New enzyme for reductive cancer chemotherapy, YieF, and its improvement by directed evolution

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Abstract

Reductive prodrugs, mitomycin C and 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), are nontoxic in their native form but become highly toxic upon reduction. Their effectiveness in cancer chemotherapy can be enhanced by delivering to tumors enzymes with improved prodrug reduction kinetics. We report the discovery of a new prodrug-reducing enzyme, YieF, from *Escherichia coli*, and the improvement of its kinetics for reducing mitomycin C and CB 1954. A YieF-derived enzyme, Y6, killed HeLa spinner cells with ≥5-fold efficiency than the wild-type enzymes, YieF and NfsA, at a variety of drug and enzyme concentrations and incubation times. With adhered HeLa cells and *Salmonella typhimurium* SL 7838 bacteria as enzyme delivery vehicle, at least an order of magnitude less of Y6-producing bacteria were required to kill >90% of tumor cells compared with bacteria expressing the wild-type enzymes, which at a comparable level killed <5% of the cells. Thus, Y6 is a promising enzyme for use in cancer chemotherapy, and *Salmonella* strain SL 7838, which specifically targets tumors, may be used to deliver the prodrug-activating enzymes to tumors. [Mol Cancer Ther 2006;5(1):97–103]

Introduction

Mitomycin C and 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), which are reductive prodrugs, are nontoxic in their native form but produce a highly toxic species when reduced. These drugs kill by generating DNA adducts and can target both growing and nongrowing tumor cells, which is advantageous because in human tumors, generally only a small fraction of cells are actively replicating at a given time (1, 2). Reductive prodrug cancer chemotherapy owes its rationale to the fact that the concentration of the enzymes that reduce them, such as mammalian DT-diaphorase (NQO1; refs. 3 and 4), increases in tumor cells. This makes the tumor cells more potent reducers of these drugs and, therefore, more susceptible to their killing effect. However, these enzymes are present also in normal mammalian cells and although their activity is lower in such cells than in tumor cells, it is high enough to produce severe side effects. A potential solution is to use suicide gene therapy (also known as gene-delivered enzyme prodrug therapy; ref. 5), which aims to deliver genes to tumors to selectively enhance their prodrug reducing activity. A class of enzymes that has been well studied in gene-delivered enzyme prodrug therapy is bacterial nitroreductases, such as NfsA and NfsB from *Escherichia coli* (6–8). These enzymes can reduce several nitrosubstituted organic compounds, including CB 1954, which is an antitumor reductive prodrug (2). Previous studies of CB 1954/bacterial nitroreductase in gene-delivered enzyme prodrug therapy have indicated the need for improving the specific activity of bacterial nitroreductases for CB 1954 (7).

In our ongoing work on bioengineering of bacteria with superior capacity to remediate Cr(VI) (chromate), which is a widespread carcinogenic environmental pollutant, we identified a new *E. coli* enzyme, YieF, which is particularly attractive toward this end (9, 10). By applying directed evolution through the technique of error-prone PCR, we have improved the capacity of this enzyme to reduce chromate by over 200-fold.[3] Based on the general characteristics of YieF (11), we tested for and confirmed that this enzyme can also reduce CB 1954 as well as another prodrug, mitomycin C. Furthermore, we discovered that improvement in chromate reductase activity of the evolved enzymes also led to improvement in their capacity to reduce the two prodrugs. Here, we report that compared with the wild-type YieF, our evolved enzymes exhibit >5-fold increased capacity to kill HeLa cells. We also show that this enzyme can be effectively delivered to tumor cells using an attenuated strain of *Salmonella typhimurium* (SL 7838). These bacteria target both the aerobic and anaerobic zones of tumors and do not infect normal tissues.[4]

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Materials and Methods

Bacterial Strains, Plasmids, Genes, and Primers

These are listed in Table 1.

Human Tumor Cell Lines

HeLa cells were used in most experiments. Freshly grown cells were kindly supplied by C. Diges (P. Sarnow Laboratory, Department of Microbiology and Immunology, Stanford University School of Medicine). They were grown in MEM (HeLa Spinners Modified, Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Gaithersburg, MD), 10 μg/mL penicillin, 100 μg/mL streptomycin, 0.292 g/L L-glutamine, and 10 mmol/L HEPES buffer (pH 7.6). Cells were routinely diluted to 10^5/mL to keep a continuous viable and active culture. When adherent HeLa cells were used, they were grown in DMEM (Life Technologies) containing the same supplements.

