Beilstein-Institut

Bozen 2006, May 15th – 19th, 2006, Bozen, Italy

# Modifying Enzyme Specificity by COmbinatorial Active Site Mutations

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*Received: 1st February 2007 / Published: 5th November 2007*

## Abstract

Recent developments in molecular biology offer new approaches for improving our understanding of enzyme-ligand interactions. The complexity of enzyme catalysis, consisting of ligand recognition and exquisite discrimination followed by rapid catalytic turnover, is now being tapped by modifying ligand specificity. While various approaches to modify specificity have been developed, we apply a 'semi-rational design' approach, whereby residues in proximity to the bound ligand are mutated. Mutations are either random (20 possible amino acids) or semi-random (a subset of amino acids is encoded), and several positions are mutated simultaneously to allow the occurrence of complementary, or compensatory, mutations.

By conducting combinatorial mutagenesis specifically directed toward the active-site area of enzymes involved in drug resistances, we are gaining insights into the nature of the enzyme-ligand interactions underlying these resistance mechanisms. We screen libraries of mutated enzymes for resistance toward their target drug(s), selecting a variety of modified active-site environments that we characterize for binding and reactivity. Here, we present modification of the specificity of a β-lactamase and of a dihydrofolate reductase. Insights into the molecular nature of these modified enzyme-ligand interactions will provide new information for design of more advanced drug gen-

erations. Our work contributes to demonstrate the generality of the combinatorial active-site mutation strategy for modifying enzyme specificity.

## INTRODUCTION

The complexity of natural evolution yields such highly efficient molecules as enzymes, the most efficient known catalysts [1]. This complexity also results in a considerable challenge in attempting to understand how enzymes work: where does their catalytic power stem from? What dictates their exquisite selectivity? We are studying a variety of enzymes by focusing specifically on the active-site environment to explore how sequence variations affect catalytic efficiency as well as specificity in ligand binding.

Natural evolution is generally thought to proceed by genetic point mutation or recombination. The resulting modified genes are conserved if the resulting phenotype, in a given environment, is neutral or beneficial. The gradual accumulation of various neutral or beneficial mutations may provide combinations of mutations that provide a greater benefit in a given environment. In a more rapid imitation of this process, our studies proceed by the application of semi-rational directed evolution to various enzymes. Directed evolution [2] consists in low frequency introduction of randomly-distributed mutations in a gene of interest, followed by selection of the mutated proteins possessing the desired properties. As a result of the random nature of the mutation process, our capacity to sample adequately across the astronomically large 'sequence space' of a protein of interest is very limited. However, it has recently been shown that the majority of mutations that beneficially affect certain enzyme properties (enantioselectivity, substrate specificity, new catalytic activities) are located in or near the active site, more specifically near residues that are implicated in binding or catalysis [3 – 6]. In agreement with these data, we specifically direct mutagenesis to residues in or near the active site, to increase the likelihood of beneficially modifying the catalytic activity relative to random mutagenesis approaches. This 'semi-rational' approach should allow building a “smarter” library than with a random whole-gene mutagenesis scheme [4] and should allow for a greater number of the interesting solutions to be identified.

Even when reducing the explored sequence space to the area encompassing the active site, the number of combinations of mutations to be tested remains extremely large. For example, mutating only 6 residues in the active site to all 20 naturally-occurring amino acids gives $20^6$ ($> 6 \times 10^7$) combinations of mutations to be tested. As a result, the main limitation we are faced with is the necessity of developing high-throughput screening methodologies to allow identification of the desired property under relevant conditions.

Selection of functional, mutated enzymes in the context of drug resistance is a simple yet powerful approach to screening. We previously applied this approach to identify heavily-mutated yet functional variants of TypeII R67 dihydrofolate reductase (R67 DHFR) [7]. R67 DHFR confers drug resistance to bacteria when exposed to the commonly-prescribed antibiotic trimethoprim. We mutated all 16 main active-site residues in a combinatorial

fashion, creating > 1500 new active-site environments within a constant framework. Only a subset of amino acids was encoded at each position to create a reasonably broad chemical diversity while keeping the number of variants relatively small. Selection was performed by challenging *Escherichia coli* harbouring individual variants with trimethoprim: functional variants allowed bacterial propagation. Importantly, the three mutated active sites we selected carried either 12 or 16 mutations relative to the native R67 DHFR; no lightly-mutated, functional variants were identified. This was not unexpected as R67 DHFR had previously been shown to be relatively intolerant to simpler schemes of active-site mutation [8].

