Published in final edited form as:
Cancer Res. 2009 June 1; 69(11): 4791–4799. doi:10.1158/0008-5472.CAN-09-0615.

Bacterial Cytosine Deaminase Mutants Created by Molecular Engineering Demonstrate Improved 5FC-Mediated Cell Killing *In Vitro* and *In Vivo*

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Abstract

Cytosine deaminase is used in combination with 5-fluorocytosine as an enzyme-prodrug combination for targeted genetic cancer treatment. This approach is limited by inefficient gene delivery and poor prodrug conversion activities. Previously, we reported individual point mutations within the substrate binding pocket of bacterial cytosine deaminase (bCD) that result in marginal improvements in the ability to sensitize cells to 5FC. Here we describe an expanded random mutagenesis and selection experiment that yielded enzyme variants which provide significant improvement in prodrug sensitization. Three of these mutants were evaluated using enzyme kinetic analyses and then assayed in three cancer cell lines for 5FC sensitization, bystander effects and formation of 5FU metabolites. All variants displayed 18 to 19-fold shifts in substrate preference toward 5FC, a significant reduction in IC_{50} values and improved bystander effect compared to wild-type bCD. In a xenograft tumor model the best enzyme mutant was shown to prevent tumor growth at much lower doses of 5FC than is observed when tumor cells express wild-type bCD. Crystallographic analyses of this construct demonstrates the basis for improved activity towards 5FC, and also how two different mutagenesis strategies yield closely related, but mutually exclusive mutations that each result in a significant alteration of enzyme specificity.

Keywords

Cytosine deaminase; suicide gene; 5-fluorocytosine; random mutagenesis; bystander effect

INTRODUCTION

Cytosine deaminase (CD; EC 3.5.4.1) is responsible for the conversion of cytosine to uracil and ammonia, providing an important mechanism for pyrimidine salvage in microbes. Because this activity is not found in mammalian cells(1,2), CD is being explored for use in suicide gene therapy (SGT) due to its ability to also convert the antifungal agent 5-fluorocytosine (5FC) to

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the potent antimetabolite, 5-fluorouracil (5FU)(3–6). Intracellularly, 5FU is subsequently metabolized by endogenous enzymes to 5FdUMP, an irreversible inhibitor of thymidylate synthetase (TS) thereby restricting the production of dTMP and downstream phosphorylated products. Depletion of dTTP pools results in inhibition of DNA synthesis and leads to apoptosis (7–9). A phenomenon known as the bystander effect (BE) provides an extension of cell killing beyond the cells expressing the suicide gene to neighboring cells(10–13). A combination of factors is thought to participate in the BE including transfer of antimetabolites and/or suicide enzymes through gap junctions, diffusion and apoptotic vesicles. An immune-related response also contributes to the BE although in a delayed fashion. A potent BE is critical to successful tumor ablation especially in light of the inefficient viral and non-viral gene delivery systems currently available. In order to support an effective BE, sufficient antimetabolites must be generated in suicide enzyme-expressing cells.

Unfortunately, wild-type CD displays relatively poor turnover of 5FC, thus limiting the overall therapeutic response. As such, high doses of 5FC are necessary and result in undesirable side effects primarily due the presence of microbes in the intestinal tract that encode CD. The rapid half-life of 5FC in blood serves to further limit the availability of 5FC at the tumor site. To overcome the constraints associated with poor 5FC activation, we sought to optimize the activity of bCD towards 5FC using regio-specific random mutagenesis by targeting two key regions of the active site. From earlier studies, residue D314 in wild-type bCD was shown to play a key role in substrate recognition(14,15). In particular, the D314G and D314S substitutions display a shift in substrate preference towards 5FC. However, these variants provide only a modest 2–4-fold decrease in IC_{50} for 5FC *in vitro* as compared to wild-type bCD-expressing tumor cells.

Using structural information as well as previous mutagenesis results, two regions lining the active site of bCD (residues 149–159 and 310–320; Fig.1) were targeted for random mutagenesis to identify mutants with further enhanced 5FC sensitivity *in vitro* and *in vivo* (16,17). Such optimized CD variants that allow lower, less toxic 5FC doses to achieve efficient cell killing and an enhanced BE will likely provide meaningful and significant clinical benefit when used in SGT protocols for the treatment of a variety of cancers.

MATERIALS AND METHODS

Materials

Oligonucleotides were obtained from IDT (Coralville, IA). Enzymes were purchased from New England Biolabs (Beverly, MA). Polyclonal bCD-antibody was generated by Harlan (Harlan, Indianapolis, IN). DNA purification was done using Wizard-PCR kit (Promega, Madison, WI), HiSpeed-Plasmid Mini Kit (Qiagen, Valencia, CA), and StrataPrep EF-Plasmid Midikit (Stratagene, La Jolla, CA). Alamar Blue was purchased from Serotec (Oxford, UK). Nickel affinity chromatography reagents were purchased from Qiagen. All cell culture reagents were purchased from Gibco (Carlsbad, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Bacterial strains

Escherichia coli strain GIA39(DE3), which is deficient in CD and orotidine 5’-phosphate decarboxylase activities, was used in the genetic complementation assays for CD activity (15). The *E. coli* strains NM522 and XL1-Blue were used as recipients for certain cloning and mutagenesis procedures. *E. coli* BL21(DE3) and BL21-RIL (Novagen, Madison, WI) were used for protein purification.

Cell lines

Rat C6 glioma cells (C6) were purchased from ATCC (Manasass, VA). Human colorectal carcinoma cells (HCT116) and human prostate carcinoma cells (DU145) were provided by Dr. Neal Davies (Washington State University, Pullman, WA. Growth conditions for the cells lines are described in detail by ATCC. Transfected cells were cultured in media supplemented with blasticidin at 4μg/ml (C6 and DU145) or 6μg/ml (HCT116).

Library construction and selection of 5FC active clones

A bCD expression library encoding variants randomized across residues149–159 was constructed, followed by insertion of randomized sequences spanning residues 310–320(Table S1). Six overlapping oligonucleotides (MB271–MB276) were used to synthesize a 256bp DNA fragment including the 11 codons (149–159) that were randomized at 9% mutation frequency. The codons for the 310–320 residues were also randomized at 9% and the 238bp DNA fragment was synthesized using the six oligonucleotides, MB384–MB387 and MB267–MB268. Plasmid DNA purified from the 149–159 mutagenesis selection and the randomized 310–320 region fragment were digested with complementary restriction enzymes and ligated together. Transformation and selection for 5FC active mutants were performed as described previously using lower 5FC concentrations (10-0.5μg/ml) in plate assays(14,15).