DNA Techniques

Small-scale plasmid DNA isolation from *E. coli* was carried out by miniprep (Qiagen, Inc., Valencia, CA). Plasmids were transformed into *E. coli* BL21 (DE3) cells (Invitrogen, Inc., Carlsbad, CA) and used for protein production. DNA sequencing was conducted by Sequetech Corporation (Mountain View, CA) using appropriate primers (Table 1).

Error-Prone PCR

The *yieF* gene (which encodes the enzyme YieF; Genbank accession no. NC 000913.2) that we have previously characterized (9, 10, 12) was used as template. Random mutations were introduced in this gene by error-prone PCR according to Chen and Arnold (13) using GeneMorph II Random Mutagenesis kit (Stratagene Corporation, La Jolla, CA) protocol. The forward and reverse *yieF* primers were used to amplify full-length hybrid products (Table 1).

Screening for Improved Enzymes

As mentioned in Introduction, the screening procedure used was aimed at identifying genes that encoded enzymes with superior chromate reductase activity. It was later serendipitously discovered that several enzymes improved in chromate reductase activity also possessed superior prodrug-reducing activity. Products generated through error-prone PCR were ligated into the pET28a$^+$ plasmid and transformed into *E. coli* BL21 (DE3). Recombinants were selected on plates containing kanamycin at 50 μg/mL. High-throughput screening of 6,000 recombinants was done by inoculating colonies into individual wells of 96-well microtiter plates, containing 200 μL Luria-Bertani medium and kanamycin. After growth to stationary phase (overnight incubation, final A_{660}, 1–1.5), 20 μL aliquots from each well were used to inoculate a second series of plates using M9 minimal medium. Each well received the same initial inoculum density. The first set of plates was stored at −80°C after addition of glycerol. Cells in the second inoculation series were allowed to grow to midexponential phase and then exposed to 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside to induce the recombinant gene expression. After an appropriate incubation

Table 1. Bacterial strains, plasmids, genes, and primers

	Relevant characteristics	Reference or source
Strain		
E. coli BL21	DE3 allowing overexpression of desired protein under IPTG-inducible T7 promoter	Novagen
S. typhimurium SL 7838	Attenuated strain containing *aroA* and *sopE* gene deletions	Thorne et al., unpublished data
Plasmids		
pET28a$^+$	pET28a$^+$ expressing the desirable gene (*Nde*I/*Bam*HI)	(9)
pCGLS1	Containing *Luciferase* gene allowing *in vivo* imaging	(16)
*pET28a$^+$::yieF**	*E. coli YieF* (Genbank accession no. NC_000913.2)	(9)
*pET28a$^+$::nfsA**	*E. coli* NfsA (Genbank accession no. P17117)	(24)
Primers		
FyieF †	5′-CGCGGGGG**CATATG**TCTGAAAAATTGCAGGT-3′	(9, 12)
RyieF ‡	5′-TTTG**GGATCC**TTAGATCTTAACTCGCTGAA-3′	(9, 12)
FA120V	5′-GTATTGATTCAGACCAGCTCAATGGGCGTGATTGG-3′	This research
RA120V	5′-CCAATCACGCCCATTGAGCTGGTCTGAATCAATAC-3′	This research
FN128Y	5′-TTGGCGGCGCGCGCTGTCAGTATCACCTGCGCCAGA-3′	This research
RN128Y	5′-TCTGGCGCAGGTGATACTGACAGCGCGCGCCGCCAAT-3′	This research
FN160T	5′-GTTGATCCGCAAACCGGAGAAGTGATTGA-3′	This research
RN160T	5′-ATCAATCACTTCTCCGGTTTGCGGATCAAC-3′	This research
RL175G	5′-TTAACTCGCTGAATAAACTCACCAAATGCGGTCAATT GCCCGGTCAGGTG-3′	This research

Abbreviation: IPTG, isopropyl-β-D-thiogalactopyranoside.
*Protein accession number in PubMed database.
† Bold underlie *Nde*I restriction site.
‡ Bold underlie *Bam*HI restriction site.

time, cells were lysed by 30 μL BugBuster (Novagen, Inc., San Diego, CA) addition and centrifuged for 20 minutes at 3,000 × *g*. One hundred microliters of the supernatant were mixed with additional 100 μL solution of the following composition: 500 μmol/L of potassium chromate, 2 mmol/L of NADH, 100 mmol/L of Tris-HCl (pH 7), and double-distilled water. Cr(VI) reduction was then determined as previously described by Ackerley et al. (9, 12).