That work illustrates the accommodation of non-conservative sequence changes within the context of multiple compensatory mutations. The approach is fundamentally different from the creation of single, active-site point mutations in that it yielded functional solutions that are too complex to have been predictable. The strategy of combinatorially exploring a restricted area of sequence space centred about an active site can allow one to jump over the non-productive exploration of single amino acid changes resulting in low function, to discover more complex enzyme mutants with high function, even in an enzyme where active-site point mutations are generally deleterious (as illustrated with R67 DHFR [7]). Different enzymes exhibit different tolerances to point mutations, and within a given enzyme, specific positions may be more or less tolerant to substitution. The tolerance to substitution of the entire sequence of *E. coli* TEM-1 β-lactamase has previously been mapped [9]. That study showed that specific residues could be classified either as invariant, as tolerant to conservative changes or as those that tolerate mutation to a broad variety of amino acids. Similarly, circular permutation conducted at each residue of *E. coli* DHFR [10] have shown that some locations can withstand permutation, while others generate inactive variants. These analyses help build a picture of the overall 'plasticity' of each enzyme [11]. In the work presented below, we explore the tolerance to combinatorial variation of residues within the active-site area of human DHFR and *E. coli* TEM-1 β-lactamase.

## Results and Discussion

### *Human DHFR combinatorial active-site mutations: increased methotrexate resistance*

The human enzyme dihydrofolate reductase (hDHFR) is essential for normal cellular proliferation. As a result, hDHFR is the target of the anti-proliferative inhibitor methotrexate (MTX) that is broadly used in cancer chemotherapy [12]. Efficiency of MTX in cancer treatment can be attributed to its high affinity for hDHFR ($K_i$ = 3.4 pM) [13]. MTX-resistant hDHFR mutants have been identified *ex vivo* in cultured cells exposed to MTX [14 – 16] and by performing site-directed mutagenesis at the active site of the enzyme [17 – 19]. However, these mutants hDHFR are not found among patients treated with the drug [20]. Most MTX-resistant point mutants identified maintain good catalytic activity while displaying moderate resistance to MTX (e. g. F31S: $K_i$ = 0.240 nM; L 22R: $K_i$= 4.6 nM) [17, 21]. The dihydrofolate (DHF) substrate and the MTX inhibitor bind to the same area of hDHFR but with distinct orientations: while the *p*ABA-Glu portions of both ligands make

similar active-site contacts, the pteroyl moiety of MTX is flipped 180 ° around the C6-C9 bond (Fig. 1), such that different contacts are formed with hDHFR. Despite these differences, mutations that confer high MTX resistance (F34S: $K_i$= 210 nM) are frequently accompanied by similarly reduced substrate (DHF) recognition and turnover [22].

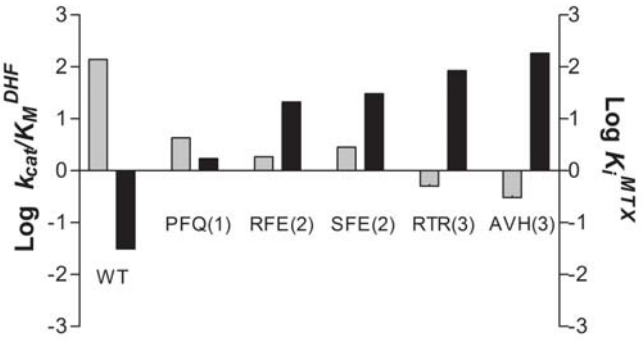



**Figure 1:** Position of the mutated active-site residues. A: Superimposition of hDHFR with bound folate (yellow; 1DRF; [57]) and its competitive inhibitor methotrexate (red; 1U72; [28]). For clarity, only hDHFR structure 1U72 is shown. The active site residues Phe 31, Phe 34 and Gln 35 are highlighted in green. B: Magnification of α-helix 1 carrying the mutated residues.

Identifying more highly MTX-resistant hDHFR mutants offers an important application. Because MTX-resistant hDHFRs have not been observed in cancer patients [23], genes encoding MTX-resistant hDHFRs have the potential to protect healthy haematopoietic stem cells from MTX-toxicity during chemotherapy, thus protecting patients from immunosuppression [24]. Gene therapy with MTX-resistant hDHFRs in mice ensured myeloprotection during MTX treatment [25, 26]. For this application, ideal candidate hDHFR mutants should have very poor affinity for MTX ($K_i$ in the high nM range), while maintaining the catalytic properties – including DHF substrate binding – required to ensure cell survival. Such a mutant was obtained by the combination of point mutants at active-site residues 22 and 31 [27]. The mutations acted synergistically (L22Y-F31G: $K_i$= 150 nM) to confer high resistance to MTX once inserted into human and mouse stem cell lines.

To obtain more highly MTX-resistant hDHFR variants for this application and to gain more insight into the nature of DHF substrate *vs* MTX inhibitor binding, we created combinatorial mutations at active-site residues of hDHFR. Here, we present concurrent mutations at residues Phe31, Phe34 and Gln35 (Fig. 1). These residues are individually known to confer MTX resistance upon mutation [17, 22, 28]. We designed oligonucleotides to introduce a restricted variety of amino acids at positions 31, 34 and 35. This approach theoretically encoded 567 different hDHFR variants. Library selection was achieved in MTX-sensitive *E. coli* SK037 [29] on M9 minimal medium containing a high concentration of MTX (1 mM). Seventy surviving clones were sequenced, revealing one novel point mutant and nine

novel combinatorial MTX-resistant mutants (double or triple mutants) (Table 1). Thus, approximately 2% (10 out of 567) of the encoded combinations of mutations provided MTX resistance in *E. coli*.

