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Development and Binding Mode Assessment of N-[4-[2-propyn-1-yl]((6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3H-cyclopenta[g]quinazolin-6-yl)amino]benzoyl]-L-γ-glutamyl-D-glutamic acid (BGC 945), a Novel Thymidylate Synthase Inhibitor that Targets Tumor Cells

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Abstract

N-[4-[2-propyn-1-yl]((6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3H-cyclopenta[g]quinazolin-6-yl)amino]benzoyl]-L-γ-glutamyl-D-glutamic acid **1** (BGC 945, now known as ONX 0801), is a small molecule thymidylate synthase (TS) inhibitor discovered at the Institute of Cancer Research in London. It is licensed by Onyx Pharmaceuticals and is in Phase 1 clinical studies. It is a novel antifolate drug resembling TS inhibitors plevitrexed and raltitrexed that combines enzymatic inhibition of thymidylate synthase with α-folate receptor-mediated targeting of tumor cells. Thus, it has potential for efficacy with lower toxicity due to selective intracellular accumulation through α-folate receptor (α-FR) transport. The α-FR, a cell-surface receptor glycoprotein, which is over expressed mainly in ovarian and lung cancer tumors, has an affinity for **1** similar to that for its natural ligand, folic acid. This study describes a novel synthesis of **1**, an X-ray crystal structure of its complex with *Escherichia coli* TS and 2'-deoxyuridine-5'-monophosphate, and a model for a similar complex with human TS.

Introduction

Thymidylate synthase (TS) (EC 2.1.1.45) has been recognized for decades as a key enzyme target for anti-cancer drugs because it plays a pivotal role in DNA replication¹⁻³ TS directly methylates the 5C of uridine in 2'-deoxyuridine-5' monophosphate (dUMP), converting it to dTMP, in the sole de novo synthetic pathway to thymidine, which is required for DNA replication. Many inhibitors that compete with either the substrate (dUMP) or the cofactor 5,10-methylene-5,6,7,8,-tetrahydrofolate (mTHF) have been developed as drug leads. Also many X-ray structures of TS complexes with such inhibitors have elucidated their

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Associated Content

The atomic coordinates 4ISK have been deposited in the Protein Data Bank.

mechanisms of inhibition⁴⁻⁹ The structures encouraged rational design of analogs and different generations of structure and mechanism-based antifolate drugs, some of which are in clinical use.^{7, 10} However, treatment is often complicated by the problems of resistance and high toxicity.^{7, 11-13}

CB3717^{14, 15} (Fig. 1) is an early quinazoline-based folic acid analogue. Its clinical development was halted because of life-threatening renal and hepatic toxicity, and poor solubility.¹³ Raltitrexed (ZD1694) is a slightly modified analogue of CB3717 that is polyglutamatable by the folylpolyglutamate synthetase, which normally acts on the cofactor mTHF. Replacing the N-10 propargyl group and the benzene ring in CB3717 with a methyl group and thiophene ring, respectively, significantly improved solubility and potency and decreased the nephrotoxicity of the compound.¹³ Importantly, intracellular polyglutamylation of raltitrexed, which allows its cellular retention, does not decrease its activity. Raltitrexed is not approved by US Food and Drug Administration (FDA). Nevertheless, it became the first new drug for treatment of colorectal cancer since the mid 1990's and it was licensed in Canada and many European countries for the treatment of metastatic colorectal cancer.^{16, 17} Subsequently, pemetrexed (LY231514), approved by the FDA in 2004, was licensed for the treatment of malignant pleural mesothelioma. Pemetrexed is another multi-targeted antifolate^{18, 19}, which in 2008 was granted approval as a first-line treatment in combination with cisplatin for treatment of locally advanced and metastatic non-small cell lung cancer. Alone or in combination with other chemotherapeutics, pemetrexed also shows activity in a number of other tumors including head and neck, breast, bladder, cervical, gastric, pancreatic, ovarian and colorectal cancers.²⁰⁻²² Pemetrexed is the first antifolate for which toxicity was reduced by a low-level folic acid and vitamin B-12 supplement.^{20, 23, 24} However, the optimization of folic acid supplementation to the level that decreases toxicity without compromising the antitumor effect of the drug still remains difficult and perhaps must be further explored.²⁵ Plevitrexed (BGC 9331, ZD9331) is a non-polyglutamatable inhibitor that was developed as a result of the raltitrexed and pemetrexed adverse clinical effects. The effectiveness of Plevitrexed demonstrates that polyglutamylation is not required for potency of antifolates. It can be transported to tumor cells *via* both the α -folate receptor (α -FR) and the physiological reduced-folate carrier system (RFC).²⁶ Clinical studies evaluating plevitrexed are still ongoing; the main interest in the drug is as an alternative treatment for gastric cancers for patients who are not able to tolerate platinum-based combination therapy.