Protein Purification

The most efficient enzymes with chromate reductase activity were purified on nickel columns as previously described (14) using inoculum obtained from the frozen plates. Several of these were subsequently tested also for CB 1954 and mitomycin C reductase activity as described in the viability determination section below.

Site-Directed Mutagenesis

Appropriate primers (Table 1) were used for site-directed mutagenesis. These were designed to create single codon mutations following the method of Kuipers et al. (15). The modified PCR products were cloned into pET28a$^+$ and transformed into *E. coli* BL21 (DE3). Verification of the desired mutations was made by sequencing.

Viability Tests

As mentioned in Results, these involved two incubation periods, prodrug activation, followed by assay of the toxic moiety generated by determining the loss of HeLa cell viability. Prodrug reduction mixture contained mitomycin C or CB 1954 and the enzyme at specified concentrations, 50 μmol/L NADPH, and HeLa cells modified MEM (see above) to a final volume of 0.5 mL. Following prodrug reduction at 37°C, 0.5 mL fresh HeLa cells (~ 0.5×10^5–1×10^5) was added. The duration of the prodrug reduction was 30 minutes in all experiments; the HeLa cell exposure periods were as specified in the text. After the latter incubation, 20 μL of the color reagent, CellTiter 96 AqueousOne (Promega, Inc., Madison, WI), were added to 100 μL aliquots of the reaction mixture. Following an additional 1 hour of incubation, A_{490} was measured in a BioTek microplate S330 reader.

Cell Survival Assay Using SL 7838 to Deliver the Wild-Type or Evolved Enzymes

SL 7838 contains deletions in the *aroA* and *sopE* genes. This strain is nonpathogenic in immunocompetent mice but can colonize and persist in solid tumors.[4] The strain was transformed with appropriate plasmids to express the *yieF, nfsA*, or theY6-encoding *yieFY6* gene. It was also transformed with a vector containing the *lux* operon, expressing the bacterial luciferase (product of the *luxR* gene) and its substrate. HeLa cells stably transfected to express firefly luciferase (16) were added to black-walled 96-well plates at a density of 1,000 per well. Once the cells had attached, a dilution series of nontransformed SL 7838 or SL 7838 transformed with plasmids expressing the appropriate genes (Table 1) was added to the plates; 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside was added to induce the genes on the plasmids. After 1-hour incubation, 15 μmol/L CB 1954 was added. Light output per well produced from bacterial luciferase (no additional substrate added) or bacterial and firefly luciferase (following addition of 2 μL of 30 mg/mL luciferin per well) was measured on an IVIS50 system (Xenogen Corp., Alameda, CA). Percentage HeLa cell survival was determined by calculating firefly luciferase expression (total light output − bacterial light output) relative to wells containing unchallenged HeLa cells or HeLa cells treated with 70% ethanol.

All experiments were done at least in triplicate; error bars in the figures are SE values.

Computer Programs

Homology searches for enzymes were done using BLASTP.[5] Sequences were aligned with Clustal W.[6]

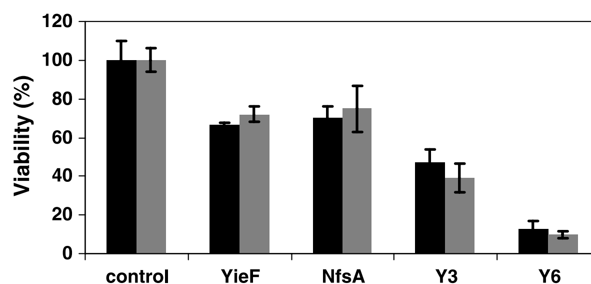

Figure 1. The effect of wild-type NfsA or YieF or the evolved enzymes (50 μg/mL) in the presence of 15 μmol/L mitomycin C (■) or CB 1954 (□) on the viability of HeLa cells. *Control,* incubation of the cells with the prodrug alone; other controls (incubation of cells alone or with just the enzyme) gave similar results.

