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Temozolomide hydrochloride dihydrate†

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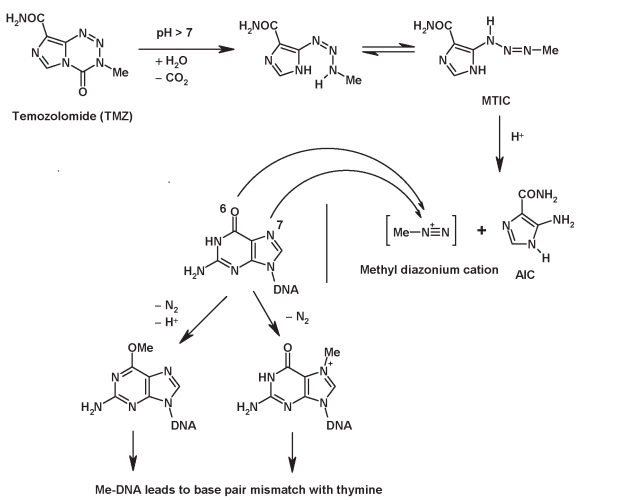
The X-ray crystal structure of temozolomide hydrochloride shows that the dihydrate salt contains one neutral temozolomide, one temozolomide-H^+Cl^-, one $H_3O^+ \cdot Cl^-$, and three water molecules. This is the first crystallographic evidence of a protonated form of the antitumour drug temozolomide. The protonation of water O and imidazole N of the drug are rationalized by calculated pK_as.

Temozolomide (TMZ) is an anticancer agent with broad-spectrum antineoplastic activity.[1] TMZ is a monofunctional alkylating agent prodrug that is able to cross the blood–brain barrier. It is the preferred treatment of choice for some of the most malignant and intractable brain tumors. Temozolomide is the most effective drug for glioblastoma multiforme, malignant melanoma, and other advanced cancers in human patients.[2] Temozolomide spontaneously hydrolyzes above pH 7 to the biochemically active species 5-(3-monomethyl-1-triazeno)imidazole-4-carboxamide (MTIC), which transforms to the 5-aminoimidazole-4-carboxamide (AIC) by-product. MTIC auto-degrades to the highly reactive methyldiazonium cation ($CH_3N_2^+$), which is the nascent alkylating agent for binding in the major groove of DNA (Scheme 1).[3] Temozolomide is an effective antitumor agent when a large population of cancer cells is actively replicating.[1b]

Several polymorphs of temozolomide are published in the patent literature[4] and X-ray crystal structures of four of these ten polymorphs (three crystalline polymorphs and a monohydrate) are reported.[5] Several hydrolytically stable cocrystals of temozolomide were recently reported.[6] Crystallization of temozolomide from conc. HCl has been known to give a hydrochloride salt whose structure was proposed by ^{15}N NMR spectroscopy (Scheme 2) in 1997,[7] but its crystal structure was elusive for almost 15 years, perhaps due to difficulty with crystal growth and quality. We have now obtained the X-ray crystal structure of TMZ·HCl which confirms that the solid crystalline salt is a dihydrate. Interestingly, two examples of salts of amide group containing active pharmaceutical ingredients (APIs), acetaminophen and dutasteride, were reported in the recently published themed issue of *CrystEngComm* on Crystal Engineering and Crystallography in the Pharmaceutical Industry.[8] Since the amide group normally is not easily protonated, even under strongly acidic conditions, and the N atoms of imidazole and tetrazinone rings in TMZ are less basic and not ionized by organic acids (pK_a 2–6),[6] the crystal structure of TMZ·HCl dihydrate is a rare example of a hydrochloride salt of a weak N base. Salt forms of APIs are generally preferred in the pharmaceutical industry and drug formulation due to their greater stability and higher solubility, and furthermore temozolomide is more stable at acidic pH.[1b,3b,6]

In a typical experiment, 150 mg of freshly purified TMZ (white color) was dissolved in 3 mL conc. HCl by slight sonication in a 50



Scheme 1 Mechanism of action for the antitumor prodrug temozolomide by DNA methylation. MTIC = 5-(3-monomethyl-1-triazeno)imidazole-4-carboxamide, AIC = 5-aminoimidazole-4-carboxamide.