**Table1.** Kinetic and inhibition constants for the selected MTX-resistant hDHFR mutants.

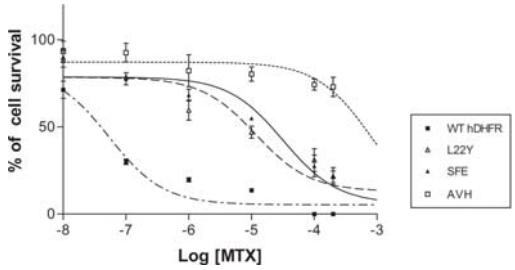
| hDHFR variant [a] | $k_{cat}$ ($s^{-1}$) | $K_M^{DHF}$ (μM) | $k_{cat}/K_M^{DHF}$ ($s^{-1}\mu M^{-1}$) | $K_i^{MTX}$ (nM) |
|---|---|---|---|---|
| WT hDHFR (FFQ) | 10 ± 2 | ≤ 0.075 | ≥ 140 | < 0.031 |
| **Point mutant** | | | | |
| **P**FQ | 6.4 ± 1.0 | 1.5 ± 0.30 | 4.3 | 1.7 ± 0.6 |
| **Double mutants** | | | | |
| **G**F**N** | 4.3 ± 1.0 | 1.7 ± 0.39 | 2.6 | 9 ± 3 |
| **P**F**H** | 1.7 ± 0.12 | 2.1 ± 0.51 | 0.80 | 10 ± 1 |
| **P**F**E** | 1.7 ± 0.05 | 2.7 ± 0.63 | 0.60 | 12 ± 4 |
| **R**F**E** | 1.3 ± 0.17 | 0.69 ± 0.13 | 1.9 | 21 ± 11 |
| **S**F**E** | 9.4 ± 0.31 | 3.3 ± 0.70 | 2.8 | 30 ± 11 |
| **Triple mutants** | | | | |
| **RAN** | 0.50 ± 0.06 | 2.2 ± 0.62 | 0.20 | 12 ± 2 |
| **RTS** | 1.0 ± 0.09 | 1.7 ± 0.27 | 0.60 | 59 ± 26 |
| **RTR** | 1.0 ± 0.10 | 2.0 ± 0.25 | 0.50 | 86 ± 49 |
| **AVH** | 1.1 ± 0.39 | 4.3 ± 1.3 | 0.30 | 180 ± 69 |

[a] hDHFR variants are designated by the one-letter code of the amino acid occurring at positions 31, 34 and 35, respectively. Mutations are shown in bold-type.

Kinetic and inhibition parameters of the purified mutants revealed that higher MTX resistance was obtained with an increasing number of mutations, the most highly-resistant mutants containing three active-site mutations ($K_i^{MTX}$ = 59 to 180 nM) (Table 1 and Fig. 2). The resistant mutants obtained all exhibited reduced catalytic efficiencies relative to the wild-type hDHFR (30 to 700-fold decrease), mainly as a result of decreased affinity for DHF (10 to 40-fold $K_M^{DHF}$ increase). The affinity for MTX was more importantly reduced by the mutations, with $K_i^{MTX}$ increased 54 to 5800-fold. Thus, we identified a variety of patterns of mutations at active-site residues 31, 34 and/or 35 that greatly reduced MTX binding while maintaining sufficient DHF affinity to provide the level of catalytic activity required for bacterial propagation. The variety of mutations obtained is indicative of significant plasticity in these active-site residues [11].

Previous studies of hDHFR point mutants at position 31 [17, 30] have shown that either the bulky, positively-charged Arg or small residues confer moderate (Gly, Ala and Ser) to good MTX resistance. We also observed these amino acids in the MTX-resistant combinatorial mutants. In addition, we observed proline at position 31 (point mutant F31P) which had not been previously reported, demonstrating that information can be generated rapidly by this selection strategy. Phe34 was the most strongly conserved native residue among the MTX-resistant mutants we identified, consistent with its important role in binding either MTX or DHF [22]. We observed Ala, Val and Thr in the four triple mutants identified, consistent

with previous reports that small amino acids (Ala, Val, Thr, Ser or Ile [19, 22]) at position 34 confer MTX resistance. A diversity of mutations was observed at position 35 in the MTX-resistant mutants (Asn, His, Glu, Ser or Arg). The Q35R and Q35P variants have been shown to confer low and elevated MTX-resistance respectively based on *in vitro* studies of murine DHFR [28]. Interestingly, the Gln to Glu substitution was observed in the three most frequently observed selected mutants: PFE, SFE and RFE, which were respectively identified 20, 18 and 14 times among the 70 clones analysed. The Glu may contribute to resistance by electrostatic repulsion of the γ-glutamate tail that binds nearby (Fig. 1). Surprisingly, positively-charged residues at position 35 also supported MTX-resistance, as observed by the substitution of Gln35 to Arg (mutant RTR) and His (mutants AVH and PFH). Gln35 has been proposed to interact with the guanidinium side chain of Arg70 in absence of ligand [31]. However, Arg70 forms a conserved salt bridge with the α-carboxylate of the bound ligands [32]. Thus, positively-charged mutations at position 35 may cause electrostatic repulsion with Arg70, decreasing ligand binding. Structural data is required to confirm these hypotheses.



