ± 0.27

Table 2. Stability data of TMZ and TMZ-SA at 30°C and 65% RH for up to 6 months. The concentration of TMZ by HPLC analysis (% relative to at $T = 0$) is mentioned in Figure 4 a

	First month	Second month	Third month	Fourth month	Fifth month	Sixth month
TMZ	96.6 ± 1.19	87.8 ± 1.93	30.8 ± 0.64	30.4 ± 0.52	29.9 ± 0.64	28.8 ± 0.39
TMZ-SA	99.9 ± 0.07	99.9 ± 0.07	99.9 ± 0.07	99.8 ± 0.07	99.8 ± 0.14	99.3 ± 0.28

of 40°C, 75% RH and 30°C, 65% RH, and quantification of TMZ and its cocrystal TMZ-SA were analysed by HPLC at monthly intervals for up to 6 months. The cocrystal was characterized by PXRD at regular intervals for up to 28 weeks¹¹. The conclusion of the previous study¹¹ was that TMZ is stable for one week under accelerated ICH conditions and transformation to TMZ hydrate started after 2 weeks with deterioration in the peak indicative of conversion to AIC hydrate after 4–5 weeks. The present study includes the percentage degradation of TMZ and TMZ-SA cocrystal with time, as detailed in Tables 1 and 2. The concentration of pure TMZ active declined to 30% of its original value after 6 months, whereas for TMZ-SA >99% active drug was present in the cocrystal by HPLC analysis at 6 months (Figure 4). Apart from chemical purity of TMZ (by HPLC) and greater physical form stability of TMZ-SA (by PXRD), the cocrystal did not show any discoloration up to 6 months in the stability chamber at 40°C, 75% RH and 30°C, 65% RH conditions. Thus the shelf life of TMZ-SA will be much longer than that of the reference drug TMZ, thereby validating the cocrystal strategy for improving the stability of TMZ. Considering colour and stability criteria, TMZ-SA cocrystal is the best lead candidate for improving the stability of TMZ.

Pharmacokinetics studies

Pharmacokinetic analysis of TMZ reference drug and TMZ-SA cocrystal test drug following oral administration of 27 and 35 mg/kg dose (300 mg/kg human dose) is summarized in Table 3 and Figure 5. In both male and female rats, TMZ-SA cocrystal concentration rapidly peaked at $T_{\max} = 1.93 \pm 0.189$ and 1.50 ± 0.289 min, while T_{\max} for TMZ in male and female rats was similar at 1.58 ± 0.204 min. The C_{\max} values for male rats of TMZ-SA cocrystal and TMZ were close at 5.63 ± 1.47 and 5.40 ± 0.96 µg/ml, whereas in female rats C_{\max} for TMZ-SA cocrystal was 4.91 ± 1.302 min and for TMZ it was 4.24 ± 0.482 min. TMZ reached the peak value

rapidly in male rats and exhibited the shortest apparent terminal half-life of 3.87 ± 0.467 min against TMZ-SA cocrystal apparent terminal half-life at 5.40 ± 0.963 min, whereas in female TMZ-SA cocrystal and TMZ exhibited $T_{1/2} = 3.99 \pm 0.463$ and 4.62 ± 0.865 min respectively. The $T_{1/2}$ of TMZ-SA is longer in the biological medium due to stabilization as a cocrystal. To summarize, we noted small pharmacokinetic variations in the behaviour of TMZ and TMZ-SA, but they were not statistically significant to suggest any trend or change in pharmacokinetic analysis between the reference drug and the test cocrystal. Thus, the absolute F^{oral} of TMZ-SA cocrystal is 102–109%. The recommended range for bioavailability bioequivalence is 80–120%. Hence the pharmacokinetics of TMZ-SA cocrystal and TMZ is bioequivalent.