Results

Prodrug Activation by Wild-Type YieF and the Evolved Enzymes

The loss of viability of HeLa cells was used to determine the capacity of different enzymes to activate the prodrugs, mitomycin C and CB 1954. The experiments involved two incubation periods. In the first period, the enzyme and the drug were incubated to generate the toxic species; in the second period, which was initiated with the addition of HeLa cells to the reaction mixture, the extent of drug activation was inferred from the loss of HeLa cell viability. In the experiments described in this section, both the prodrug reduction and the subsequent cell exposure periods were of 30-minute duration and the enzyme and drug concentrations were 50 μg/mL and 15 μmol/L, respectively. HeLa cell survival was compared between reaction mixtures containing the wild-type *E. coli* YieF in the presence of mitomycin C or CB 1954, and controls from which the drug, the enzyme, or both were excluded. YieF/mitomycin C and YieF/CB 1954 were both 30% to 40% more effective than the controls in reducing the viability of the HeLa cells (Fig. 1). This is the first demonstration that YieF has prodrug reducing activity. We also tested NfsA in this system; its effects were comparable with YieF with either of the drugs (Fig. 1). Given this and the fact that

[5] http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi.
[6] http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html.

NfsA prodrug reduction is well characterized (1, 2, 7), the data presented below will compare the activity of our evolved enzymes to that of the wild-type NfsA.

The above experiments were also conducted using a single incubation period in which HeLa cells were added at the same time as the drug and the enzyme. Very similar results were obtained, suggesting that the cytotoxic reduction products of the prodrugs were stable (data not shown). Subsequent experiments used the two incubation method.

Error-prone PCR was carried out using the *E. coli yieF* gene and the evolved genes were screened for improved chromate reductase activity as described in Materials and Methods. Selected enzymes showing superior chromate reductase activity were also tested for prodrug reduction. Two of these enzymes, Y3 and Y6, exhibited improved activity for prodrug reduction, with Y6, the more active of the two, showing some 90% greater reduction in HeLa cells survival compared with the controls (Fig. 1).

Effect of Changing Experimental Parameters

To test that the results reported above were not confined to the conditions used, the effect of changing drug or enzyme concentration, or the duration of HeLa cell exposure period, were investigated. Neither of the drugs produced much killing of the HeLa cells at a concentration of 1 or 3 μmol/L when NfsA was used as the activating enzyme (Fig. 2). However, at these drug concentrations, Y6 gave a 30% to 40% reduction in viability. At 5 and 10 μmol/L, both drugs produced appreciable cell killing with NfsA, but this was still markedly less than that generated by Y6. Increasing NfsA concentration from 10 to 30 μg/mL increased the killing of HeLa cells from around 5% to 25% (Fig. 3). In contrast, killing by Y6 in this enzyme concentration range was between ~70% to ~90%. The effect of varying the exposure period of cells gave similar results. With either of the prodrugs present, NfsA caused little or no reduction in cell viability during the first 20 minutes of incubation (Fig. 4). Y6 enzyme, however, generated some 40% reduction in this period. With further incubation of up to 180 minutes, although NfsA produced up to 50% reduction, Y6 generated up to 90% reduction of HeLa cell viability, showing that during this prodrug incubation period, Y6 was able to generate more of the activated drug than NfsA. The fact that increasing incubation times generated progressively greater killing of HeLa cells is consistent with the stability of the cytotoxic reduction products as noted above.