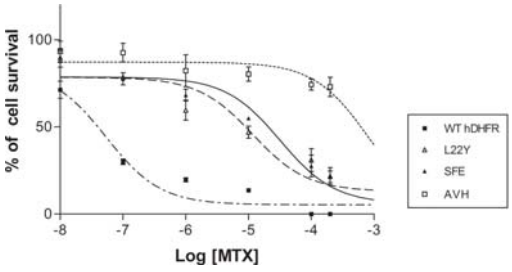

| Transfected variants | $EC_{50}^{MTX}$ (μM) |
|---|---|
| Null | 0 |
| WT hDHFR (FFQ) | 0.054 |
| L22Y | 12 |
| RFE | 31 |
| SFE | 32 |
| RTS | 56 |
| AVH | > 200 |

**Figure 2:** Relation between the number of hDHFR mutations and $k_{cat}/K_M^{DHF}$ or $K_i^{MTX}$. Mutants are designated by the one-letter code of the amino acid occurring at positions 31, 34 and 35, respectively. Numbers in parentheses correspond to the number of mutations in the variant. Log $k_{cat}/K_M^{DHF}$ values are shown in grey while log $K_i^{MTX}$ values are shown in black.

How effective was the cumulation of mutations in providing increased MTX resistance? All selected double mutants were mutated at positions 31 and 35 while retaining the native Phe34. Comparing $K_i^{MTX}$ for known F31X mutants with the related double mutants (i.e. comparing point mutant F31S with the double mutant SFE) revealed that the additional mutations at position 35 increased $K_i^{MTX}$ 3- to 125-fold [17, 22, 30]. The selected triple mutants provided further evidence of the impact of multiple mutations on resistance: the most highly MTX-resistant hDHFR variants were all triple mutants (Table 1, Fig. 2). While the F31R point mutant alone provides good MTX resistance ($K_i^{MTX}$ = 7.2 nM, [30]), additional mutations increased resistance, as evidenced by triple mutants RTS ($K_i^{MTX}$ = 59 nM) and RTR ($K_i^{MTX}$ = 86 nM). Triple mutant AVH displayed the weakest MTX binding out of the selected mutants ($K_i^{MTX}$ = 180 nM), an almost 6000-fold increase in $K_i^{MTX}$ relative to WT. This combinatorial mutant nearly matches the best-reported $K_i^{MTX}$ (F34S = 210 nM [22]). However, it boasts a catalytic efficiency that is almost 20-fold superior to the F34S point mutant, demonstrating the efficiency of the semi-rational evolution approach used.

The current data set is consistent with synergistic effects of many of the mutations toward MTX binding, as opposed to additive effects. For example, point mutants Q35E and Q35 H were encoded in the library but were not selected, suggesting that alone, they do not confer a high level of resistance. Nonetheless, these mutations increased the $K_i^{MTX}$ of the point mutant F31P 6 to 7-fold in mutants PFE and PFH. The same Q35 H mutation, combined with F31A ($K_i^{MTX}$ = 0.27 nM; [22]) and F34V ($K_i^{MTX}$ = 10 nM; [22]), also contributed to the highest resistance in mutant AVH ($K_i^{MTX}$= 180 nM). In a similar fashion, the Q35R mutation (the murine Q35R mutant displays only a 10-fold increase in $K_i^{MTX}$; [28]), combined with F31R ($K_i^{MTX}$ = 7.2 nM; [30]) and F34T ($K_i^{MTX}$ = 9.6 nM; [22]), contributed to resistance in mutant RTR ($K_i^{MTX}$= 86 nM), again consistent with synergistic rather than additive effects.

To verify the efficiency of eukaryotic cell protection, CHO DUKX B11 cells (*dhfr*$^-$) were transfected with MTX-resistant mutants. As these cells have no intrinsic DHFR, their survival is conferred by the transfected gene in the absence of nucleotides in the growth medium; in the presence of MTX, the DHFR must be MTX-resistant. The combinatorial mutants RFE, SFE, RTS and AVH were chosen because they displayed the highest $K_i^{MTX}$ values while maintaining catalytic efficiency comparable to MTX-resistant hDHFRs used in similar studies [33, 34]. All mutants tested conferred MTX-resistance to CHO DUKX B11 cells (Fig. 3). The control, point mutant L22Y provided a 220-fold increase in $EC_{50}^{MTX}$, while the double mutants RFE and SFE provided a 570-fold increase in $EC_{50}^{MTX}$ relative to WT hDHFR. The triple mutant RTS provided a 1030-fold increase in $EC_{50}^{MTX}$, a 2-fold increase in resistance relative to the double mutants. The $EC_{50}^{MTX}$ for the triple mutant AVH was greater than the maximal concentration of MTX that could be included in the medium (> 4000-fold increase). Thus, mutant AVH allowed survival of 73% of the cell population at 200 µM MTX. Cells containing wild-type hDHFR were sensitive to low concentrations of MTX, ruling out gene amplification.