1 (BGC 945, ONX 0801), Fig. 1, was designed to further reduce toxicity by more effectively targeting cancer cells that overexpress the α -FR.²⁷ The α -FR is overexpressed in certain epithelial tumors, particularly ovarian cancer cells (more than 90% overexpress α -FR), and also lung, endometrial and mesothelioma tumors.^{28, 29} Importantly, the inhibitor is selectively transported *via* the α -FR and has reduced affinity for the widely expressed bidirectional RFC.²⁷ The RFC is ubiquitously expressed and responsible for the uptake of conventional antifolates into normal tissues, and hence can cause TS-related toxicities in the bone marrow and gut. **1** emerged from a lead series of potent inhibitors that displayed low and high affinity for the RFC and the α -FR respectively ($K_D \sim 1\text{mM}$ and 1nM).³⁰ The compound has an L-Glu- γ -D-Glu moiety that enhances binding to TS, and mimics the diglutamate metabolites of mTHF and certain conventional antifolates. The stereochemistry around the D-amino acid α -C prevents cleavage by peptide hydrolases. In the α -FR expressing human epidermoid (KB) or ovarian (IGROV-1) tumor cells its IC_{50} for inhibition of proliferation is $\sim 1\text{-}10\text{ nM}$ and for cells that do not express the FR it is in low μM range.²⁷ Thus some non-FR-mediated uptake into cells occurs at higher concentrations. However, short exposure to these higher concentrations, as observed in the plasma of KB or IGROV-1 tumor-bearing mice dosed with a single bolus injection of **1**, leads to tumor selective TS

inhibition because of rapid clearance from normal tissues. Compound **1** is licensed by Onyx Pharmaceuticals and is in Phase 1 clinical studies (ISRCTN 79302332).

We present a synthetic route for **1** and subsequent structural biology for the 1.7 Å co-crystal structure of the ternary complex of *E. coli* TS (*ec*TS) with dUMP and **1**. Further a model of human TS (hTS) in complex with dUMP and **1** is reported, highlighting the impact of chemical modifications on protein binding.

Results

Development of compound **1**

The inhibitor **1** (Fig. 1 and Scheme 1A) belongs to a series of cyclopenta(*g*)quinazoline-based antifolates for which synthesis and development have been described previously.^{31, 32} These compounds were synthesized by stepwise addition of a *p*-aminobenzoate moiety, an N10-substituent, and a dipeptide moiety to a cyclopenta[*g*]quinazoline ring. Importantly, an L-Glu- γ -D-Glu dipeptide moiety replaces the glutamate moiety present in other antifolates.^{33, 34} Further intracellular polyglutamylolation of TS antifolate inhibitors by folate polyglutamate synthase increases their affinity for TS.^{33,34} The L-Glu- γ -D-Glu dipeptide moiety of **1** serves the same purpose as the polyglutamate tail, increasing affinity for TS through electrostatic interactions with the positively charged polyglutamate-binding groove on the enzyme. The D-Glu protects **1** from peptide hydrolases in the cell. As mentioned above, several routes of synthesis of **1** are reported.^{31, 32} For preparation of the sample for X-ray crystallography as well as the initial Phase I clinical study drug substance, an innovative four-stage synthetic route was evolved and conducted under Good Manufacturing Practices at Regis Technologies, Inc. (Morton Grove, IL) (Scheme 1).^{35, 36} Amongst other improvements, this route did not require the cobalt catalyst system used in earlier routes.^{31, 32} A molar yield of 13.3 % with respect to the tricyclic acid starting material was achieved of very good quality. The purity results were: 99.5 % of area percent basis (a/a) by achiral HPLC, 99.6 % enantiomeric excess (ee) 6-S chiral purity, and 99.8 % L, D diastereomeric purity (see Methods). This process has subsequently been further improved for larger scale pharmaceutical production with increased robustness.