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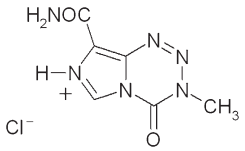
† Electronic supplementary information (ESI) available: CIF files, RLATT map, hydrogen bonding in temozolomide–formic acid monohydrate (2 : 1 : 1), and IR spectra. CCDC 895908 and 895909. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ce26528a



Scheme 2 Chemical structure of temozolomide hydrochloride salt.

mL beaker. The mixture was kept for 1 day at room temperature, after which a few plate morphology crystals appeared in the flask, which were characterized as TMZ·HCl dihydrate by single crystal X-ray diffraction.‡ Most of the crystals were of poor quality. The best single crystal which gave the data set reported in this paper was obtained after numerous crystallization attempts, several of which gave weakly diffracting or highly twinned crystals. Visualization of the diffraction spots of the best crystal data in RLATT (Reciprocal Lattice program, ver 3.0, part of Bruker AXS 2000 package)[9] suggested that the single crystal is twinned. Minor twin domain reflections were discarded leaving only major twin domain reflections for accurate unit cell determination and thereafter crystal structure solution (see RLATT spots in Fig. S1, ESI†).

The crystal structure was solved in the triclinic space group $P\bar{1}$. All heavy atoms were located in the difference electron density maps. Thermal ellipsoids indicated the absence of disorder in the drug molecule. H atoms bound to N and O were located in difference Fourier maps, however, their bond distances were constrained during the structure refinement. Out of 10750 reflections collected, 4115 were unique and 2606 of these were strong ($I > 2\sigma(I)$). With 360 parameters, the final *R*-factor of the crystal structure is 0.1274. There are two temozolomide, two HCl, and four water molecules in the asymmetric unit. Protonation resulted in one TMZ-H^+·Cl^- cation (protonation at imidazole N2 of TMZ1) while the other TMZ is a neutral molecule (TMZ2). The proton from the second HCl was transferred to one of the four water molecules (to O8 water) resulting in a hydronium chloride salt (H_3O^+·Cl^-), as shown in Fig. 1. Overall, the chemical formula of the asymmetric unit of TMZ·HCl dihydrate is $(C_6H_7N_6O_2^+\cdot Cl^-)\cdot(C_6H_6N_6O_2)\cdot 3(H_2O)\cdot(H_3O^+\cdot Cl^-)$. The successful structure solution of TMZ·HCl dihydrate is a response to the appeal by Bond[10] for a deeper analysis of X-ray data and

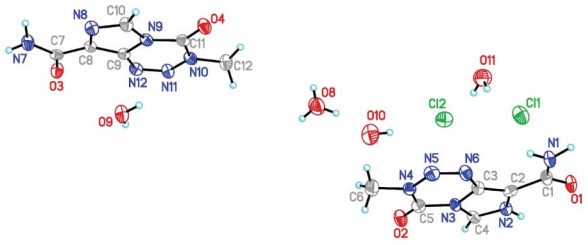
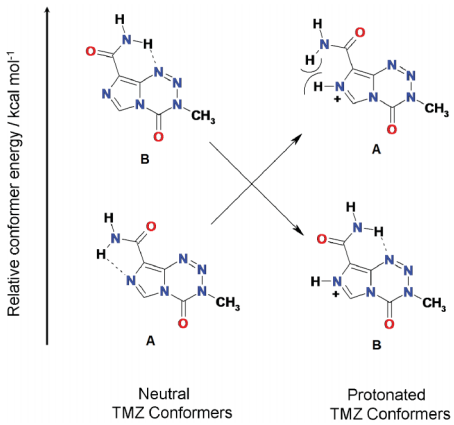


Fig. 1 ORTEP diagram of temozolomide hydrochloride salt dihydrate. There are two temozolomide molecules of which one is protonated at the imidazole N2, four water molecules of which one is a hydronium ion (O8 water), and two chloride ions in the asymmetric unit. Thermal ellipsoids are drawn at 30% probability of the atoms. Hydrogen atoms are given as spheres of arbitrary radii.