| Transfected variants | $EC_{50}^{MTX}$ (µM) |
|---|---|
| Null | 0 |
| WT hDHFR (FFQ) | 0.054 |
| L22Y | 12 |
| RFE | 31 |
| SFE | 32 |
| RTS | 56 |
| AVH | > 200 |

**Figure 3:** Survival of CHO DUKX B11 cells transfected with native hDHFR or mutants L22Y, RTS, SFE, RFE and AVH over a range of MTX concentrations. Percentage of cell survival represents the ratio of cells counted in absence and in presence of various MTX concentrations for 48 hrs in α-MEM media containing no nucleotides, ensuring that cell survival was solely based on activity of transfected hDHFRs. The cell survival data was analyzed with a non-linear sigmoidal fit, generating $EC_{50}^{MTX}$ values for each variant. For clarity, curves are shown only for certain mutants. Combinatorial mutants are designated by the one-letter code of the amino acid occurring at positions 31, 34 and 35, respectively. Native hDHFR (WT) served as

a negative control. The MTX-resistant point mutant L22Y served as a positive control because it confers good MTX resistance in mammalian cells as a result of its catalytic efficiency ($12\,s^{-1}\mu M^{-1}$) and moderately high $K_i^{MTX}$ (10.9 nM) [19]. The mock experiment consisted in transfecting pcDNA 3.1 vector (= Null).

In conclusion, we developed a rapid and efficient system for identifying highly MTX-resistant variants of human DHFR. Our results support the application of a semi-rational directed evolution strategy for evolution of a desired property in an existing enzyme.

### *TEM-1* β*-lactamase active-site mutations*

β-Lactamases are drug-resistance enzymes belonging to several classes, among which class A β-lactamases are greater than 300 in number (http://www.lahey.org/Studies/). β-Lactamases are not primary drug targets. Instead, bacterially-produced β-lactamases efficiently break down penicillin and cephalosporin-type drugs such that they never reach their primary target, allowing bacteria to evade drug action. As a result of mutations, each β-lactamase has a specific recognition spectrum for penicillins and cephalosporins; new drugs are rapidly matched by new mutations [35, 36]. In order to better understand the protein-ligand interactions that define the recognition spectrum of different β-lactamases, we turned toward the well-characterized class A *E. coli* TEM-1 β-lactamase.

We previously investigated the role of the TEM-1 β-lactamase active-site residue Tyr105 using saturation mutagenesis, enzyme kinetics and *in silico* molecular dynamics simulations [37]. Our results show that Tyr105 is involved in substrate discrimination and stabilization at the active site of TEM-1: Y105X mutations affect catalysis ($k_{cat}$) only up to 5-fold but productive binding ($K_M$) up to 40-fold. While conservative mutation to other aromatic amino acids at position 105 preserved activity, most other residue replacements were found to possess too many degrees of freedom for appropriate substrate stabilization. Interestingly, the small-residue replacements Y105G and Y105A allowed discrimination between penicillin and cephalosporin substrates. Thus, TEM-1 position 105 acts as a 'gatekeeper': the identity of the amino acid at this position provides ligand discrimination.

We then probed for potential cooperativity between TEM-1 active-site residues. Five residues with side chains pointing toward the substrate binding pocket were targeted for combinatorial mutagenesis: Glu104, Tyr105, Gly238, Glu240 and Arg244 (Fig. 4; numbering according to Ambler [38]). Residues 104 and 105, carried on a loop, are relatively permissive to mutagenesis [9, 37]. On the opposite face of the active site lie β-strands β3 and β4, which harbour Gly238, Glu240 and Arg244. Gly238 is notorious for increasing resistance toward third-generation cephalosporins, particularly when mutated to Ser, although Asn or Thr also confer good levels of resistance [39 – 41]. The E240K mutation also increases resistance to third-generation cephalosporins [42]. Finally, though Arg244 has not been involved in cephalosporin resistance, we also mutated it because of its proximity to the active site and because of the inhibitor-resistance reported upon its mutation [43 – 45].