Overall structure of the TS-dUMP-inhibitor complex

The structure of the TS ternary complex with the substrate dUMP and inhibitor **1** elucidates the binding mode of **1** and may be useful in further evolution of this class of drugs. We attempted to co-crystallize **1** with hTS and substrate, dUMP, but after extensive screening were only able to obtain small needles that did not diffract well. We were unable to optimize these crystal hits, so instead focused on the *ec*TS complex. The justification for this approach is that the active sites of hTS and *ec*TS are highly conserved and high affinity hTS inhibitors have been developed based on the high-resolution crystal structure of an *ec*TS complex.³⁷

We determined the crystal structure of *Escherichia coli* TS (*ec*TS) in a complex with dUMP and **1** to a resolution of 1.7 Å. The crystals contained eight independent molecules (four obligate homodimers) in the asymmetric unit (Table 1). The second Glu (the D-Glu) of the di-Glu moiety of each inhibitor protrudes from the protein surface and contributes to the packing interface with an adjacent pseudo-two-fold related dimer. A similar interface cannot be formed in hTS because hTS has inserts with respect to *ec*TS in the vicinity of the *ec*TS interface. In the case of hTS, the di-Glu moiety may actually interfere with formation of well-ordered crystals, especially if it is conformationally disordered.

Both protomers contribute to each active site of a dimer. A molecule of **1** and dUMP are found in each active site. As expected, the *ec*TS enzyme is in an *active* conformation, also referred as a *closed* conformation. This is typical of TS ternary complexes with substrate and cofactor (or cofactor analogue) (Fig. 2A).^{8, 9, 38}

Substrate dUMP binding mode

The substrate dUMP and the inhibitor tricyclic ring system are bound in the same orientation and binding site in each of the subunits in the asymmetric unit. In five of the subunits there is a covalent bond between the S γ of the catalytic Cys146 and ⁶C of the dUMP pyrimidine ring. Despite this result, in three of the four dimers the Cys146 adopts a different rotamer in one of the two active sites and is not covalently linked to dUMP ⁶C. This asymmetry in dUMP binding has been seen in other TS ternary complexes such as the ternary complex of hTS with raltitrexed and dUMP⁶ and is consistent with the half-of-the-sites reactivity of *ec*TS.³⁹ A covalent bond between the catalytic cysteine and the substrate dUMP is not required for potent TS inhibition by cofactor analogs.

The *ec*TS ternary complex with dUMP and **1** has a novel magnesium cluster

The binding mode of **1** is well defined by the electron density and is schematized in Fig. 3 and shown in three-dimensions in Figs. 4 and 5A. There are at least three direct polar contacts between the ligand and the protein observed: the nitrogen at quinazoline position 3 forms a hydrogen bond with Asp169 (2.6 Å) and the carbonyl oxygen at position 4 makes a hydrogen bond with Gly173 (3.0 Å). The 2-hydroxy-methyl group is within hydrogen bonding distance of the backbone nitrogen of Ala263 (2.8 Å) and the carboxyl oxygen of Asp169 (2.9 Å). The nitrogen at position 1 hydrogen bonds with a conserved structural water molecule (Wat477). The cyclopenta moiety displaces Trp80 and the propargyl group is oriented towards Asn177 (Fig. 5B). The benzoate ring of the inhibitor makes a T-shaped edge-to-face π - π stacking interaction with Phe176.

The L-Glu- γ -D-Glu moiety is a novel feature of **1** whose binding mode to TS has not been previously characterized. It binds to *ec*TS analogously to the polyglutamyl moiety of polyglutamylated raltitrexed⁴⁰ except that the D-Glu is rotated 180° about its N- α -C bond. This rotation switches the orientations of D-Glu's carboxyl group and side chain compared to the second Glu moiety (Glu-2) of the raltitrexed polyglutamate (Fig. 4). Thus the carboxyl group lies in the polyglutamate-binding groove of the protein, whose floor is formed by His51, Arg53 and Ser54. The major protein contact with Glu-2 in the raltitrexed complex is a van der Waals interaction with His51 in which the plane of the His51 imidazole ring is parallel to the plane of the Glu-2-Glu-3 amide linkage. His51 is in van der Waals interaction with the D-Glu carboxyl of **1**, with the plane of its imidazole ring parallel to the plane of the D-Glu carboxyl (Fig. 4).

There are no direct hydrogen bonds between the L-Glu- γ -D-Glu moiety and the protein, but there are several water-mediated hydrogen bonds to the protein and two intramolecular polar hydrogen bonds (N-H – O-C=O) between the amides and the carboxyl groups. **1** also makes four hydrogen bonds with water molecules from a newly observed hexahydrated magnesium ion cluster. The Mg²⁺ cluster is sandwiched between the second glutamate side chain carboxylate of **1** and the side chain of protein residue Glu82, thereby forming ionic interactions with both the ligand and the protein (Fig. 2B, 3).