Enzyme assays

The activity of wild-type and variant bCD lysates was assayed by monitoring the absorbance change due to the consumption of cytosine at 286nm and the production of 5FU at 316nm using an HP8452A Diode Array Spectrophotometer (Olis, Bogart, GA) at room temperature for 10min following a protocol adapted from Hayden *et al.*(18). Cleared cell lysates were prepared as described previously(14). Selected bCDs were expressed in *E. coli* BL21(DE3) and enzymes were purified by Ni-NTA chromatography and quantified as described(14). The kinetic constants were determined by plotting initial reaction rates fitted to the Michaelis-Menten equation using KaleidaGraph software (Synergy Software, Reading, PA).

Construction of mammalian expression vectors

The wild-type and mutant bCD were subcloned as *Nco*I(blunt-ended)/*Xho*I fragments into pCDNA6/*myc*-HisB (Invitrogen, Carlsbad, CA) digested with *Eco*RV and *Xho*I. Site-directed mutagenesis was performed to overlay D314S mutation into pCDNA:1525 using QuikChange mutagenesis according to the manufacturer's protocol (Stratagene, La Jolla, CA).

In vitro cytotoxicity assays

One μg of each plasmid DNA was used to transfect 1×10^5 C6, HCT116 and DU145 cells by lipofection using FuGENE 6 transfection reagent (Roche, Penzberg, Germany) at a 3:1 ratio according to the manufacturer's instructions. Protein expression level was determined by immunoblot analysis as previously described(19). For *in vitro* cytotoxicity assays pools of transfectants were transferred to 96-well microtiter plates at an initial density of 500 (C6) or 1000 cells/well (HCT116/DU145). Following cell adherence overnight, 5FC (0–30mM) was added and the cells were incubated for 6 days, at which time the redox indicator dye Alamar Blue was added. Cell survival was determined by fluorescence recorded at a 530/590nm as described by the manufacturer and data were plotted with the SD. At least three replicates were performed.

In vitro Bystander Effect (BE) assays

C6, HCT116 and DU145 cells stably transfected with pCDNA were mixed at different ratios with stable transfectants harboring either pCDNA:bCD, pCDNA:1246, pCDNA:1525 or

pCDNA:1779. The mixed cells were transferred to 96-well microtiter plates at final density as described above. Following cell adherence overnight, 5FC (4mM (C6) or 10mM (HCT116/DU145) was added and the cells were incubated for 6 days. Cell viability was determined as described above. At least three replicates were performed.

HPLC metabolite analysis

Stably transfected cells (HCT116) were plated in 6-well dishes (Corning, New York, NY) at a concentration of 4×10^4 cells/well. The reaction was stopped at various hours post-dose (2.5mM 5FC) by adding 200μl/well of 94:6 (v/v) acetonitrile/glacial acetic acid and the level of 5FU in cell lysates was detected using an isocratic, reverse-phase high performance liquid chromatography (HPLC) method(20). Metabolite separation was carried out on a Phenomenex Luna C18(2) analytical column (250mm ×4.6mm, 5μm particle size) (Phenomenex, Torrence, CA) at a flow rate of 1ml/min. The mobile phase was formic acid and water (1:99, v/v). 5FU was detected at an ultraviolet wavelength of 285nm and eluted at 5.8min. Total metabolite levels were calculated relative to a known concentration of an internal standard (5-chlorouracil).

Introduction of individual mutations at residues 316 and 317

The QuikChange kit was used to create amino acid substitutions F316C (MB480–481), F316L (MB482–MB483), F316V (MB484–MB485), D317G (MB478–MB479) F316C/D317G (MB490–MB491), F316L/D317G (MB492–MB493), F316V/D317G (MB494–MB495). After DNA sequence confirmation, the resulting plasmids were designated pETHT:F316C, pETHT:F316L, pETHT:F316V, pETHT:D317G, pETHT:F316C/D317G, pETHT:F316L/D317G and pETHT:F316V/D317G.

Xenograft tumor model

Pools of HCT116 cells stably transfected with pCDNA, pCDNA:bCD or pCDNA:1525 (0.5×10^6 cells in 200μl of PBS (pH 7.2)) were injected subcutaneously into the flanks of 5–6 week old female nude mice (n=5)(BALB/cAnNCr-nu/nu; National Cancer Institute, Fredrick, MD). When tumors reached 3–4mm (day 0), PBS or 5FC (375mg/kg) was administered by intraperitoneal injection twice/day for 21 days. Starting at day 0, the tumor volume was monitored using caliper measurement every other day, calculated using the formula: $4/3\pi$ ((width × length × height)/2) and analyzed for statistical significance using Student's t-test.

X-ray crystallography

Three sequential mutations (V152A, F316C and D317G) were introduced by site-directed mutagenesis into the wild-type bCD. The expression and purification of the resulting construct (1525) was carried out as previously described(21). Crystals of 1525 were grown in sitting drops by vapor phase equilibration against reservoirs containing 10%–15% PEG 6K, 200mM $MgCl_2$ and 100mM HEPES (pH 7~8). Crystals were grown using microseeds of wild-type enzyme crystals (transferred by streaking drops with a fiber) and grew within one week to approximately 500μm in each dimension.

Crystals were cryo-preserved as previously described and found to be isomorphous with crystals of wild-type enzyme(16). Data were collected on a Rigaku RAXIS IV^{++} area detector, using X-rays produced by a Rigaku MicroMax™-007 HighFlux(HF) microfocus X-ray generator (λ=1.54Å). Data collection and reduction were performed using the CrystalClear™ software package (Rigaku, The Woodlands, TX). The structures were refined using the CCP4 crystallographic software suite(22).

RESULTS AND DISCUSSION

Many factors limit the overall efficacy of current SGT approaches for cancer when CD and 5-FC are used. These include rapid turnover and clearance of 5FC in serum, inefficient gene delivery efficiency such that only a small population of tumor cells expresses the suicide gene, and poor enzyme activity towards the prodrug coupled with efficient competition for the enzyme's active site by the cells' endogenous pool of cytosine(1,23–25). As a means to overcome these obstacles, we sought to create novel CD variants with significantly improved kinetic preferences towards 5FC. We employed random mutagenesis to introduce multiple amino acid substitutions in two regions lining the active site of bCD and used both positive and negative selection in *E. coli* to identify variants with the ability to confer enhanced 5FC sensitivity to three cancer cell lines *in vitro* and *in vivo*. A similar strategy was used to identify several Herpes Simplex Virus thymidine kinase (HSVTK) mutants with enhanced tumor ablation capabilities(26,27). One such variant, SR39, is currently being used in a phase III clinical trial for prostate cancer. While HSVTK is widely used, there are two key reasons for optimizing additional suicide genes: 1) not all cancers are equally responsive to the same drug and 2) should treatment with one suicide gene fail, alternate suicide genes that the immune system has not been exposed to previously, could be used in additional rounds of therapy to ablate tumors.