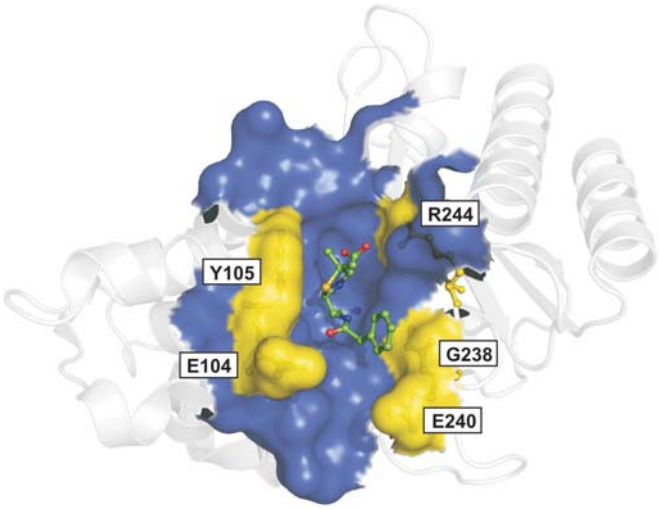



**Figure 4:** Schematic representation of the wild-type TEM-1 β-lactamase acyl-enzyme intermediate complexed with benzylpenicillin. Residues delineating the walls of the active site cavity are in blue surface representation while the residues selected for mutagenesis are colored yellow with underlying balls-and-sticks. Benzylpenicillin, a classical substrate of TEM-1 β-lactamase is colored by atom and shown in balls-and-sticks representation. Brookhaven Protein Data Bank entry 1FQG [58].

We designed oligonucleotides to simultaneously introduce all 20 natural amino acids at each of the 5 selected positions in TEM-1 (104, 105, 238, 240 and 244). Two separate libraries (104 – 105 and 238 – 240 – 244) were individually synthesized and subsequently recombined using a restriction site located between the two mutated regions. This approach theoretically yielded $20^5$ different β-lactamase variants. Selection on media containing the third-generation cephalosporin cefotaxime (CTX; 250 ng/mL) yielded a number of CTX-resistant mutants. Mutants were named after the one-letter amino acid code at positions 104, 105, 238, 240 and 244, respectively, such that the WT enzyme is 'EYGER'.

Among the 34 CTX-resistant clones we analysed, 11 unique sequences were identified. The most frequently observed was the double mutant G238S/E240K (EYSKR), representing over 40 % of the sequenced clones. This particular mutant has been extensively studied and therefore was not further analysed [40, 46 – 48]. None of the other mutant sequences occurred more than 4 times, although most exhibited similar features. For instance, Arg244 remained unchanged among the 34 mutants, consistent with previous observations suggesting that this residue is essential for appropriate hydrogen bonding with the substrate in both the ground and transition states in TEM-1 [49]. In addition, all but one of the selected mutants encoded the well-documented G238S mutation conferring CTX resistance [35, 50].

Amino acids introduced at positions 104, 105 and 240 may act cooperatively with the G238S mutation to alter resistance, either synergistically, additively or antagonistically [51]. To examine TEM-1 tolerance to multiple active-site mutations, we analysed mutants displaying a wide variation in amino acid identity at the selected positions (Table 2). We determined the *in vivo* vulnerability of the mutants toward various β-lactam antibiotics by determining their minimum inhibitory concentrations (MICs): the lowest concentration of antibiotic at which bacterial growth was inhibited. The mutants that had been selected against CTX considerably gained resistance toward CTX at the expense of penicillin resistance (Fig. 5). In contrast, effects on resistance to the first-generation cephalosporins cephalothin and cefazolin were mitigated and kept within the wild-type range (data not shown). Thus, selection for resistance toward the third-generation cephalosporin CTX did not result in cross-resistance to a similar level toward first-generation cephalosporins nor toward penicillins.

**Table 2.** Kinetic parameters for wild-type TEM-1 β-lactamase and for selected mutants that include the G238S mutation.

| Substrate | TEM-1 variant [a] | $k_{cat}/K_M$ | $k_{cat}/K_M$ |
|---|---|---|---|
| Cefotaxime | **Wild-type (EYGER)** | **1** | **$6.8 \times 10^{-3}$** |
| | VNSLR | 818 | 5.5 |
| | VNSTR | 364 | 2.5 |
| | PHSER | 586 | 4.0 |
| | SWSSR | 557 | 3.8 |
| | **G238S** [b] | **148** | **1** |
| Cephalothin | **Wild-type** | **1** | **1.1** |
| | VNSLR | 7.44 | 8.4 |
| | VNSTR | 1.49 | 1.7 |
| | PHSER | 10.3 | 11.8 |
| | SWSSR | 5.81 | 6.6 |
| | **G238S** [b] | **0.88** | **1** |
| Benzylpenicillin | **Wild-type** | **1** | **82** |
| | VNSLR | 0.07 | 6.0 |
| | VNSTR | 0.04 | 3.3 |
| | PHSER | 0.02 | 1.9 |
| | SWSSR | ND [c] | ND |
| | **G238S** [b] | **0.01** | **1** |

[a] Variants are identified according to the amino acid present at positions 104, 105, 238, 240 and 244, respectively.
[b] Data from Cantu & Palzkill [39].
[c] Not determined.

*In vitro* kinetic parameters were determined (Table 2) to verify whether they paralleled the *in vivo* MICs. Not unexpectedly, the most important kinetic differences observed concerned improvement of CTX hydrolysis. Catalytic efficiency ($k_{cat}/K_M$) improvements of nearly three orders of magnitude were observed for the mutants relative to the wild-type enzyme. In addition, all mutants characterized were more efficient than the G238S point mutant (Table 2), indicating that increased efficiency toward CTX hydrolysis results from the additional mutations. However, the additional improvements over G238S were all modest (2.5 to 5.5-fold).