Clinical chemistry

Liver function test: Liver function test was performed on the rats after administration of TMZ and TMZ-SA. The first intervention was taken at 0 min, second at 60 min, and third at 720 min. In this study, several differences were noted between TMZ and TMZ-SA cocrystal (Table 4). For TMZ assigned rats, ALT levels were elevated significantly ($P < 0.0001$) up to 60 min and at 720 min, a significant drop ($P < 0.0001$) in ALT level was observed. In the case of TMZ-SA cocrystal the trend remained the same, but at 720 min there was lowering of ALT levels ($P < 0.0001$). The trend lines of ALT levels in TMZ are higher than those for TMZ-SA cocrystal (at 60 min). The AST level of TMZ assigned subjects significantly increased ($P < 0.0001$) at 60 min, but decreased at 720 min ($P < 0.0001$). In case of TMZ-SA cocrystal, however, the AST levels observed at 60 and 720 min were significantly lower ($P < 0.0001$) than TMZ. Thus the AST levels of TMZ were higher than TMZ-SA cocrystal.

The ALP levels of TMZ assigned rats decreased up to 720 min (at 60 and 720 min fall is significant with $P < 0.0001$). For TMZ-SA cocrystal assigned rats the level at 60 min was slightly lower but at 720 min there

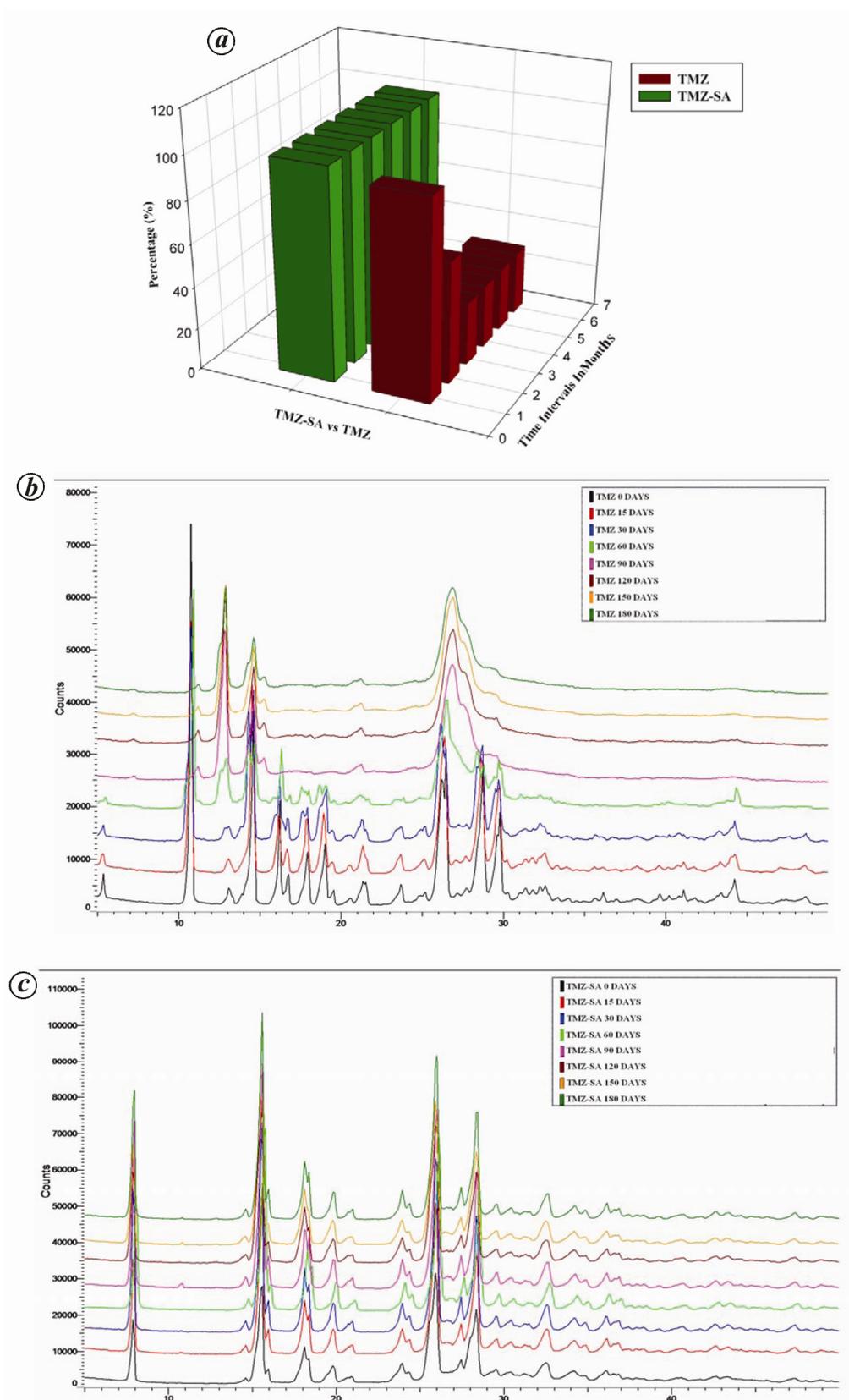


Figure 4. *a*, Chemical stability of pure TMZ and TMZ-SA cocrystal (for temozolamide) by HPLC analysis stored at accelerated conditions. TMZ showed degradation to 30% of the original purity in the 6-month period. TMZ-SA test cocrystal remained stable during the entire 6 months of study with final drug concentration of over 99%. *b*, Overlay of powder XRD to compare the physical form stability of TMZ and TMZ-SA over six months. New peaks for impurities and by-products start to appear for TMZ at $2\theta = 11^\circ$, 13° and product peaks start to decrease in intensity at $2\theta = 21^\circ$, signifying decomposition. *c*, Stacked PXRD plots for TMZ-SA relatively unchanged during the 6-month period.