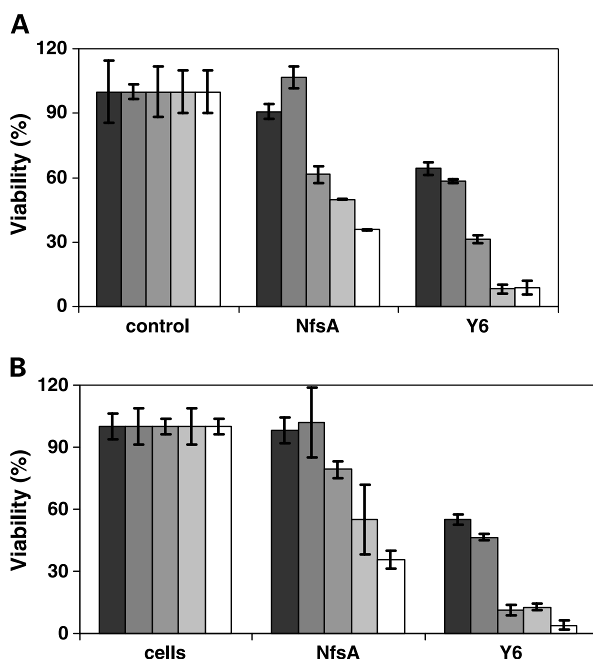

Figure 2. The effect of different mitomycin C (**A**) or CB 1954 (**B**) concentrations on the viability inhibition of HeLa cells in the presence of the wild-type or the evolved enzymes (50 μg/mL). The drug concentrations were 1 μmol/L (■), 3 μmol/L (▩), 5 μmol/L (▩), 10 μmol/L (▩), or 30 μmol/L (□).

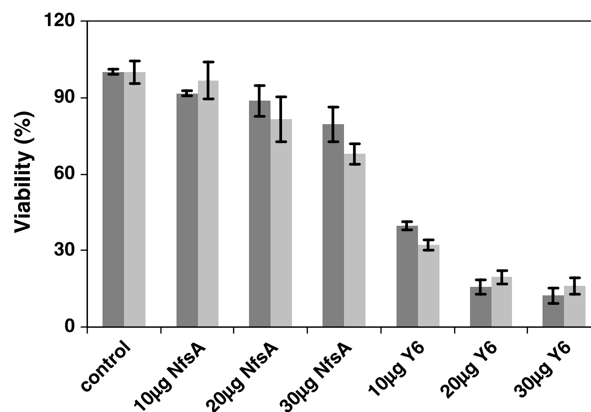

Figure 3. The effect of different NfsA or Y6 enzyme concentrations on the viability of HeLa cells in the presence of 15 μmol/L mitomycin C (▩) or CB 1954 (▩).

SL 7838 as a Delivery Vehicle to Tumor Cells

For Y6 to be effective in cancer therapy, it is necessary to find a means of targeting it to the tumor cells. We tested the *S. typhimurium* strain 7838 (SL 7838), transformed with different plasmids (ref. 17; see Materials and Methods) as a potential vehicle to attain this objective. Isopropyl-β-D-thiogalactopyranoside was used to induce the wild-type and mutant enzymes encoded by the plasmids. In these experiments, the prodrugs were activated by bacteria producing the specified enzymes instead of, as in previous experiments, the added enzymes. Only CB 1954 was used because mitomycin C was toxic to SL 7838 at even low concentrations (5 μmol/L). In the presence of CB 1954, SL 7838 bacteria alone produced 10%; those expressing wild-type NfsA or YieF, 10% to 25% killing of HeLa cells. On the other hand, bacteria expressing Y6 produced 90% reduction in survival of HeLa cells (Fig. 5). This is consistent with the data presented above for the superiority of Y6 over the wild-type enzymes and shows that SL 7838 could be used as an effective delivery vehicle.

Further analysis was done to test the efficiency of SL 7838 to deliver the enzymes to surface-adhered HeLa cells.