Scheme 3 Temozolomide exists in two conformation, A and B, the former is assisted by a five membered intramolecular N–H···N between amide and imidazole N while the latter is assisted by a six membered N–H···N between amide and tetrazinone N. The conformer stability order is reversed upon imidazole N protonation to avoid repulsion between atoms. As a neutral species, the energy of the conformer A is more stable than B by 1.4 kcal mol^{-1}, and as a protonated species, conformer B is more stable than A by 11.2 kcal mol^{-1}.

diffraction spots in pharmaceutical crystallography, beyond routine structure solution and automated CIF analysis.

Normally, temozolomide molecule resides in the A conformation, stabilized by a five membered intramolecular (amide)N–H···N(imidazole) interaction.[5] However, since imidazole N2 of TMZ1 is protonated, the TMZ-H^+ ion resides in the B conformation with a six membered intramolecular (amide)N–H···N (tetrazinone) interaction (Scheme 3). The second TMZ2 neutral molecule is in the stable A conformation. It is interesting to note that the stability order of TMZ conformations is reversed upon protonation. In a neutral state, conformer A is more stable than B by 1.4 kcal mol^{-1},[5b] whereas conformer B is more stable than A by 11.2 kcal mol^{-1} for the protonated species (DFT calculations at the B3LYP/6-31G(d,p) level in Gaussian 03).[11] The protonation at imidazole N in conformer A results in significant repulsion between amide NH and imidazole NH and also between carbonyl O and tetrazinone N atom which causes the amide group to deviate considerably from the plane of the molecule (by about 30°), and this further disrupts the extended conjugation of the molecule. These repulsions are avoided in the protonated conformer B and it is further stabilized by an intramolecular N–H···N interaction. The protonation of temozolomide was ascertained by comparing the C–N–C bond angle of imidazole (110.1° in protonated TMZ1 and 106.9° for neutral TMZ2), the slightly obtuse angle being consistent with a charged N in the ring. Computations support the same trend of angles in the geometry optimized structures (111.8° for protonated TMZ and 107.5° for neutral TMZ).

The protonated imidazole is hydrogen-bonded to O9 water by $N2^+$–H2···O9 H bond (1.59 Å, 2.596(12) Å, 177°), which in turn is hydrogen bonded to two temozolomide molecules (Fig. 2) through the donor capabilities of water. One donor forms an H-bond with the amide oxygen of an inversion-related TMZ1 (O9–H9B···O1, 1.77 Å, 2.703(11) Å, 157°) to form a centrosymmetric hydrogen

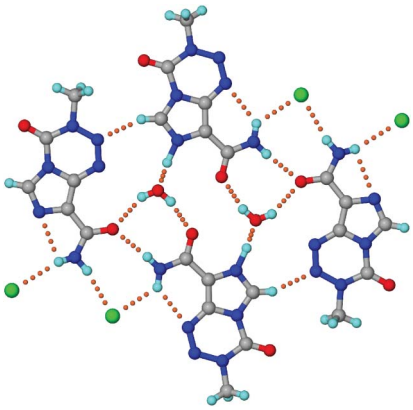


Fig. 2 A tetrameric unit of temozolomide in the crystal structure. The amide dimer synthon is absent. Instead, the amide groups is N–H···O hydrogen-bonded and also to chloride ion. The H bond from the protonated imidazole N^+–H to the water O is short, 2.58(1) Å.

bond ring motif $R_4^4(14)$ and the second donor makes bifurcated interactions with tetrazinone N and amide carbonyl O of neutral TMZ1 (O9–H9A···O3, 2.00 Å, 2.872(11) Å, 147°; O9–H9A···N12, 2.41 Å, 3.060(11) Å, 123°). Surprisingly, the amide $CONH_2$ group does not form the $R_2^2(8)$ dimer motif, a common synthon of the amide group[12,13] that was also observed in the crystal structures of TMZ polymorphs.[5] Instead, the amide group prefers to form an O–H···O bond with water and N–H···Cl^- interactions with chloride ions (N1–H1A···Cl1, 2.45 Å, 3.201(11) Å, 131°; N7–H7A···Cl1, 2.37 Å, 3.333(11) Å, 160°; N7–H7B···Cl2, 2.52 Å, 3.401(11) Å, 146°). There is also a single point N–H···O interaction between two amide groups ((TMZ2)N1–H1B···O3(TMZ2), 2.01 Å, 2.960(12) Å, 155°). The network of these hydrogen bonds generates a tetrameric unit of temozolomide which extends into a 1D ribbon along the *b*-axis *via* $R_2^2(10)$ C–H···O centrosymmetric dimer motif (C10–H4···O4 2.21 Å, 3.193 (11) Å, 150°) (Fig. 3). Parallel sets of such ribbons are connected by strong hydrogen bonds to water and chloride ions.