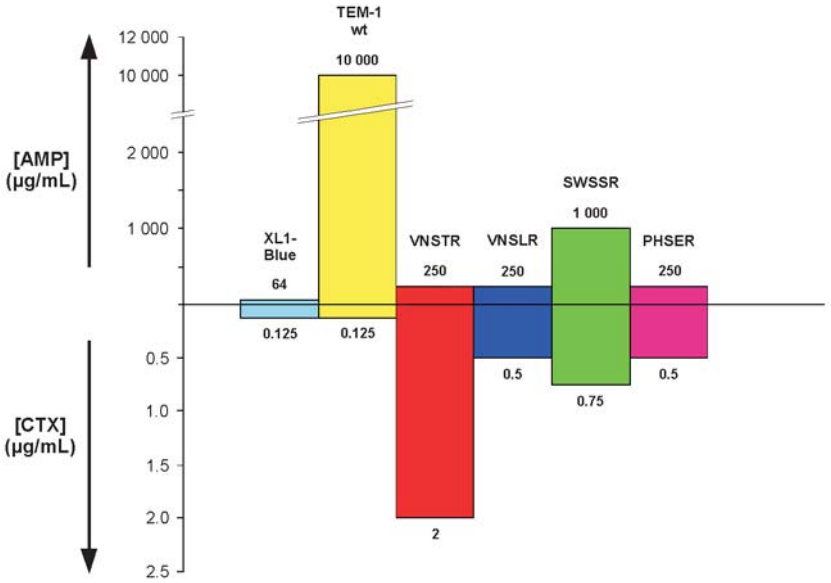



**Figure 5:** Minimum inhibitory concentrations (MICs) of *E. coli* XL 1-Blue cells expressing native TEM-1 β-lactamase and selected mutants (VNSTR, VNSLR, SWSSR and PHSER) tested against the penicillin substrate ampicillin (AMP), and the third-generation cephalosporin substrate cefotaxime (CTX). MICs were determined by broth microdilutions with two-fold dilutions of the antibiotics tested according to Cantu *et al.* (1996) [44]. XL 1-Blue corresponds to the strain devoid of TEM-1 β-lactamase expression.

Mutant VNSLR exhibited the highest catalytic efficiency. Comparison of mutants VNSLR and VNSTR was instructive. Both encode Val104: out of all reported natural isolates or following mutagenesis, this mutation was identified only in the CTX-hydrolysing class A β-lactamase D 488 from *Klebsiella oxytoca* [52]. Asn105 is compatible with both penicillin and first-generation cephalosporin hydrolysis [37], mutation G238S confers CTX resistance [35, 50] and Arg244 is the native residue. The mutants differ by only the amino acid at position 240: E240L *vs.* E240T. Mutation E240L has not been previously characterized; mutation E240T has been observed only in the class A PC 1 β-lactamase from *Staphylococcus aureus* [38, 53, 54] but has never been characterized in detail in the context of other class A enzymes. It has been proposed that an exposed hydrophilic residue at position 240 could potentially H-bond with the acylamide substituent of a cephalosporin such as CTX [42, 55]. Thus, one would expect that mutant VNSTR exhibiting a polar side chain at position 240 should manifest the best productive affinity for the cephalosporin substrates. However, while VNSTR and VNSLR featured similar $k_{cat}$ for the three cephalosporins tested (Table 2; data not shown for cefazolin), VNSLR exhibited the lowest $K_M$ value (3-fold lower than VNSTR), in apparent contradiction with the proposed role of position 240 [55]. The non-native, combinatorially mutated environment that serves as a backdrop for

the E240 mutations likely accounts for the apparent discrepancy. This result highlights the additional phenotypic diversity that can arise from creating multiply mutated active-site areas.

Mutant PHSER exhibited the greatest increases in $k_{cat}$ for the cephalosporins tested (data not shown). This mutant combined two previously characterized point mutations with substitution G238S. E104P is active toward a range of substrates and is particularly advantageous toward CTX hydrolysis [56] while Y105 H exhibits native-like resistance toward penicillins and first-generation cephalosporins [37]. Finally, mutant SWSSR is also a combination of G238S with two characterized point mutations advantageous for catalytic efficiency toward penicillins and cephalosporins (E104S and Y105W) [37, 56]. These two substitutions appear to display cooperativity (or 'complex additivity'; see below) with G238S and the uncharacterized E240S to provide SWSSR with increased catalytic efficiency toward cephalosporins.