Construction of random library and mutant selection

Previous studies revealed that individual point mutations in the substrate binding pocket of bCD, that confer sensitivity to *E. coli* at 1μg/ml 5FC, do not achieve substantial *in vitro* activity. We therefore aimed to direct the bCD variants to evolve more efficiently towards 5FC by heavily randomizing the two regions lining the active site in a stepwise approach (Fig.1). A single-targeted library randomized at the 149–159 residue region was initially generated and ~590 *E. coli* that harbored functional CD were pooled. A second randomized coding region, corresponding to the incorporation of mutations across residues 310 to 320, was then introduced and approximately 3700 enzymatically active variants were identified from an estimated 1.35×10^6 total number of transformants. Of those, 849, 365, 62, 20 and 12 clones conferred sensitivity at 20, 10, 2, 1 and 0.5μg/ml 5FC, respectively. This lowest 5FC dose is 40-fold lower than the sublethal dose for *E. coli* expressing wild-type bCD.

Sequence analysis

Sequences of 47 variants sampled from each round of screening (data not shown) revealed that all targeted codons had at least one amino acid substitution. The average number of substitutions was 2.6, close to the theoretical average number of 2.1 for 9% randomization. Sequences of the 12 variants identified at 0.5μg/ml 5FC were somewhat unexpected (Supplementary Table S2) because substitutions in the 149–159 residue region occurred infrequently and appeared unrelated to the degree of 5FC activity, and many of the same substitutions were observed in mutants with less or no 5FC activity. In contrast, the substitutions at the 310–320 residue region showed a strong relationship with 5FC sensitivity: in particular, substitutions at positions D317 and F316 with smaller hydrophobic residues were observed for the most active constructs. Few substitutions were observed at F310, G311, P318, W319 and Y320, suggesting these sites are important for overall catalytic activity or for structural stabilization. Sequence analysis reveals that the most active 12 mutants all have substitutions at D314, F316 and/or D317. -As described in detail below, three mutants (referred to as constructs 1246, 1525 and 1779) were identified as conferring sensitivity at significantly lower concentrations of 5FC, and were chosen for further characterization.

To elucidate the participation of individual amino acid substitutions at F316 and D317 found in all three constructs (1246=D314E/F316L/D317G, 1525=V152A/F316C/D317G and

1779=V315L/F316V/D317G), site-directed mutagenesis was used to generate the substitutions: F316L, F316C, F316V, D317G, F316L/D317G, F316C/D317G and F316V/D317G. Results from *E. coli* complementation assays indicate that all mutants display CD activity and that the individual D317G substitution and the double F316C/D317G substitutions found in 1525 appears to be responsible for conferring the greatest degree of sensitivity to 5FC.

Enzyme assays of select mutants

To further characterize the 12 mutants identified by growth inhibition on plates containing 0.5μg/ml 5FC, cell lysates were used initially to assess cytosine and 5FC conversion levels. Lysates of the mutants 1246, 1525 and 1779 showed the largest 5FC conversions of the variants and the wild-type, while the other variants displayed weak or no activity (data not shown). These three mutants and wild-type bCD were then purified to near homogeneity using a Ni-NTA chromatography column and their kinetic constants for cytosine and 5FC conversion were determined.

All three random mutants display reduced kinetic parameters towards cytosine (Table 1). Mutant K_m values are increased 3.7–10.7-fold relative to wild-type bCD and k_{cat} values for the mutants range from 16.6–29-fold lower, corresponding to a 82.2–305.4-fold reduction in catalytic efficiency (k_{cat}/K_m). When 5FC is used as the substrate, K_m values are modestly increased 1.8–3.3-fold and k_{cat} values are increased 4.3–5.9-fold, to yield an overall improvement in k_{cat}/K_m for 5FC of 1.5–2.9-fold as compared to wild-type bCD. The combination of reduced activity towards cytosine, and increased k_{cat} for 5FC, results in a significant (18.2 to 19.2-fold) shift in the relative substrate specificity towards 5FC relative to wild-type bCD.

In vitro analysis of mutant bCDs

Mutant and wild-type bCD genes were cloned into the expression vector, pCDNA, and used to stably transfect C6, HCT116 and DU145 cell lines. Results from prodrug sensitivity assays demonstrate that all three variant bCD constructs confer increased sensitivity to 5FC, albeit to varying degrees in the different cell lines (Fig.2A). In C6 transfectants, only a modest reduction in IC_{50} was observed compared to wild-type bCD-transfected cells (1.3–2.7-fold). The IC_{50} values of mutant bCD-transfected DU145 cells were 2.1–5-fold lower than wild-type bCD-transfectants. The mutants displayed the greatest reduction in IC_{50} values in HCT116 transfectants at 2.4–17-fold lower values than bCD. In all cell lines examined, 1525 consistently displayed the lowest IC_{50} values of the three mutants with the best response observed in HCT116 cells. We observed some cell line variance in the degree of 5FC sensitivity. Clinical studies indicate 5FU is an effective chemotherapeutic agent for colorectal, pancreatic and breast cancer, but is less effective for other types of cancers(28). Therefore, the observed cell line differences may simply be a reflection of inherent variations in drug sensitivity exhibited by individual cell types.

Previously we reported the construction and characterization of single site mutations at residue 314 (D314)(14). Here we sought to compare these D314 variants (D314G, D314A and D314S) to the randomly derived mutants (1246, 1525 and 1779) and to explore the possibility that combinations of the best mutants from both series would yield an additive effect. Initially D314G, D314A and D314S and wild-type bCD-transfected HCT116 cells were evaluated for 5FC sensitivity (Fig.2B). Of the three D314 substitutions, D314S displays the greatest response to 5FC with an IC_{50} approximately 3.4-fold lower than that of wild-type bCD and ~5-fold higher than 1525-transfected cells. To assess the impact of the combination mutant, cells transfected with pCDNA, pCDNA:bCD, pCDNA:1525, pCDNA:D314S and pCDNA:1525/D314S were subjected to *in vitro* 5FC sensitivity assays as described above. Results from this comparison indicate that the 1525/D314S overlay does not yield an increase in 5FC sensitivity

over 1525 or the individual D314S mutant or wild-type bCD-transfected cells (Fig.2C). Thus, the mutational routes towards optimal 5FC activation, generated by site-directed or randomizing mutagenesis, yield mutually exclusive sets of mutations that do not act synergistically. Below we provide a possible explanation for this lack of additive response based on details that emerged from 1525 structure determinations.