Structural differences between the *ec*TS complex with **1** and dUMP and its complex with raltitrexed and dUMP

1 differs from raltitrexed in five regions of the molecule: 1). the glutamate moiety of raltitrexed is replaced by a L-Glu- γ -D-Glu moiety; 2). the methyl at ²C of the quinazoline

ring in raltitrexed is replaced by a hydroxymethyl; 3). in **1**, a cyclopentan group is fused to the quinazoline ring system; 4). the ^{10}N methyl is replaced by a propargyl group in **1**; and 5). the thiophene ring is replaced by a benzene ring (Fig. 1). These molecular differences make **1** (MW 646) a larger molecule than raltitrexed (MW 458) and the active site has to expand to accommodate the larger ligand. Thus, superposition of the structures of the two *ec*TS ternary complexes reveals minor changes in the active site residue side chains (Fig. 5B), such as movements of Trp80, Asn177, Leu172 and His51. However, the backbone atoms of the two complexes closely overlap: the rmsd for the α -Cs of the complexes after they are superposed is 0.4 Å. These mean deviations are similar to the rmsds between any two complexes in the crystal structure of the *ec*TS ternary complex with **1**, which has four dimers per asymmetric unit.

Implication for binding of **1** to hTS (model)

There is no X-ray structure of **1** bound to hTS available. To understand how **1** could bind to hTS, the hTS-dUMP-**1** complex was modeled (Fig. 5C) based on the structure of *ec*TS complex reported here. The suitability of this model is supported by the similarity of the crystal structures of hTS and *ec*TS ternary complexes with dUMP and raltitrexed (rmsd = 0.53 Å over 1500 atoms) (Fig. 5E), and the high conservation of the residues within the folate binding site (12 out of 14 residues in a 5 Å radius around the ligand are identical, which corresponds to a sequence identity of 86 %).

To accommodate **1** within the hTS active site, hTS residues within a radius of 5 Å of the ligand were minimized (see Methods). The binding mode of **1** in the model of the hTS complex is very similar to the binding mode of **1** in the crystal structure of the *ec*TS complex (Fig. 5F). The most profound changes during minimization were observed for residues Trp109 and Asn226, which rotated by 20 and 30 degrees respectively, to accommodate the cyclopentyl and propargyl groups (Fig. 5D). Also, the benzoate ring of **1** displaces L221 and F225. These rearrangements are similar to the structural differences observed between the *ec*TS ternary complex with **1** and *ec*TS-dUMP-raltitrexed (Fig. 5B). The hydrogen-bonding network of the quinazoline ring of **1** is highly conserved between human and *E. coli* TS. Subtle differences are observed in the interactions of the diglutamate moiety. One of two sequence differences in the binding site of **1** is that His51 in *ec*TS is Phe80 in hTS. In *ec*TS, the C-terminal glutamate carboxylate makes an anion π - π stacking interaction with His51. In theory, such an interaction is also possible with Phe80 in hTS, but in the hTS model the carboxylate shifts by 2 Å towards Arg49. Also, while the diglutamate moiety of **1** makes exclusively water-mediated hydrogen bonds with *ec*TS, Lys77 and Lys308 side chains interact directly with **1** in the model of hTS (Fig. 5C). These changes in protein-ligand interactions are likely observed because the energy minimization was carried out in the absence of water, but they could also reflect potential ionic interactions.

Discussion and Conclusions

Novel drug discovery strategies are pivotal for developing better cancer therapies. Effectiveness of antifolate drugs used in cancer treatment is limited by toxicity and mechanisms of drug resistance, including over-expression of hTS. The thymidylate synthase inhibitor **1** was developed to reduce toxicity toward non-tumor cells by specifically targeting the α -folate receptor, which is overexpressed in certain tumor cells. The IC_{50} for growth inhibition of α -FR-negative cells by **1** is $\sim 7 \mu\text{M}$ compared to IC_{50} s in the 1-300 nM range for cells over-expressing the α -FR receptors.²⁷

1 was designed based on experience with a series of predecessors that bind to TS in a similar manner, primarily pemetrexed, a multitargeted drug licensed for lung cancer and

mesothelioma treatment, and raltitrexed, which is specific for colorectal cancer. The novel features of **1** are the cyclopenta[g]quinazoline ring system and the side chain L-Glu- γ -D-Glu moiety. The purpose of this study was to elucidate how these novel structural features are accommodated in the active site of hTS to give a potent ($K_i \sim 1.2$ nM) inhibitor. The crystal structure of **1** and dUMP bound to *ec*TS has been determined at a resolution of 1.7 Å and used to evaluate the structural determinants of binding affinity. The high resolution of the crystal structure allowed accurate description of the inhibitor binding mode and interactions with the protein.