Table 3. Pharmacokinetics data of TMZ reference drug and TMZ-SA test cocrystal

	TMZ-SA (M)		TMZ (M)		TMZ-SA (F)		TMZ (F)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
T_{max} (h)	1.93	0.189	1.58	0.204	1.50	0.289	1.58	0.204
C_{max} ($\mu\text{g/ml}$)	5.63	1.474	5.40	0.963	4.91	1.302	4.24	0.482
$T_{1/2}$ (h)	5.00	0.697	3.87	0.467	3.99	0.463	4.62	0.865
$AUC_{(0-24)}$ ($\mu\text{g h/ml}$)	21.74	2.508	24.00	2.962	21.85	2.488	18.66	3.257
$AUC_{(0-\infty)}$ ($\mu\text{g h/ml}$)	21.99	2.462	24.13	2.967	21.99	2.446	18.84	3.265
K_e (h^{-1})	0.14	0.018	0.18	0.022	0.18	0.018	0.15	0.026

Table 4. Differences in clinical chemistry parameters between TMZ and TMZ-SA

Exposure	Compound	0 min	60 min	720 min
Liver function				
ALT (IU/l)	TMZ	78.92 \pm 8.34	114.33 \pm 12.18	67.00 \pm 8.40
	TMZ-SA	81.43 \pm 6.81	78.86 \pm 9.85	61.36 \pm 5.84
AST (IU/l)	TMZ	170.08 \pm 21.61	212.25 \pm 12.42	116.83 \pm 12.32
	TMZ-SA	169.50 \pm 23.33	120.79 \pm 11.32	96.64 \pm 7.20
ALP (IU/l)	TMZ	711.50 \pm 45.18	502.00 \pm 13.24	375.00 \pm 17.65
	TMZ-SA	727.93 \pm 46.61	598.14 \pm 12.82	290.43 \pm 20.72
TBILI (mg/dl)	TMZ	0.40 \pm 0.17	0.37 \pm 0.14	0.43 \pm 0.08
	TMZ-SA	0.42 \pm 0.09	0.34 \pm 0.05	0.21 \pm 0.07
Renal function				
Creatinine (mg/dl)	TMZ	0.45 \pm 0.15	0.54 \pm 0.20	0.70 \pm 0.09
	TMZ-SA	0.44 \pm 0.16	0.48 \pm 0.08	0.57 \pm 0.07
Urea (mg/dl)	TMZ	25.41 \pm 2.19	23.33 \pm 1.87	35.75 \pm 3.55
	TMZ-SA	25.14 \pm 2.68	30.36 \pm 2.56	29.71 \pm 2.13

Values are mean \pm SD ($n = 14$) pooled data.