Bystander effect

The three key mutants were further characterized for their ability to influence the killing of non-bCD expressing cells via the BE. Towards that end, populations of vector and bCD-transfected cells were mixed at various ratios and subjected to either 4mM (C6) or 10mM (HCT116 and DU145) 5FC for six days. The percentage of bCD-expressing cells needed to achieve 50% cell killing ranges from 20–50%, 20–60% and 10–80% for C6, DU145 and HCT116 cells, respectively, when mutant bCDs were examined (Fig.3A–C). In contrast, mixed populations containing wild-type bCD-transfectants displayed no cell killing effect in HCT116 cells and slight to modest effects in DU145 and C6 cells, respectively, and were unable to achieve 50% cell killing in any of the cell lines tested. Mutant 1525 displayed the greatest BE in every cell line examined. In HCT116 cells this is most evident; when only 10% of 1525-expressing cells are present, 50% of the population is killed at a 5FC concentration at which 100% wild-type bCD population remains fully viable.

HPLC analysis of 5FU metabolites

Low transfection efficiencies in SGT impose a reliance on a robust BE for tumor ablation. While results from our kinetic studies suggest an improvement in 5FC deamination activity, we sought to more directly assess the production of 5FU by bCD-transfected cells by high performance liquid chromatography (HPLC). Starting on day 3 the level of 5FU begins to accumulate in lysates from 1525-transfected cells and continues to increase from about 100μg/ml at day 3 to ~400μg/ml on day 7 (last time point) and reflects a 35.8-fold difference in 5FU levels between 1525 and wild-type bCD (Fig.3D). This is in contrast to vector and bCD-transfected cell lysates that show no significant change in 5FU levels over the time course. Bystander experiments with 5FC revealed that all three variant bCDs demonstrated a more robust cell killing in all three transfected cell lines as compared to wild-type bCD-transfected cells. We suggest this is a direct reflection of the amount of 5FU produced in the transfected cells.

In vivo xenograft tumor model

Mutant 1525 demonstrates the best cell killing and BE *in vitro* and was selected for further analysis in an *in vivo* xenograft tumor model for human colorectal cancer. Tumor growth in all groups treated with PBS was indistinguishable with the exception of later points (Fig.4). This statistically insignificant difference in the tumor volume near the end of the time course is likely due to the amorphous nature of the large tumors that made it difficult to obtain consistent measurements. While no difference in tumor volume was observed between mice bearing vector or wild-type bCD tumors throughout or following the 5FC treatment period, the lack of significant tumor growth observed in mice bearing 1525 tumors is in stark contrast. From the time 5FC administration began to the maximum size, wild-type bCD tumors increased in volume from 34.4mm^3 to 1154 mm^3 (30.8-fold) whereas the 1525 tumors increased only from 32.5mm^3 to maximum of 185mm^3 (5.7-fold). Other studies using wild-type bCD show a reduced tumor growth rate using comparable doses of 5FC(29–31). In our hands, no significant antitumor response with wild-type bCD is observed at 375mg/kg twice a day whereas this dose provides a substantial growth restriction in 1525 bearing tumors. As such, 1525 provides significant improvement in tumor growth inhibition using a 5FC dose at which wild-type bCD tumors is completely unresponsive to.

Crystallographic analysis of bCD 1525

Crystals containing the enzyme in complex with the mechanism-based inhibitors 4-(R)-hydroxyl-3,4-dihydropyrimidine (DHP) or 5-fluoro-4-(S)- hydroxyl-3,4-dihydropyrimidine (5F-DHP) were generated out by soaking crystals in buffer containing 10mM 2-hydroxypyrimidine or 5-fluoro-2-hydroxypyrimidine (Aldrich, St. Louis, MO), respectively. In these experiments, the pyrimidine compounds are converted enzymatically to a tightly-bound hydrated adduct that mimics the enzyme transition state. The statistics for data collection and refinement are summarized in Supplementary Table S3.

Unbiased Fourier difference (F_{obs}-F_{calc}) maps were calculated using diffraction data of 1525 soaked in DHP and 5F-DHP (Fig.5a), respectively, using the phase information calculated from a protein model consisting of the wild-type bCD enzyme (PDB accession code 1K70) with residues 152, 316 and 317 changed to alanine and the bound ligand DHP removed. The density of the bound compounds clearly demonstrated the presence of bound compounds in positions nearly identical to those observed in the wild-type enzyme. The orientation of the pyrimidine ring within the density maps is confirmed by the presence of additional density in the 5F-DHP complex, corresponding to the additional fluorine substituent. However, as compared to the wild-type enzyme, the compounds appear to exhibit significant conformational heterogeneity in the active site, particularly evident as disorder and minimal density at the C1 carbon position of both compounds. As a result, coordinates of the bound compounds were not included in the final refined models of 1525. These structural models, along with the X-ray diffraction amplitudes, have been deposited in the RCSB structural database (accession code 3G77) in order to allow independent calculation of difference maps by interested investigators.

In the previously described structure of D314A mutant, the D314A substitution creates a hydrophobic pocket to accommodate the fluorine atom of 5F-DHP(16). This mutation not only eliminates a spatial clash with the fluorine atoms, but also provides a favorable van der Waals contact with the bound 5F-DHP. In contrast, none of the three side chains mutated in the active site of 1525 are in direct contact with bound substrate. However, as a result of the mutation of D317 (which eliminates a carboxylate side chain), the wild type residue D314, which is the side chain nearest to the 5'-position of the substrate, swings away from the active site (Fig.5b), creating a neighboring cavity and reducing the local negative charge. This movement produces a similar chemical and structural effect as observed in D314A mutant.

Two other mutations (V152A and F316C) do not seem to affect the active site directly. This is further supported by experiments in which the substitutions found at F316 and D317 in 1246, 1525 and 1779 were individually examined for the ability to confer 5FC sensitivity in complementation assays. Close examination of the sequences at position D314 and D317 of the 12 bCD variants identified from the genetic complementation studies reveals D317G substitutions (but no D314A, S or G substitutions) in nine of the isolates, whereas the remaining three clones have A or G substitutions at D314 but none at D317. Combining 1525 (V152A/F316C/D31G) and D314S substitutions resulted in a reversal of 5FC sensitivity rather than an additive effect. Taken together, these results support the notion that enhancement of 5FC activity occurs when mutations exist at D314 or the D317G substitution is present but that these substitutions are mutually exclusive.