Two crucial observations emerge from this study. Firstly, the novel diglutamate moiety binds in a solvent-mediated association with the surface of TS, analogously to the polyglutamate moieties of polyglutamylated antifolates. Crystal structures of ternary complexes of *ec*TS with polyglutamylated TS inhibitors have shown that the polyglutamylate tails bind to an electropositive binding groove on the enzyme surface, making mainly water-mediated hydrogen bonds with the protein.^{7, 40} Residues lining the groove are poorly conserved, which is consistent with the small number of direct hydrogen bonding interactions. Hence, there is considerable plasticity at the polyglutamate-protein interface; it is not surprising that the groove can bind a dipeptide with novel stereochemistry. The binding site for L-Glu- γ -D-Glu dipeptide, with the D-enantiomer stabilizing **1** against hydrolysis, overlaps the binding site for the first two glutamates in the polyglutamate tails, but with the carboxyl and side chain groups of the D-Glu in reversed positions. Thus, the carboxyl of the D-Glu is in the binding groove while the side chain is oriented away from the binding groove and towards the protein surface. The D-Glu makes water-mediated intermolecular hydrogen bonds with adjacent dimers in the crystal. hTS is not conserved in this region, and we speculate that if the D-Glu were oriented in a different conformation, or disordered, these stabilizing crystal packing interactions could not occur, which may explain our failure to co-crystallize hTS with **1**.

As co-crystallization of hTS with **1** was not successful, we used modeling to investigate how **1** may bind to this enzyme. Human and *E.coli* TS active sites are highly conserved (86 %), and comparison of crystal structures of *ec*TS- and hTS- with raltitrexed (Fig. 5E) showed that the folate inhibitor binding to both species was almost identical. Thus, similar changes to those observed in the crystal structure of *ec*TS-dUMP-**1** were also predicted in a model of hTS-dUMP-**1** (Fig. 5F). Analysis of this model revealed that hTS may accommodate the inhibitor by minor side chain movements of a few active site residues, mainly to harbor the cyclopentyl and propargyl groups. Furthermore, the model predicted two additional direct polar interactions between the C-terminal glutamate tail and the human protein (K77 and K308) (Fig 5C). The caveat with the modeling is that the tail could equally well make water mediated contacts with side chains in human TS, but water molecules were not included in the minimization.

The second key observation is a magnesium ion cluster linking a loop containing the invariant active site residue Trp80 with the end of the polyglutamate moiety. There is evidence in the literature that magnesium enhances catalysis by *ec*TS, but the basis for this effect was not known.⁴² Our structure is the first to show a hexa-hydrated Mg^{2+} cluster unambiguously bound to *ec*TS. It is consistent with the proposal that Mg^{2+} binding to a surface groove remote from the active site can enhance catalysis indirectly, by reducing the entropy of active site residues.⁴³ The Mg^{2+} cluster also likely increases affinity of *ec*TS for the inhibitor by hydrogen bonding to the D-Glu. The loop residues that hydrogen bond to the cluster are not conserved in hTS, thus a Mg^{2+} cluster would not likely bind to the same site in hTS.

The potent and preferential transport of **1** by a receptor that is over-expressed in cancer cells strongly suggests that **1** might be the first highly-targeted antifolate for cancer treatment. Due to the well-characterized transport of **1** via α -FR, appropriate patient selection might benefit from use of diagnostic techniques to assess α -FR receptor level prior to initiation of treatment with this agent.³⁰ This approach would facilitate a personalized identification of patients most suitable to the intrinsic cell targeting capability of **1**. Our studies have developed a novel synthetic path to **1** and shown how this inhibitor binds to the validated cancer drug target TS. The novel di-Glu moiety on the inhibitor binds in the polyglutamate-binding groove and its mode of binding includes an ionic magnesium cluster coordinated by structural water, the inhibitor, and multiple residues on the TS enzyme.