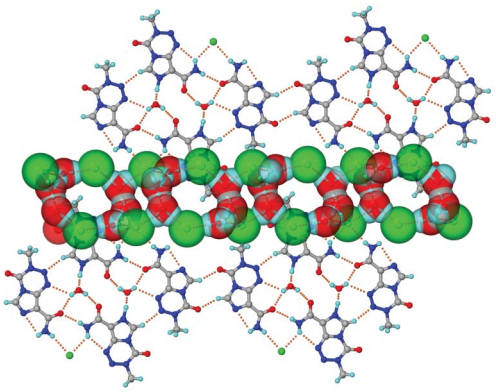


Fig. 3 The tetrameric unit shown in Fig. 2 is extended into a one dimensional ribbon of tetramers along the *b*-axis through a centrosymmetric $R_2^2(10)$ C–H···O motif. The water–hydronium–chloride ions form an infinite network of hydrogen bonds in a channel (highlighted). The channels link parallel sets of TMZ tetramer ribbons.

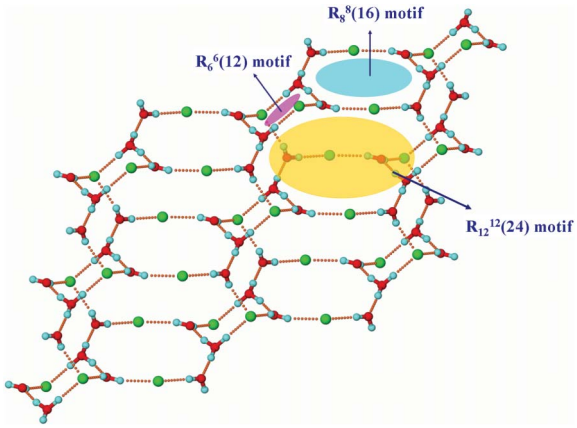
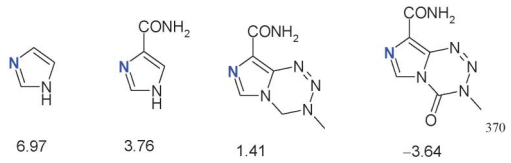


Fig. 4 Two water molecules, two chloride ions, and a hydronium ion are interlinked by a network of hydrogen bonds to form three types of hybrid cyclic clusters, $R_8^8(16)$, $R_6^6(12)$ and $R_{12}^{12}(24)$ motifs. These motifs are periodically repeated to form channels along the *a*-axis.

An interesting feature of the present crystal structure is the identification of three types of novel hybrid clusters of water–hydronium–chloride ions,[14] which appear to be as such unprecedented.[15] These clusters include an eight-membered $R_8^8(16)$ motif, a six-membered $R_6^6(12)$, and a twelve-membered $R_{12}^{12}(24)$ motif,[13] and these are all interlinked by a continuous network of O–H···O and O–H···Cl^- H bonds to form a water-ion channel along the *a*-axis (Fig. 4). The hydrogen bond network of water and chloride ions contains water molecules in four, three and two coordination geometry. A better understanding of hybrid water clusters, especially with HCl, has implications in the stabilization of certain supramolecular structures in the stratosphere for ozone layer depletion.[16] The fundamental chemical process of acid dissociation on the surface of ice crystals in ultracold conditions has remained a puzzle for many years until it was recently shown that this process actually occurs *via* an aggregation induced acid dissociation phenomenon, wherein four H bonded water molecules induced proton transfer from HCl to water.[17]