Cancer chemotherapy treatments can result in significant and often debilitating side effects due to the absence of distinct biochemical differences that occur between normal and neoplastic cells. SGT provides an exceptional opportunity to introduce unique biochemical characteristics to cancer cells that can then be exploited for a safer, less toxic and more effective treatment. Two suicide gene/prodrug systems have garnered significant attention and are undergoing preclinical and clinical evaluations; the widely used HSVTK/ganciclovir and the CD/5FC systems(30). Despite initial enthusiasm for the use of these suicide gene/prodrug approaches,

limitations such as insufficient gene delivery to the tumor site and poor prodrug conversion properties of the suicide enzymes have restricted the full potential of SGT. From over a million bCD mutants we have identified one bCD variant (1525) that displays superior 5FC kinetic, cell killing, bystander and tumor growth restriction activities. Collaborative studies using adenoviral vectors expressing mutant D314A in combination with radiotherapy to treat pancreatic cancer suggested that even the slight improvement of D314A provided enhancement of 5FC-mediated cytotoxicity *in vitro* and *in vivo*(32). Therefore, mutant 1525 will likely provide a significant advantage over wild-type bCD or D314A in at least three important ways: 1) by enhancing 5FC-mediated cell killing through both direct and bystander mechanisms; 2) by allowing the use of lower, less debilitating doses of 5FC to achieve effective cell killing and; 3) by providing an optimized alternative to the use of wild-type CD or HSVTK suicide gene therapy approaches. Furthermore mutant 1525 may be used in a variety of different applications including for restenosis, non-invasive tumor imaging and in negative selection systems.

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ACKNOWLEDGMENTS

This work was supported by NIH grants CA85939 (MEB) and CA97380 (BLS and MEB).

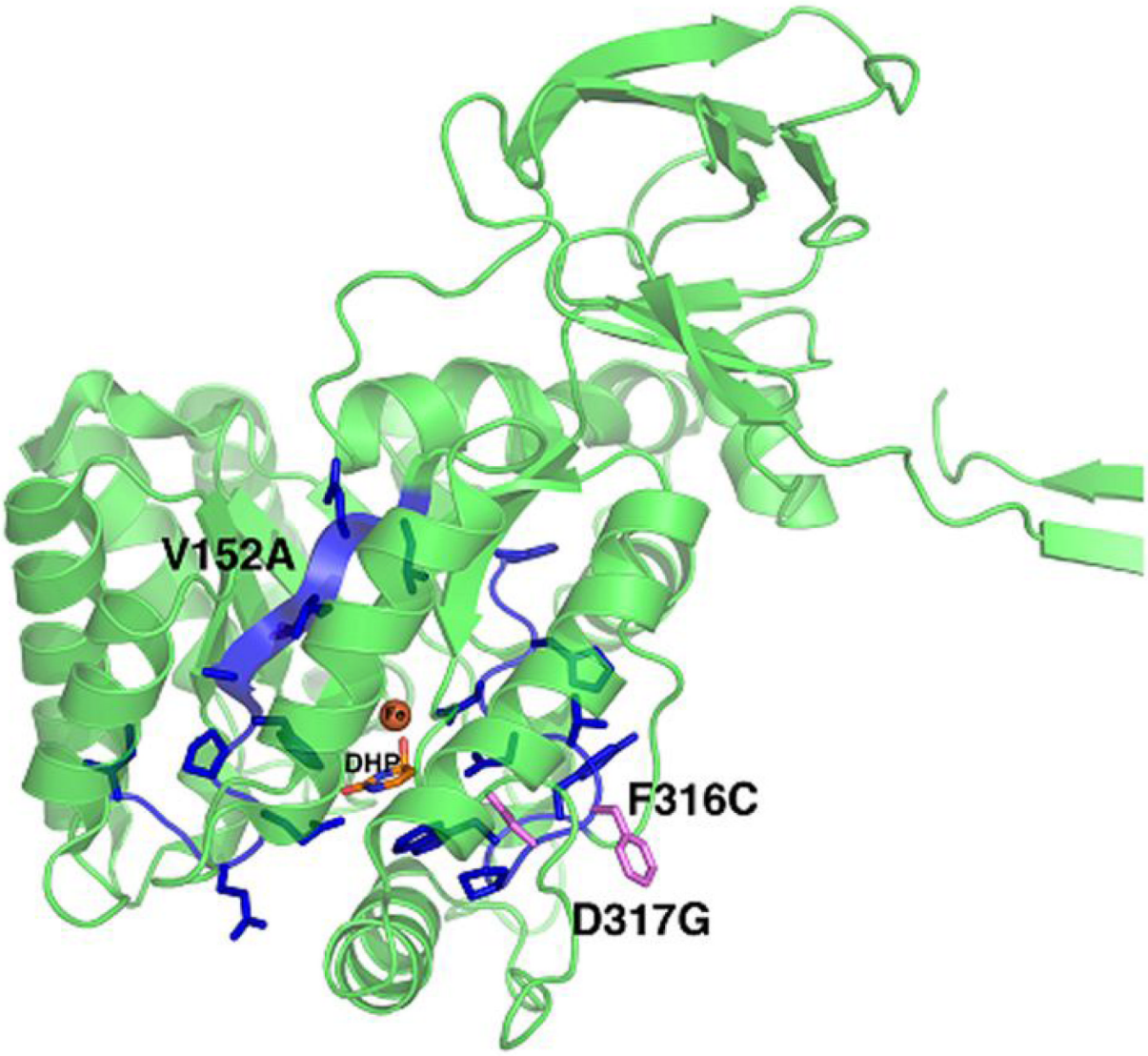

Figure 1.
Cartoon diagram of wild-type bCD monomer. The 22 residues that were subjected to randomization and selection for 5FC sensitization are in blue. The location and identity of mutations (V152A, F316C and D317G; 1525) that gave rise to highest specificity and activity toward 5FC are labeled.