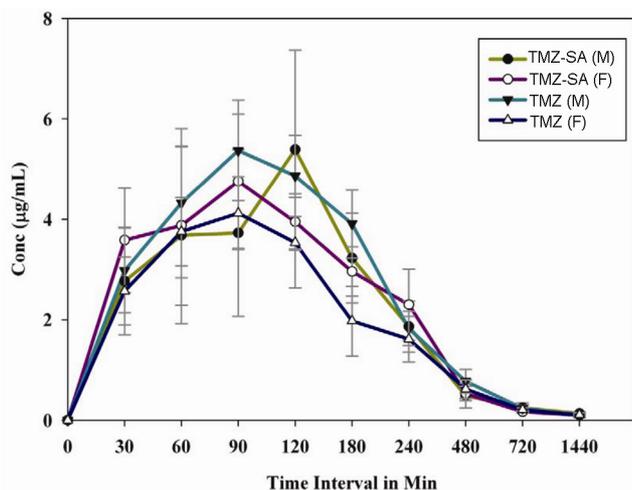


Figure 5. Mean plasma concentration versus time profile of TMZ reference drug and TMZ-SA test cocrystal.

was a significant drop in ALP levels ($P < 0.0001$). The trend observed in TMZ-SA cocrystal at 720 min was lower than to that of TMZ. Total bilirubin (TBILI) levels in TMZ assigned rats dropped at 60 min and but recovered back at 720 min. In case of TMZ-SA cocrystal, TBILI levels continued to drop to half the original value at 720 min ($P < 0.0001$). To summarize, TMZ-SA test co-

crystal exhibits lower hepatotoxicity than the standard drug TMZ.

Kidney function test: Creatinine levels of both TMZ and TMZ-SA cocrystal assigned rats increased up to 720 min. Urea levels for TMZ assigned rats declined ($P < 0.02$) at 60 min; thereafter, the levels increased elevated with significance ($P < 0.0001$). In TMZ-SA cocrystal assigned rats in the first phase, that is, up to 60 min urea levels were elevated significantly ($P < 0.0001$); thereafter, a decrease in levels with no significance was observed till 720 min interval. Thus the trend line of urea in TMZ-SA cocrystal assigned rats is lower when compared to TMZ assigned rats. To summarize, TMZ-SA cocrystal is less nephrotoxic than the standard drug.

Hematology

Haematological investigation was performed on all assigned rats. Two readings were recorded for each parameter, first at 0 min and second at 60 min. Both TMZ and TMZ-SA cocrystal, exhibited similar changes (Table 5). The RBC count decreased significantly ($P < 0.001$) in TMZ-SA cocrystal, whereas in TMZ the RBC count increased with no significance. The platelets count decreased in TMZ assigned rats and this decrease was significant