As observed with the *in vivo* MIC results, *in vitro* effects of the mutants toward the first-generation cephalosporins cephalothin and cefazolin were mitigated. Mutants respectively displayed increased catalytic efficiencies up to 10-fold and 3-fold relative to wild type (Table 2, CZ results not shown). Since the G238S point mutation confers no improvement in efficiency toward these substrates [39, 57], the improvements must be attributed to the additional mutations at positions 104, 105 and 240 (see Table 2, $k_{cat}/K_M$ mutant relative to G238S). It is remarkable that, although the mutants were selected for CTX hydrolysis, the beneficial effect of the additional mutations – excluding effects of G238S – toward cephalothin hydrolysis (a first-generation cephalosporin) was similar to their effect toward CTX hydrolysis (a third-generation cephalosporin). This suggests that the effects of the mutations were additive, as opposed to being synergistic or antagonistic.

We also assayed the mutants for hydrolysis of a penicillin, benzylpenicillin (Table 2). In all cases, efficiency was reduced by one to two orders of magnitude relative to the wild-type enzyme. This reduction in efficiency is likely attributed in great part to the detrimental effect of the G238S mutation toward penicillin hydrolysis, particularly affecting $k_{cat}$. Similar observations have been reported for this mutation [39, 41] and other point mutants at positions 104, 164 and 240 [42, 57]. The additional mutations we introduced at positions 104, 105 and 240 reversed the detrimental effect of the G238S point mutant, allowing for a 10-fold increase in $k_{cat}$ for benzylpenicillin hydrolysis (data not shown).

Because the magnitude of the improved efficiency, excluding the effect of the G238S mutation, is similar for all cases reported in Table 2, it is tempting to speculate that the additional mutations ensure improved β-lactam hydrolysis *via* a relatively non-specific mechanism that applies indiscriminately to cephalosporins and penicillins. However, for both cephalosporins, there was a slightly greater improvement of $K_M$ than $k_{cat}$ relative to the wild-type enzyme, while $k_{cat}$ was the sole contributor in the case of the penicillin. Importantly, there was less difference when comparing the various mutants than when comparing the different substrates, despite the variety in the additional mutations.

In contrast to the synergy observed between the mutations identified in MTX-resistant hDHFR variants, the mutations identified in the antibiotic-resistant TEM-1 β-lactamase variants appear to have additive effects. Importantly, mutant catalytic efficiencies were increased in a similar fashion toward the third-generation cephalosporin they were selected against and toward first-generation cephalosporins (and to a lesser extent toward penicillins). This effect appears to be the result of additivity of mutations at positions 104, 105 and 240 to the G238S mutation.

## CONCLUSION

By combinatorially mutating active-site residues that are in contact with the ligands, we have obtained highly drug-resistant variants of two unrelated enzymes: hDHFR and TEM-1 β-lactamase. In both cases, previously reported as well as novel mutations were identified. Also, in both cases, specific combinations of additional mutations proved advantageous. Synergistic effects were observed in the case of hDHFR variants while additivity was observed in the case of TEM-1 β-lactamase. It is possible that synergistic effects of mutations occur more readily when the target property is decreased binding (toward MTX, in the case of hDHFR) than when the target is a more complex property, such as improved catalytic efficiency (toward β-lactam substrates, in the case of TEM-1 β-lactamase). The selection stringency we applied may have amplified this outcome: the synergistic mutations identified in hDHFR conferred a great selective advantage that is not likely to have been matched by the effect of additive mutations. Thus, additive mutations in hDHFR may exist but were neglected under the stringent selection conditions we applied.

In addition, the intrinsic properties of the enzyme under study (fold, internal dynamics, interactions of the active-site residues, etc.) may determine the potential for mutations to act in a synergistic rather than an additive fashion. It has been previously proposed that simple additivity generally results from distant independent mutations that affect substrate binding, protein-protein interactions and protein stability, as long as the overall structure remains unchanged [58]. Discrepancies from simple additivity have been observed when residues share physical interaction. Such “complex additivity” occurs either when the residues act cooperatively or when mutated residues make direct or indirect contacts, e.g. *via* electrostatic bonding [58]. When synergy applies to $k_{cat}$ (or to $k_{cat}/K_M$ by extension), it is generally an indication that the mutated residues function independently and in a concerted fashion in the same step of the catalytic reaction if the enzyme structure is maintained [59]. Future work will be directed at gaining a better understanding of the nature of interactions between mutations.

Globally, our work provides additional information relative to the plasticity of enzyme active sites, where multiple solutions can satisfy the requirement for a new binding specificity. Applications include directing future drug design to reduce the rate at which mutations allow drug resistance, and developing highly resistant enzymes for protection of healthy cells during chemotherapy.

## Acknowledgments

We are grateful to Gwen S. Knapp and James C. Hu (Texas A&M) for the *E. coli* SK037 cells, Ingrid Remy and Stephen W. Michnick (Université de Montréal) for donating CHO DUKX B11 cells, Frédérick-Antoine Mallette and Gerardo Ferbeyre (Université de Montréal) for guidance concerning cell culture and Stéphane Roy (Université de Montréal) for the use of cell culture facilities. This work was supported by Natural Sciences and Engineering Research Council grant #227853 and Canada Institutes for Health Research grant #68851.

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