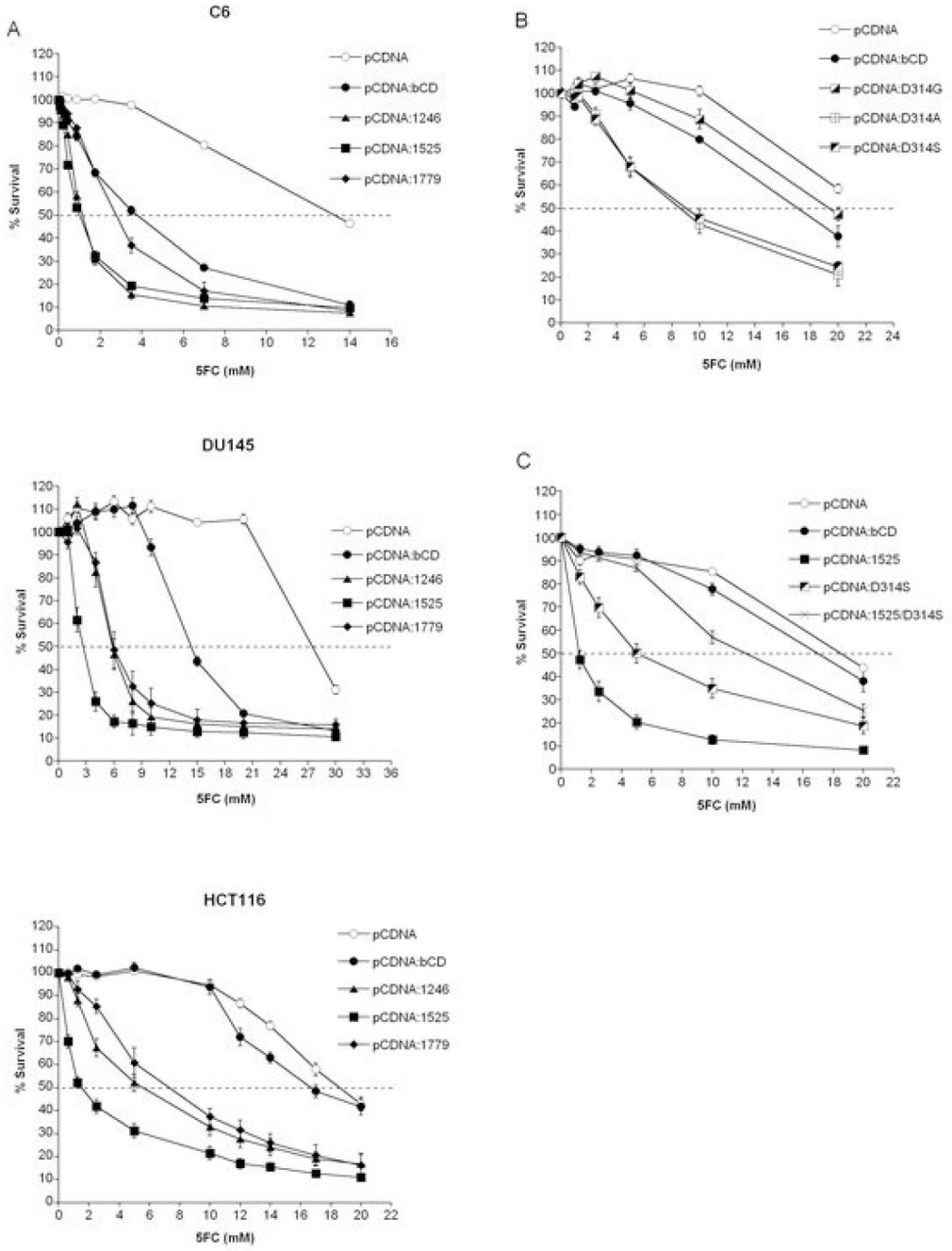

Figure 2.

5FC sensitivity assays of cells stably transfected with wild-type or mutant bCDs. Pools of stably transfected **(A)** C6, DU145 and HCT116 cells containing pCDNA, pCDNA:bCD, pCDNA:1246, pCDNA:1525 and pCDNA:1779 were evaluated for 5FC sensitivity. Pools of stably transfected HCT116 cells containing pCDNA, pCDNA:bCD, **(B)** pCDNA:D314G, pCDNA:D314A and pCDNA:D314S; or **(C)** pCDNA:1525, pCDNA:D314S and pCDNA: 1525/D314S were evaluated for 5FC sensitivity. After 6 days of 5FC treatment, cell survival was determined using Alamar Blue according to the manufacturer's instructions. Each data point (mean ± SEM; n=3; performed with 24 replicates) is expressed as a percentage of the value for control wells with no 5FC treatment. Student's t-test analysis determined that the

differences in IC_{50} values between mutant bCD-and wild-type bCD-transfectants are statistically significant ($P \leq 0.05$).