Table 5. Differences in hematology parameters between TMZ and TMZ-SA

Blood	TMZ		TMZ-SA	
	0 min	60 min	0 min	60 min
WBC ($10^3/\text{mm}^3$)	8.73 ± 1.341	4.12 ± 1.045	8.75 ± 1.309	5.94 ± 1.561
RBC ($10^6/\text{mm}^3$)	6.98 ± 0.598	7.01 ± 0.358	6.96 ± 0.595	5.97 ± 0.292
Hb/HGB (g/dl)	12.87 ± 1.013	13.59 ± 0.444	12.86 ± 0.966	11.17 ± 0.264
HCT (%)	34.38 ± 2.083	42.44 ± 1.437	34.37 ± 2.017	32.49 ± 2.654
MCV (μm^3)	49.40 ± 1.599	60.71 ± 3.285	49.51 ± 1.680	54.12 ± 4.549
MCH (pg)	18.44 ± 0.414	19.46 ± 1.289	18.49 ± 0.472	18.48 ± 0.726
MCHC (g/dl)	37.36 ± 0.939	32.04 ± 0.627	37.36 ± 0.867	34.25 ± 2.542
PLT ($10^3/\text{mm}^3$)	952.50 ± 99.621	824.64 ± 101.967	955.93 ± 92.551	899.79 ± 60.85
Neutrophils (%)	12.92 ± 3.528	26.09 ± 5.890	13.29 ± 3.384	22.30 ± 3.31
Lymphocytes (%)	82.67 ± 3.551	68.91 ± 6.008	82.21 ± 3.468	73.43 ± 8.582
Monocytes (%)	3.00 ± 0.426	2.73 ± 0.647	3.07 ± 0.475	2.86 ± 0.770
Eosinophils (%)	1.42 ± 0.515	2.27 ± 0.650	1.43 ± 0.514	1.43 ± 0.514
Basophils (%)	0	0	0	0

Values are mean ± SD ($n = 14$) for sex pooled data.

For basophilis, 0 is normal value. A positive number indicates adverse drug reactions.

($P < 0.01$), whereas in TMZ-SA assigned rats the decrease was not significant. We conclude that TMZ-SA cocrystal has less negative effect on the platelet count than the standard drug. Similar effects on other hematological parameters were observed between TMZ and TMZ-SA cocrystal.

Measurement of TMZ and TMZ-SA in brain tissue

Brain tissue samples of rats treated with TMZ and TMZ-SA were analysed for drug uptake at the target site. These results demonstrate the important requisite of pharmaceutical cocrystal as required by the recent guidance of the US-FDA¹⁷. The drug cocrystal TMZ-SA must deliver the active drug species after crossing the blood–brain barrier and the free drug must be present at the site of biochemical action. The peak concentration of temozolomide which reaches the target brain tissue from the reference drug TMZ and the test cocrystal TMZ-SA is comparable at 0.147 $\mu\text{g}/0.145 \mu\text{g}$ for 0.6 g of brain tissue. The mean peak TMZ concentration in the brain is reported as $0.6 \pm 0.3 \mu\text{g}/\text{ml}$ and the mean time to reach the peak level in brain is $2.0 \pm 0.8 \text{ h}$ (ref. 18).

The recovery of drug active in the brain tissue was analysed by oral administration dose at 1X level of TMZ and TMZ-SA cocrystal, which was given to six rats of each group for 4 weeks. The rats were left for 15 days without exposure to the drug and then necropsy was carried out. The resulting samples extracted showed no drug-active species in the brain tissue of either sex group of rats.

Conclusion

Pharmaceutical cocrystal is a relatively recent strategy to systematically modulate the physico-chemical and pharmacodynamic properties of drugs. In this article, we

have successfully addressed the hydrolytic stability problem of an active potent drug, TMZ by crystal engineering its succinic acid cocrystal. Whereas the formation of cocrystals can now be engineered using the supramolecular synthon concept, the specific cocrystal which will exhibit the desirable profile of high bioavailability, drug kinetics and transport must be established in pre-clinical studies. The succinic acid cocrystal of TMZ is a case study to illustrate solving stability issues in high humidity and tropical conditions typical of Asia and the Far East regions. The fact that the drug dissociates for the cocrystal and reaches the brain tissue means that TMZ-SA cocrystal complies with the US-FDA guidance on pharmaceutical cocrystals. Excellent physico-chemical stability along with comparative absorption, bioavailability, and excretion of the cocrystal drug are the main advantages of the improved oral formulation. This study demonstrates a successful case of pharmaceutical development and drug translation in university–industry collaboration and partnership model.

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