Experimental Section

1. Cells and cell culture, protein expression and purification

*ec*TS was expressed in DH5 α cells at 37 °C, induced at OD₆₀₀ 0.6-0.8 with 1 mM IPTG for 4 hours. Enzyme was purified by ion exchange chromatography (Q column) as described by Boles *et al.*⁴⁴ and subsequently dialyzed into 20 mM KPO₄ pH 7.4, 100 mM NaCl, and 2 mM DTT. The purification was then confirmed by SDS-PAGE gel.

2. Protein crystallization

An N-terminally His-tagged construct that was previously co-crystallized with inhibitors⁴⁵ was used for screening crystallization conditions for the ternary complex of hTS with dUMP and **1**. Several published protocols for co-crystallizing hTS with dUMP and antifolate inhibitors were tried, and small crystals were obtained using the protocol used by Almog *et al.*⁶ However, these diffracted to less than 5 Å resolution or were twinned. Some crystal hits were also obtained from commercial crystallization screens, but these could not be optimized. Therefore, **1** was co-crystallized with *ec*TS and dUMP.

*ec*TS was concentrated to around 6 mg/mL, dUMP was added to a final concentration of 3.3 mM, and the inhibitor **1** was added to a final concentration of 3.3 mM. Additional inhibitor was added right before the crystal set-up. The optimized final crystallization condition was 20-21 % PEG 8000, 0.1 M TRIS pH 7.9, 0.2 M MgCl₂, and 5 mM DTT at room temperature. To obtain better crystals conditions were optimized using additive screens. Finally, much better diffraction with MnCl₂ and Ethanol (added separately) was observed and data were collected. Crystals were grown in hanging drops by the vapor diffusion method using a Mosquito nanoliter-scale robotic workstation (TTP Labtech). Crystals reached final size in ~15 days.

3. X-ray data collection and structure determination

X-ray diffraction data were collected from cryo-protected crystals (in 20 % ethylene glycol) at Beamline 8.3.1 of the Advanced Light Source (Lawrence Berkeley National Laboratories) on a 315r CCD detector. The monoclinic crystals diffracted to 1.75 Å resolution. Data were indexed, reduced, and scaled with XDS.⁴⁶ The structure was determined by molecular replacement using Phaser⁴⁷ and using liganded *ec*TS with the ligands removed (1AXW) as a search model. Restrained positional refinement and refinement of isotropic B-factors, and TLS refinement were carried out using Phenix.⁴⁸ NCS restraints were not used because of small but significant conformational differences between the subunits in the asymmetric unit. Manual building was done using Coot.⁴⁹ Data collection, processing statistics, *R* factors and statistics for the final structure are listed in Table 1.

4. Modeling of the hTS complex with dUMP and 1

Inhibitor **1** was modeled in the structure of hTS using the program PLOP (Protein Local Optimization Program).⁵⁰ **1** and dUMP were prepared using Schrodinger's hetgrp_ffgen utility bundled with PRIME.⁵¹ The structure of hTS bound to raltitrexed (PDB ID: 1HVY)³⁸ served as a template for the hTS model. One conserved water molecule (Wat980) from 1HVY was included in the minimization. **1** was placed in the hTS active site by superposing the *ec*TS-dUMP-**1** complex (chain A) on hTS (1HVY, chain A). Protein residues within 5 Å radius of **1** were minimized to remove clashes of **1** with hTS. After the protein minimization, **1** was minimized in the enlarged binding site. dUMP was modeled without a covalent link to Cys195, as was observed in several subunits of the *ec*TS-dUMP-**1** structure.

4. Synthetic chemistry and development of 1

Inhibitor **1** is D-Glutamic acid, N-[4-[2-propyn-1-yl][(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-, sodium salt (1:3); CAS registry number 1097638-00-0. The chemical structure of **1** is shown in Fig. 1 and Scheme 1A. The molecular formula of the trisodium salt is C₃₂H₃₀N₅Na₃O₁₀ and has a molecular weight of 713.58 g/mol. The solid form is amorphous, hygroscopic, and with a glass transition temperature of approximately 120.6 °C.

Synthetic chemistry. General Information

A synthesis of **1** Drug Substance was established *via* a four-stage process referred to as the SR-1 process, and performed under contract by Regis Technologies (Morton Grove, IL). Chemicals and reagents were purchased from commercial suppliers and were used without further purification.