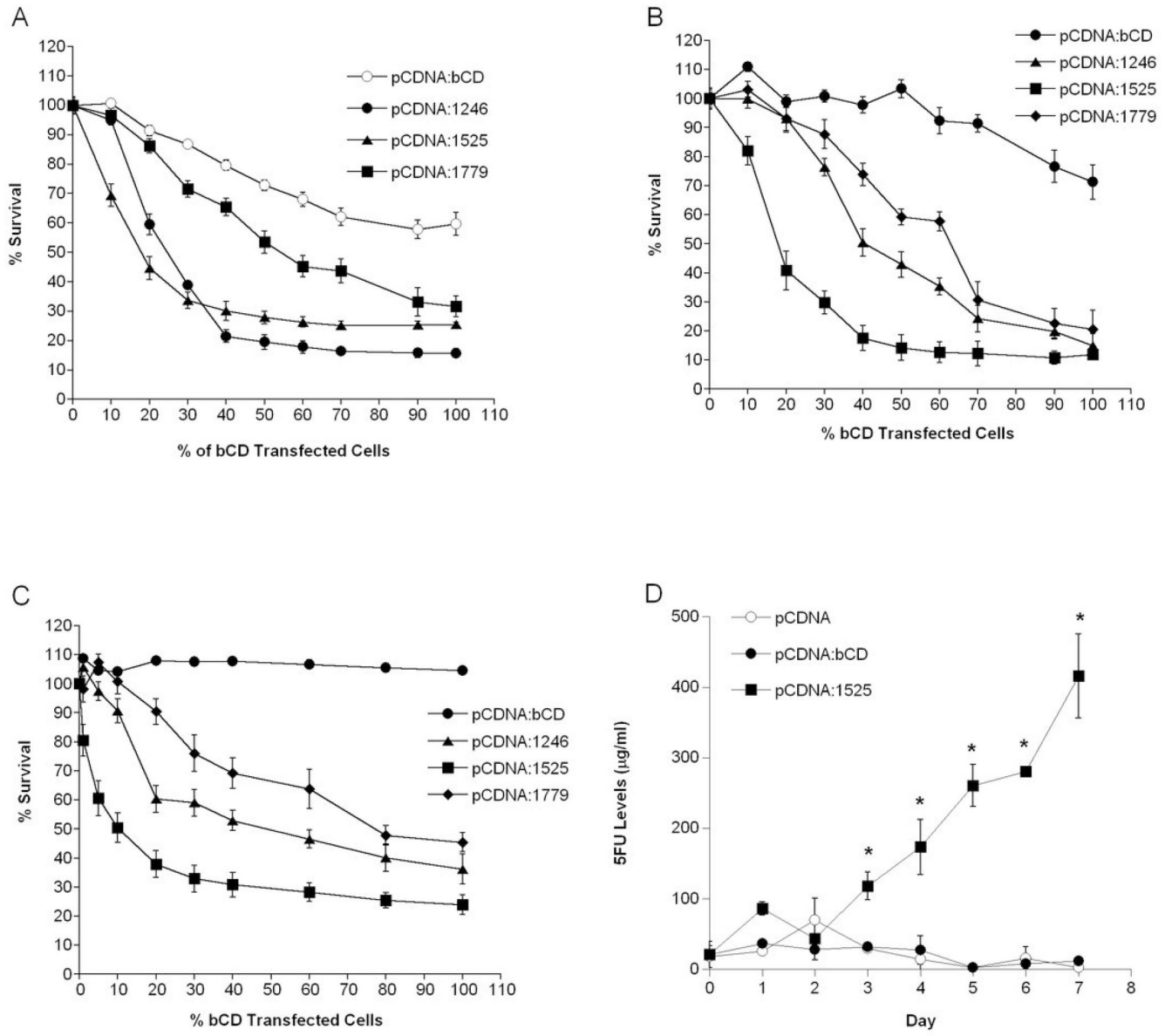

Figure 3.

Bystander analysis of stable transfectants exposed to 5FC. Pools of **(A)** C6, **(B)** DU145, **(C)** HCT116 cells containing pCDNA were mixed with cells harboring either pCDNA:bCD, pCDNA:1246, pCDNA:1525 or pCDNA:1779 at different ratios and were subjected to 4mM (C6) or 10mM (DU145 and HCT116) 5FC for a period of 6 days and cell survival was determined using Alamar Blue. Each data point (mean ± SEM; n=3; performed with 24 replicates) is expressed as a percentage of the value for control wells with vector-transfected cells. Student's t-test analysis determined that the differences between percentage of mutant bCD-and wild-type bCD-transfectants needed to achieve 50% tumor cell killing are statistically significant ($P \leq 0.05$). **(D)** HPLC analysis of 5FU levels in transfected cells. Stably transfected HCT116 cells were incubated with 2.5mM 5FC for up to seven days and metabolite levels calculated relative to a known concentration of an internal standard. Student's t-test was done to compare 1525 versus wild-type bCD transfected cell lysates. Asterisks denote $p \leq 0.005$ (days 3–4) and $P \leq 0.0003$ (days 5–7).

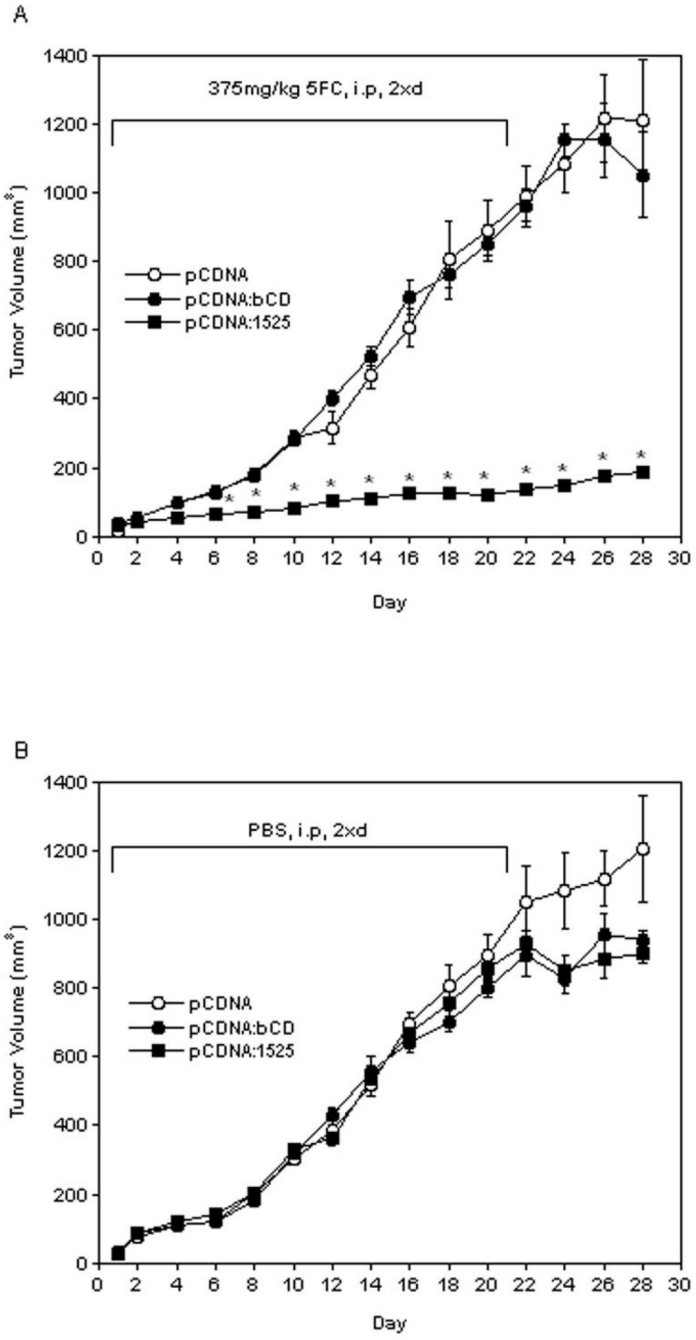

Figure 4.

Tumor growth during and after 5FC treatment in a xenograft model. Pools of HCT116 cells transfected with pCDNA, pCDNA:bCD or pCDNA:1525 were used to seed tumors in nude mice (n=5). When tumor size reached 3–4mm (day 0), **(A)** 5FC (375mg/kg) or **(B)** PBS was intraperitoneally administered twice a day for 8 days. Tumor growth was measured every other day for the duration of the experiment. Tumor volume was calculated using the formula $4/3\pi$ ((width ×length ×height)/2), plotted and analyzed for statistical significance using Student's t-test. Asterisks denote statistical significance ($p \leq 0.05$) in tumor sizes between mice harboring 1525-expressing tumor cells and those that received either empty vector- or wild-type bCD-expressing tumor cells in the presence of 375mg/kg 5FC.