The migration of the proton from HCl to water in the present crystal structure is reflected by the presence of a very short hydrogen bond between two oxygen atoms (O8–H8B···O10, 1.46 Å, 2.427(14) Å, 167°). Such short hydrogen bonds (O···O distance less than 2.5 Å) are normally not observed unless there is a charge assistance.[18] In addition to the short hydrogen bond, the hydronium ion is involved in two more hydrogen bonds, one with chloride ion ($O8^+$–H8C···$Cl1^-$, 2.02 Å, 2.994(12) Å, 173°) and another with a neutral water ($O8^+$–H8A···O11, 1.55 Å, 2.505(14) Å, 164°). Hydrogen bonds at the hydronium ion are relatively shorter than neutral water H bonds. Thus, the assignment of the HCl proton on water O8 (Fig. 4) is consistent with charge-assisted H bond shortening, hydrogen bond geometry, number of near neighbor atoms, and chemical equation balance.

The protonation sites on water O8 and imidazole N2 were rationalized by acidity and basicity values using the Marvin pK_a calculator.[19] The calculated pK_as are HCl – 7.0, imidazole N – 3.64, and water – 1.80. The imidazole N in temozolomide is far less



Scheme 4 Calculated pK_a values of N from imidazole to temozolomide. The basicity decreases by over 10 log (pK_a) units from left to right.

basic than a normal unsubstituted imidazole, by more than 10 log (or pK_a) units due to electron-withdrawing amide and urea groups (Scheme 4), and hence is not easy to protonate. The order of protonation in conc. HCl medium should be water and then temozolomide based on calculated pK_as, which explains the H_3O^+ and TMZ-H^+ ions in the crystal structure. Incidentally, the crystal structure of TMZ–formic acid monohydrate (data collected at 293 K)‡ showed proton migration from formic acid (pK_a 4.27) to water resulting in hydronium and formate ions (Fig. S2, ESI†). The near equibond C–O distances of carboxylate (1.213 (7) Å and 1.230(9) Å) are indicative of deprotonation. Proton transfer from formic acid to water was however not observed when the crystal data were collected at 100 K.[6] Five other hydrated crystal structures of TMZ with carboxylic acid coformers, such as fumaric acid, *p*-aminobenzoic acid, acetic acid, and *p*-hydroxybenzoic acid are all neutral in nature (pK_a 2–6) with no proton transfer.[6] Such an unpredictable behavior of cocrystal-salt states is not uncommon.[20] A very recent survey of over 6000 cocrystals and salts[21] in the Cambridge Structural Database[15] suggested a quantitative classification in the three zones of the oft-quoted ΔpK_a rule:[22] zone 1 is $\Delta pK_a < -1$ of neutral cocrystals, zone 2 is $-1 < \Delta pK_a < 4$ of cocrystals and salts, and zone 3 is $\Delta pK_a > 4$ of ionic salts. The ionization states in TMZ·HCl dihydrate conform to zone 3 (actually borderline of zones 2 and 3) in which proton transfer occurs from the strong acid to the weak bases water and temozolomide. We are aware that pK_a values are highly solvent dependent,[23] and that the actual acidities and basicities in the conc. HCl medium of crystallization could be very different from the calculated values quoted above.

An early hint that the water molecule is protonated in TMZ·HCl·2H_2O came from thermogravimetric analysis (TGA, Fig. 5), which indicated that the observed water loss (6.57%) was about half that of the value calculated for a dihydrate material (13.65%). It is likely that O9 water is bound very strongly to the protonated imidazole along with O8 hydronium ion, which is surrounded by strong chloride ion acceptors. Thus two of the four water molecules are trapped in the crystal lattice *via* ionic H bonds and hence the experimental moisture content matched with that of a monohydrate. Differential scanning calorimetry (DSC) of TMZ·HCl dihydrate (Fig. 5) indicates loss of water from the crystal at 114.6 °C endotherm (T_{peak}), followed by a sharp exotherm at 184.3 °C (T_{peak}) due to decomposition/dissociation of the salt as free TMZ (T_{decomp} 210 °C). PXRD of TMZ hydrochloride dihydrate salt kept for 1 week in accelerated stability conditions of 40 °C and 75% RH (Fig. 6) indicated a phase transformation back to neutral temozolomide form 1, or the commercial material[5] (Fig. S3, ESI†). ^{1}H NMR and IR of the product at 1 week matched with that of