SR-1 Stage 1; L-γ-glutamyl-D-glutamic acid tris(1,1-dimethylethyl) ester (Scheme 1B)

N-[N-[(phenylmethoxy)carbonyl]-L-γ-glutamyl]-D-glutamic acid, tris(1,1-dimethylethyl) ester, 3.5 kg, was dissolved in ethanol and hydrogenated in the presence of 5% Palladium on Carbon under a 20 pound per square inch (gauge) hydrogen atmosphere. After the uptake of hydrogen ceased, the reaction was filtered through celite to remove the catalyst, and the catalyst cake was washed with methylene chloride. The resulting solution was stripped to dryness, then dissolved in ethylene dichloride, and stripped to dryness under reduced pressure to give 2.54 kg (approximately 94 % yield) Stage 1 product as a clear yellow oil. Ethylene dichloride was removed through subsequent processing in the remainder of the synthesis and verified by Gas Chromatography of the Drug Substance to be below < 5 ppm.

SR-1 Stage 2; N-[4-[2-propynyl]-[(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid tris(1,1-dimethylethyl) ester (Scheme 1C)

4-[2-propyn-1-yl]-[6S-4,6,7,8,-tetrahydro-2-(hydroxylmethyl)-4-oxo-3H-cyclopenta[g]quinazolin-yl]amino]-benzoic acid, 1.52 kg, was charged to 32 kg dimethylformamide (DMF) solvent. To this mixture was added a solution of 2.53 kg L-γ-glutamyl]-D-glutamic acid tris(1,1-dimethylethyl) ester (Stage 1) dissolved in 10.1 kg of DMF. To this combined mixture was added 0.79 kg of 1-hydroxybenzotriazole hydrate, 0.87 kg of N-methylmorpholine and 0.82 kg of dimethylaminopropyl ethylcarbodiimide hydrochloride. This mixture was stirred at room temperature for 12 hours, and then poured into water, and extracted into ethyl acetate. The product containing ethyl acetate portion was stripped to dryness to obtain 3.13 kg of oil. This material was purified using silica gel chromatography, eluting with methanol/methylene chloride. The eluent was stripped to dryness to give 1.22 kg (38 % yield) of the Stage 2 product.

SR-1 Stage 3; N-[4-[2-propynyl][(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid (Scheme 1D)

N-[4-[2-propynyl][(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid tris(1,1-dimethylethyl) ester (Stage 2), 407 g, was dissolved in 4.1 L of trifluoroacetic acid and stirred at 0 to 5 °C for about 2 hours. The trifluoroacetic acid was removed by vacuum distillation, and replaced with 17 L of methylene chloride. The methylene chloride was removed by vacuum distillation, and a red oil resulted. Methyl tert-butylether (MTBE, 3.4 L) was charged to the oil and the mixture was stirred until a solid resulted. The solid was isolated by filtration, rinsed on the filter with MTBE, and slurried in acetonitrile, filtered and dried to give 292 g (77 % yield) of Stage 3 product.

SR-1 Stage 4; N-[4-[2-propynyl][(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L-γ-glutamyl-D-glutamic acid sodium salt (1:3), ONX 0801 Trisodium Salt Amorphous Drug Substance synthesis (Scheme 1E)

The Stage 3 product, 557 g, was slowly added to a solution of 278 g sodium bicarbonate and 5.5 L water, resulting in a clear solution. This solution was added to a column containing 32 kg of Sepabeads SP-207SS (Resindion, Mitsubishi Chemical Corporation) and eluted with water, followed by dilute acetonitrile in water. The pure product containing fractions were combined, filtered through a 2 micron cartridge, and lyophilized. The lyophilized solid was redissolved in 20 L water and ultra-filtered using a 5,000 MW cut-off cartridge. The filtrate was lyophilized to give 370 g (67 % yield) of a light-beige powder, ONX 0801 trisodium salt amorphous Drug Substance with a purity by HPLC of 99.5 % a/a, by achiral HPLC 99.6 % ee 6-S chiral purity, and 99.8 % L, D diastereomeric purity.

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Abbreviations

TS	thymidylate synthase
hTS	human thymidylate synthase
<i>ec</i>TS	<i>Escherichia coli</i> thymidylate synthase
dUMP	2'-deoxyuridine-5'-monophosphate
TMP	thymidine-5'-monophosphate
mTHF	N5,N10-methylene-5,6,7,8-tetrahydrofolate
α-FR	α -folate receptor
RFC	reduced-folate carrier system
DDT	dichlorodiphenyltrichloroethane
PMSF	phenylmethylsulphonyl fluoride
a/a	area percent basis
ee	enantiomeric excess

Figure 1.
Chemical structures of the cofactor folic acid and thymidylate synthase inhibitors: CB3717, raltitrexed, pemetrexed, plevitrexed and **1**.