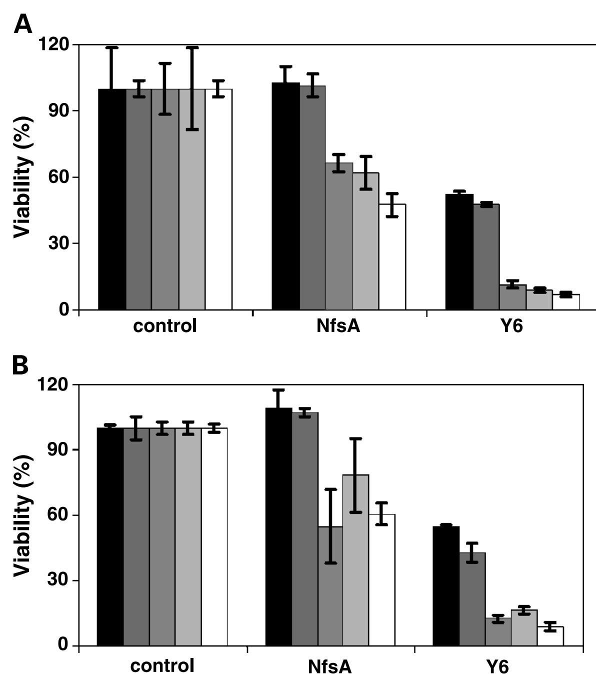

Figure 4. The effect of different durations of cell exposure on viability in the presence of NfsA or Y6 enzyme (50 μg/mL) and 15 μmol/L mitomycin C (**A**) or CB 1954 (**B**). The duration of the incubation periods were 10 min (■); 20 min (▪); 60 min (▪); 120 min (▪), or 180 min (□).

Firefly luciferase-expressing HeLa cells (16) were incubated with different colony-forming units/mL of the various SL 7838 strains for 1 hour before addition of CB 1954 and *in situ* production of toxic products. After a further 3-hour incubation period, the firefly luciferase substrate (luciferin) was added and the light output was used to quantify HeLa cell survival (Fig. 6). It was found that CB 1954 did not inhibit replication of SL 7838 and that neither expression of wild-type or evolved enzyme reduced the

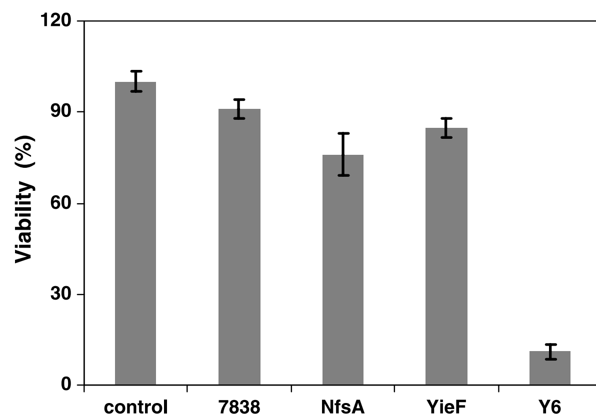

Figure 5. Viability of HeLa cells after exposure to 15 μmol/L CB 1954 alone (*control*); CB 1954 and untransformed SL 7838; or CB 1954 and SL 7838 expressing NfsA, YieF, or Y6.

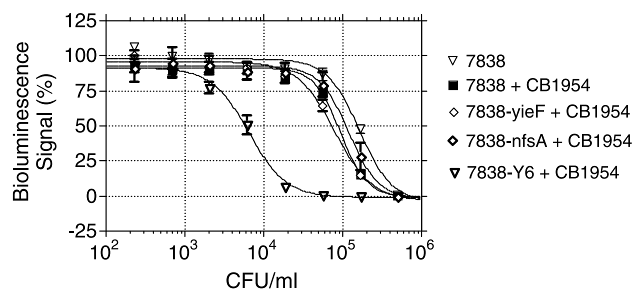

Figure 6. The use of SL 7838 to deliver wild-type or evolved enzymes to adherent HeLa cells. Survival of HeLa-*luc* cells was measured via light output after addition of luciferin (see Materials and Methods). Data are presented as percentage luminescent signal relative to control wells containing *luc*-transfected HeLa cells only (100%) or following treatment with 70% ethanol (0%). *Points,* averages of three replicates. CB 1954 concentration was 15 μmol/L.

replication capacity of the bacteria (data not shown). SL 7838 expressing Y6 enzyme required at least an order of magnitude less bacteria for effective HeLa cell killing than cells expressing the wild-type enzymes, YieF or NfsA. In fact, the killing by the latter was scarcely greater than that seen in controls using untransformed bacteria with or without CB 1954.