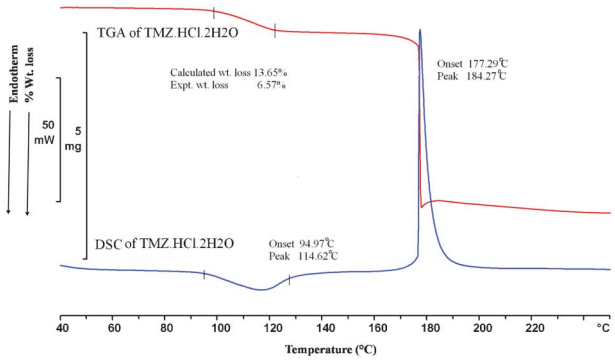


Fig. 5 TGA (red trace) of TMZ·HCl dihydrate salt bulk material. Water loss occurred at 100–120 °C. The endothermic peak at 114.6 °C in DSC (blue trace) is for water loss and the dehydrated product decomposed upon further heating to 200 °C.

starting TMZ. The accelerated humidity conditions result in dissociation of TMZ·HCl·2H_2O salt hydrate to $H_3O^+ \cdot Cl^-$ by-product and finally neutral temozolomide.

That the bulk material from the crystallization batch corresponds to the single crystal X-ray structure of TMZ·HCl·2H_2O was confirmed by an excellent match of the experimental powder X-ray diffraction pattern with the calculated XRD lines from the crystal structure (Fig. 7). The Rietveld refinement of the bulk powder pattern with the single crystal X-ray structure model confirmed good agreement§ (R_p 0.075, R_{wp} 0.108).[24] The characteristic PXRD lines for TMZ·HCl dihydrate are at 2θ 5.28, 9.69, 14.15, 14.41, 18.91, 20.40, 28.32, 28.61, and 35.57 $\pm$ 0.2° and the unit cell dimensions after the Rietveld refinement are near identical to the crystal structure (a = 6.4527, b = 10.3692, c = 17.8401 Å, α = 98.0212, β = 90.3461, γ = 98.7121, V = 1167.9 Å^3). There was no identifiable trace of any of the known TMZ polymorphs or TMZ monohydrate in the experimental powder pattern of TMZ·HCl dihydrate by an eye comparison with the reported PXRD patterns.[4–6] The IR spectra of TMZ·HCl dihydrate and temozolomide are visibly different (Fig. S4, ESI†) with their signature peaks.

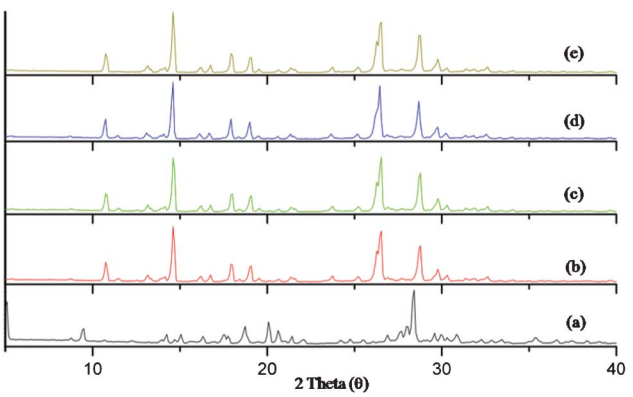


Fig. 6 PXRD plots of TMZ·HCl·2H_2O exposed to accelerated humidity conditions. (a) Initial, (b) 1 week, (c) 2 weeks, (d) 3 weeks, and (e) 6 weeks. There was no further change in PXRD after 1 week. The product after 1 week is neutral temozolomide (see Fig. S3, ESI†).