Figure 2.

A) Cartoon representation of crystal structure of the *ecTS* homodimer (blue and grey) with substrate dUMP (green carbons) and inhibitor **1** (pink carbons) bound at the active site shown in CPK representation. **B)** A hydrated magnesium-cluster is found between enzyme and the second glutamate side chain of inhibitor **1**. Final 2Fo-Fc electron density for the inhibitor and Mg²⁺-water cluster (Mg ion and hydrogen bonds depicted in green) is shown in blue.

Figure 3.

Schematic representation of **1** binding interactions with *ecTS* generated by the program LigPlot+. ⁴¹ The covalent bonds of BGC 945 are depicted in purple, and orange for the protein. Hydrogen bonds are illustrated in green and hydrophobic interactions are represented as red eyelashes around the protein residue names and ligand atoms. Only a few waters are shown for clarity (cyan spheres), for example the water molecules of the Magnesium-cluster (Mg^{2+} in green) near the diglutamate moiety.

Figure 4. Stick representation of the inhibitor-binding site in the *ecTS* complex focusing on the di-Glu environment. Water-mediated hydrogen bonds connecting the inhibitor to the protein are shown as yellow dashes. Waters involved in the network are shown as red spheres. The inhibitor is shown with pink carbons. The magnesium cluster is shown with cyan (for water) and green (for Mg²⁺) spheres. For comparison, polyglutamylated raltitrexed from an *ecTS*-dUMP-raltitrexed complex (PDB 2BBQ) is shown as thin green lines overlaid on **1**.

Figure 5. Views of *ec*TS and *h*TS active sites bound to folate mimetic inhibitors

A) Crystal structure of *ec*TS bound to **1** and dUMP. **B)** Crystal structure of *ec*TS bound to **1** (green carbon atoms) compared to the crystal structure of *ec*TS bound to raltitrexed (grey carbon atoms, PDB ID 2KCE). **C)** Model of *h*TS bound to **1**. A crystallographic water molecule (Wat980) included in the modeling is depicted with hydrogens. **D)** Model of *h*TS bound to **1** (yellow carbon atoms) compared to the crystal structure of *h*TS bound to raltitrexed (cyan carbon atoms, PDB ID 1HVV). **E)** Superposition of crystal structure of *ec*TS bound to raltitrexed (grey carbon atoms) on crystal structure of *h*TS bound to raltitrexed (cyan carbon atoms). **F)** Superposition of the crystal structure of *ec*TS bound to **1** (green carbon atoms) on the model of *h*TS bound to **1** (yellow carbon atoms).

Scheme 1.**A)** Molecular Structure of **1** Trisodium Salt Drug Substance.

B) L- γ -glutamyl-D-glutamic acid tris(1,1-dimethylethyl) ester. **C)** N-[4-[2-propynyl]-[(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L- γ -glutamyl-D-glutamic acid tris(1,1-dimethylethyl) ester. **D)** N-[4-[2-propynyl]-[(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L- γ -glutamyl-D-glutamic acid. **E)** N-[4-[2-propynyl]-[(6S)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-1H-cyclopenta[g]quinazolin-6-yl]amino]benzoyl]-L- γ -glutamyl-D-glutamic acid sodium salt (1:3), ONX 0801 Trisodium Salt Amorphous Drug Substance.

Table 1

Data collection and refinement statistics.

PDB code	4ISK
Data Collection	
Space group	$P2_1$
N molecules per AU	8
Cell dimensions (Å)	
a,b,c (Å)	95.9, 85.5, 134.3
β (deg)	109.4
Resolution (Å)	30.0 - 1.75
Total reflections	602132
Unique reflections	200975
Completeness (%)	99.8 (97.8) ^a
R_{merge} (%)	7.0 (65.3) ^a
Redundancy	2.98 (3.04) ^a
$I/\sigma(I)$	8.79 (1.59) ^a
Wilson B-factor	20.41
Refinement	
Resolution (Å)	29.8 - 1.75
No. of reflections (test set)	200689 (4014)
R_{work}/R_{free} (%)	0.18 / 0.23
No. atoms	37536
protein	35916
ligand (inhibitor 1)	368
ligand (UMP/UMC)	245
ions (Mg ²⁺)	7
water	1000
Average B -factor (Å)	34.40
rms deviations	
bond length (Å)	±0.013
bond angles (deg)	±1.44

^a Statistics for the highest resolution shell are shown in parentheses.