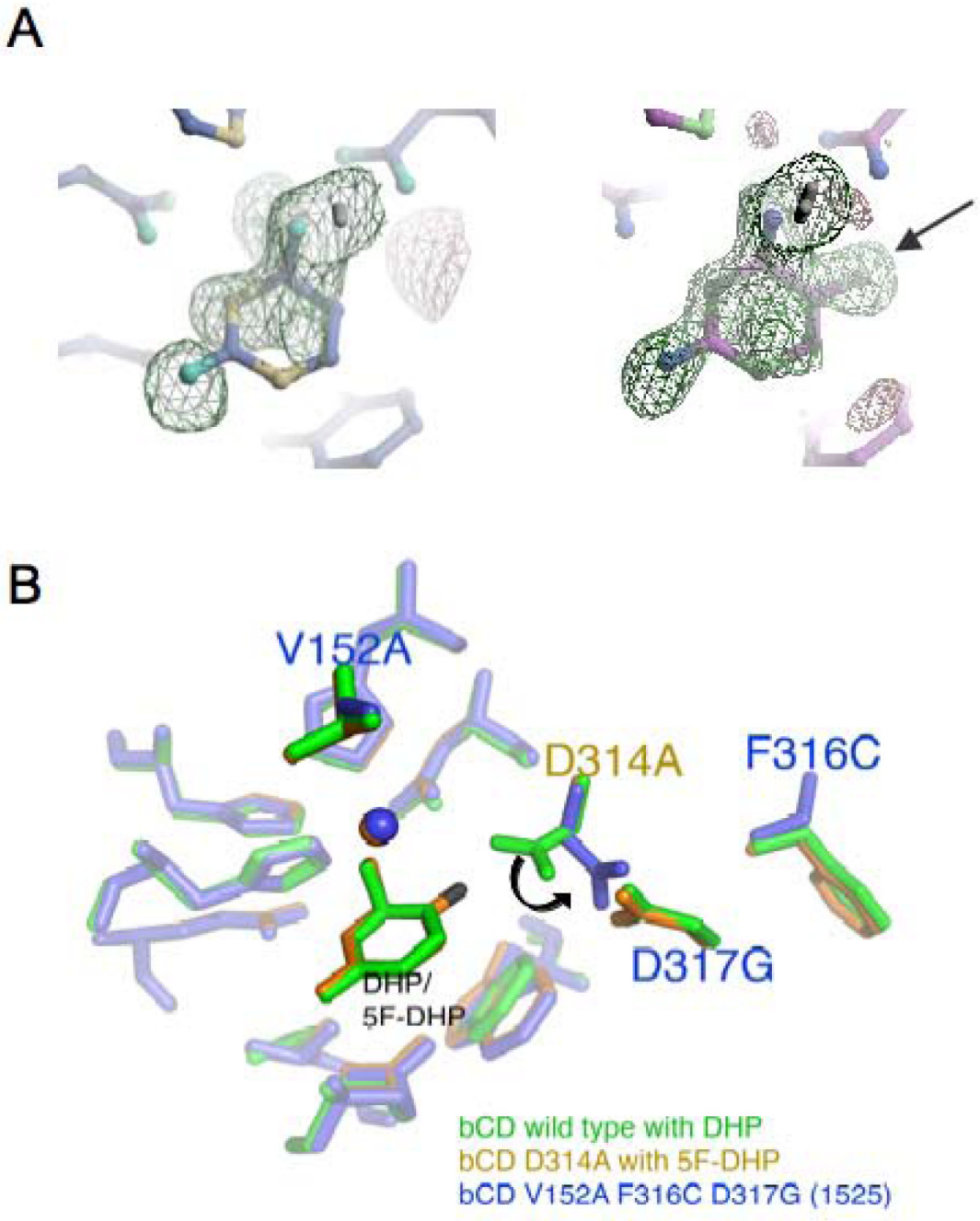

Figure 5.

(A) Electron density for bound substrate analogue 2-hydroxypyrimidine (or dihydroxypyrimidine, DHP) (left) and 5-fluoro-DHP (right) bound in the active site of bCD. As noted in the text, the density for the bound compound indicates disorder, particularly near the C1 carbon. However, the appearance of additional difference density corresponding to the 5-fluoro substituent (arrow) unambiguously confirms the orientation of the inhibitor. **(B)** Comparison of the active sites of wild-type and mutant bCD enzymes. Superposition of the active sites of wild-type bCD in complex with DHP (PDB accession code-1K70), D314A mutant in complex with 5F-DHP (PDB accession code-1RA5) versus 1525 soaked with 5F-DHP. The motion of residue D314 (arrow), into the cavity vacated by removal of a side chain

in the D317G mutation, mimics the structural effect of the previously described D314A and D314G mutations.

Table 1

Enzyme kinetics

	WT-bCD		1246		1525		1779	
	cytosine	**5FC**	**cytosine**	**5FC**	**cytosine**	**5FC**	**cytosine**	**5FC**
K_m(mM)	0.46(0.05)[b]	3.76(0.04)	1.65(0.34)	6.73(2.07)	4.9(1.11)	12.69(4.53)	3.16(1.8)	7.55(0.84)
$k_{cat(sec^{-1})}$	49.68(2.14)	19.71(0.74)	2.13(0.26)	83.68(12.16)	1.69(0.45)	101.74(21.72)	2.98(0.72)	115.24(6.28)
$k_{cat}/K_{m(mM^{-1}sec^{-1})}$	106.85(39.3)	5.24(1.87)	1.29(0.76)	12.44(5.88)	0.35(0.4)	8.02(4.8)	0.94(0.46)	15.27(7.47)
Relative efficiency to the wild type	1	1	0.01	2.37	0.003	1.53	0.009	2.91
Relative substrate specificity[a]	0.95	0.05	0.09	0.91	0.04	0.96	0.06	0.94
Relative substrate specificity to the wild type	**1**	**1**	**0.09**	**18.2**	**0.04**	**19.2**	**0.06**	**18.8**

[a] Cytosine:[k_{cat}/K_m(cytosine)] / [k_{cat}/K_m(cytosine)]+[k_{cat}/K_m(5FC)];5FC: [k_{cat}/K_m(5FC)]/[k_{cat}/K_m(cytosine)]+[k_{cat}/K_m(5FC)]

[b] Standard error