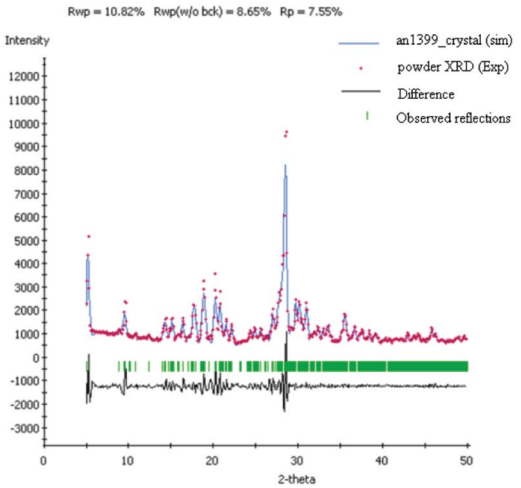


Fig. 7 Comparison of the experimental powder pattern with the calculated lines from the X-ray crystal structure. Rietveld refinement using the crystal structure model indicated a good match between the two patterns to confirm the identity of the bulk material with the single crystal structure.

In conclusion, the single crystal X-ray diffraction analysis and thermal characterization of the hydrochloride salt of antitumour drug temozolomide confirms the stoichiometry of the salt as TMZ·HCl dihydrate. Protonation at imidazole N of TMZ1 and O8 of water resulted in TMZ-H^+ and H_3O^+ ions. There is only one X-ray crystal structure in the CSD, that of ethenocytidine HCl (refcode ETCYTC),[25] whose heterocycle bicyclic skeleton and hydrogen bonding match with that in TMZ·HCl dihydrate. Since the amide group is normally not protonated even under strongly acidic conditions, and the N atoms of imidazole and tetrazinone rings in temozolomide are extremely weak bases to be ionized, a hydrochloride crystal structure of protonated temozolomide is as such rare and difficult to structurally characterize.

Acknowledgements

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‡ Crystal data for TMZ·HCl·2H_2O: $C_{12}H_{22}Cl_2N_{12}O_8$, M = 533.32, colorless needle, 0.32 × 0.18 × 0.14 mm^3, triclinic, space group $P\bar{1}$ (no. 2), a = 6.453(15), b = 10.37(2), c = 17.84(4) Å, α = 97.80(4), β = 90.44(4), γ = 98.27(4)°, V = 1171(5) Å^3, Z = 2, D_c = 1.513 g cm^{-3}, F_{000} = 552, Mo Kα radiation, λ = 0.71073 Å, T = 100(2) K, $2\theta_{max}$ = 50.0°, 10750 reflections collected, 4115 unique (R_{int} = 0.1006). Final GoF = 1.181, R_1 = 0.1274, wR_2 = 0.2894, R indices based on 2606 reflections with $I > 2\sigma(I)$ (refinement on F^2), 360 parameters, μ = 0.342 mm^{-1}. Crystal data for 2TMZ·formic acid·H_2O: crystals were obtained by dissolving 50 mg of TMZ in 4 mL formic acid and allowing slow evaporation. $C_{13}H_{16}N_{12}O_7$, M = 452.38, colourless needle, 0.32 × 0.14 × 0.06 mm^3, triclinic, space group $P\bar{1}$ (no. 2), a = 8.173(12), b = 11.626(17), c = 11.735(17) Å, α = 64.73(2), β = 74.09(3), γ = 89.31(3)°, V = 963(2) Å^3, Z = 2, D_c = 1.561 g cm^{-3}, F_{000} = 468, Mo Kα radiation, λ = 0.71073 Å, T = 293(2) K, $2\theta_{max}$ = 49.4°, 8751 reflections collected, 3278 unique (R_{int} = 0.0641). Final GoF = 0.947, R_1 = 0.0587, wR_2 = 0.1243, R indices based on 1769 reflections with $I > 2\sigma(I)$ (refinement on F^2), 319 parameters, μ = 0.129 mm^{-1}. Both crystal data were collected on Bruker Smart diffractometer and solved using SHELX-TL package. DFIX command was applied to constrain O–H and N–H distances in TMZ·HCl·2H_2O.

§ Full-profile structure refinement of the collected powder diffraction data for TMZ·HCl·2H_2O was performed using the Materials Studio Reflex program suite. During each refinement cycle, the scale factor, peak profiles, background parameter, and cell parameters were allowed to optimize until a best fit was achieved between the experimental pattern and the crystal structure model.

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