Amino Acid Sequence of the Y6 Enzyme

Four substitutions were found in Y6 protein: V120A, Y128N, T160N, and Q175L. When each altered amino acid was individually reverted to its original residue (see Materials and Methods), only the N128 to Y reversion diminished Y6 activity (Table 2). To gain an understanding of the chemical basis of these findings, attempts are currently under way to crystallize the YieF and Y6 proteins as well as to apply computational models that predict protein sequence-function relationships.[7]

Discussion

Prodrug cancer chemotherapy has attracted considerable interest. Examples of such drugs currently in use in cancer treatment include 5-fluorocytosine, cyclophosphamide, capecitabine, mitomycin C, and AQ4N. Many other prodrugs, such as CB 1954, although not yet proven to be effective, are being intensively studied. The side effects of prodrug therapy remain a serious concern, however; they arise from the fact that noncancerous cells also possess the capacity to reduce them. This has created the incentive to generate more effective prodrug-activating enzymes so that prodrugs can kill tumor cells at concentrations that generate little or no side effects, to design more powerful and tumor-specific drugs, and to devise more effective methods for delivering them specifically to tumors (6, 18–20).

[7] Y. Barak, Y. Nov, A. Matin, unpublished data.

Table 2. Killing of HeLa cells by Y6 reversion enzymes

Variant	MMC viability (%)	CB 1954 viability (%)
Cells	100 ± 8	100 ± 5
YieF	65 ± 9	72 ± 2
Y6	26 ± 3	7 ± 3
A120V	24 ± 4	4 ± 5
N128Y	95 ± 3	102 ± 8
N160T	30 ± 9	12 ± 2
L175Q	26 ± 2	9 ± 4

NOTE: HeLa cells were incubated in the presence of 15 μmol/L of one of the prodrugs and 50 μg/mL of the enzyme.
Abbreviation: MMC, mitomycin C.

Several methods have been used to increase the affinity of the prodrug-reducing enzymes. For example, Grove et al. (7) generated mutant enzymes by amino acid substitutions around the active site of NfsB whose crystal structure is known (21); some of these showed improved kinetics for reducing CB 1954. Similarly, Mahan et al. (22) markedly increased *E. coli* cytosine deaminase activity toward 5-fluorocytosine by alanine-scanning mutagenesis. The improved enzymes were more effective in sensitizing tumor cells to prodrugs. In the present work, we used directed evolution to improve the activity of a newly discovered prodrug-reducing enzyme of *E. coli*, YieF, for mitomycin C and CB 1954.

Our most evolved version of YieF, Y6, shows marked improvements over the activity of the wild-type enzyme in prodrug-mediated HeLa cell killing. It reduced the viability of HeLa tumor cells by >40% at the clinically relevant prodrug concentration of 1 μmol/L; neither the wild-type *E. coli* YieF nor the previously studied prodrug-reducing enzyme, the *E. coli* NfsA, had any effect on HeLa cells survival at this prodrug concentration. When a series of enzyme concentrations was used, the superiority of Y6 was again evident. For instance, at 10 μg/mL, the wild-type enzymes had no effect but Y6 reduced tumor cell survival by nearly 70%, indicating that lower expression levels of Y6 can still be effective. This is relevant with respect to the bystander effect, which arises from the fact that the methods of delivery of genes or enzymes to tumors do not transform every tumor cell. Thus, the effectiveness of prodrug chemotherapy, when practiced in conjunction with gene/enzyme delivery to tumors, relies on activated prodrug leakage from the transformed cells to kill the neighboring nontransformed cells; an enzyme effective at lower concentrations will, therefore, be more useful.

Y6 also reduced HeLa cell survival more markedly compared with the wild-type enzymes at all HeLa cell exposure periods tested, as well as when SL 7838 was used as the vehicle of enzyme delivery to tumor cells. When adhered HeLa cells were used as an *in vitro* model, at least an order of magnitude fewer Y6-producing bacteria were required to destroy the tumor cells compared with the YieF- or NfsA-producing bacteria. Taken together, these findings indicate that Y6 is a promising enzyme for use in cancer chemotherapy. We have recently devised a direct (instead of chromate reduction) method for screening shuffled genes encoding superior prodrug-reducing enzymes.[8] Given the track record of dramatic improvements that directed evolution can achieve (23), we expect to generate even more effective enzymes.

[8] Y. Barak, S. Jaiswal, J. Rao, A. Matin, unpublished data.

Acknowledgments

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