Supporting Information

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SI Materials and Methods

Materials. The National Cancer Institute/Developmental Therapeutics Program Open Chemical Repository diversity set II (http://dtp.cancer.gov/) of 1,364 compounds was used in the inhibitor screen. DNA standards and DNA loading dye [10 mM Tris·HCl (pH 7.6), 0.03% (wt/vol) bromophenol blue, 0.03% (wt/vol) xylene cyanol, 60% (vol/vol) glycerol, and 60 mM EDTA] were from Fermentas. A 1× working solution of Tris/acetate/EDTA (TAE) buffer contained 40 mM Tris·acetate and 2 mM EDTA and was pH-adjusted to 8.0.

Determination of protein concentration was performed according to the method of Bradford (1) with BSA as standard or by measuring the absorbance at 280 nm.

PCR with a Limiting Amount of dCTP. PCR samples were analyzed by agarose gel electrophoresis and ethidium bromide staining or by fluorescence intensity measurement after mixing with SYBR green. For fluorescence intensity analysis, a 10-μL aliquot from the PCR samples was mixed with 190 μL of SYBR green (1× working solution per the manufacturer's instructions) dissolved in 1× TAE buffer and incubated for ∼5 min in the dark. Fluorescence intensity was recorded at 520 nm (10-nm bandwidth) with excitation set to 485 nm (10-nm bandwidth) with the use of a microplate reader fluorometer (Polarstar Omega plate reader; BMG Labtech Gmbh).

Assay of RNR Enzyme Activity. The reaction volume was 50 μL, and reactions were started by addition of ^{3}H-CDP (12,000 counts per minute/nmol) to give a concentration of 100 μM (this corresponds to ∼3·K_m for CDP for *Pseudomonas aeruginos*a RNR). The samples were incubated for 45 min (giving a near-linear substrate consumption progress curve and about 30% substrate turnover), quenched by addition of 50 μL of ice-cold buffer [15 mM $MgCl_2$ in 25 mM ammonium carbonate/bicarbonate (pH 8.9)], and immediately placed on ice, or frozen if subsequent analysis was not performed directly. For comparison, a dose–response experiment with hydroxyurea was performed with identical reaction conditions except that the highest concentration tested was 1.75 mM. Formed dCDP was purified by boronate affinity chromatography and quantified by liquid scintillation counting (2, 3).

Antimicrobial Activity. Compounds with confirmed activity in the dose–response analysis were tested for bacterial growth inhibition at 100 μM in LB (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 mmol of NaOH per liter) and in a disk diffusion test (4) with 400 nmol of substance loaded onto paper discs (6-mm antibiotic assay discs; Whatman) placed onto LA plates (LB with 15 g of agar per liter, 4-mm media depth in plates).

For the growth test, 2 mL from an overnight culture of *P. aeruginosa* PAO1 in LB was inoculated into 48 mL of fresh LB. The culture was grown at 37 °C until the OD_{600} reached ∼0.6 and was then diluted 600-fold in 1-mL aliquots. Test substance (final concentration of 100 μM) was added, and cultures grown for 18 h under shaking at 37 °C. For disk diffusion tests, 100 μL from the bacterial culture with OD_{600} ∼ 0.6 was evenly spread on LA plates. Paper discs containing 400 nmol of test substance were placed on top of the solid media, and plates were incubated for 18 h at 37 °C. Reference tests with hydroxyurea, carbenicillin, and tetracycline were also performed.

Compounds that prevented the increase in OD_{600} in liquid bacterial cultures or that gave rise to a zone of inhibition in the disk diffusion test were used to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (5). For determination of MIC, 1-mL aliquots of bacterial cultures with OD_{600} ∼ 0.001 (600-fold dilution from stock culture as discussed above) containing 400 μM test substance (dissolved in DMSO) were prepared. Dilution series (2-fold dilution steps) from 400 μM down to 0.39 μM, along with a control without test substance, were prepared, and the resulting cultures incubated under gentle shaking for 18 h at 37 °C. All samples contained the same amount of solvent [2% (vol/vol) DMSO]. The lowest concentration of test substance that significantly prevented an increase in the OD_{600} of the bacterial culture was taken as the MIC. To determine an MBC, a sterilized inoculation loop was dipped into cultures without sign of growth and the inoculum was streaked onto a fresh LA plate without inhibitory substance. After incubation at 37 °C for 18 h, plates without visible bacterial growth were taken to define the MBC.

Bacterial Cultivation for Real-Time PCR Analysis. Overnight cultures of *P. aeruginosa* PAO1 in minimal media [standard recipe per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 3 mg $CaCl_2$, 1 mM $MgSO_4$, and 0.4% (wt/vol) glucose] enriched with 1 μM vitamin B_{12} were diluted 1:15 into fresh vitamin B_{12}-enriched minimal media and cultured at 37 °C under shaking (200 rpm) in conical flasks. When the OD_{600} reached 0.15, RNR inhibitors dissolved in DMSO were added to give subbactericidal concentrations; streptonigrin, 20 μM; toluidine blue, 100 μM; NSC228155, 100 μM; and NSC361666, 100 μM. DMSO was only added to control samples. For preparation of mRNA, samples were withdrawn immediately after the first addition of inhibitor/DMSO and at several time points during the following 3 h.

mRNA Isolation and RT. Immediately after withdrawal, samples were mixed with RNAprotect Bacteria Reagent (Qiagen). Bacterial cells were then concentrated by centrifugation and stored at −70 °C until further processed. mRNA was prepared using the RNeasy Mini Kit (Qiagen). Genomic DNA was removed from RNA preparations using DNase I (Fermentas), and cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers.

Real-Time PCR. For quantitative real-time PCR, the Maxima Probe/ROX qPCR kit (Fermentas) was used. The investigated genes of interest were RNR of class I (*nrdA*) and II (*nrdJ*), and *rpoD* was used as the housekeeping control gene (6). The specific primer sequences were as follows:

i) nrdA:
CCAAGCTGAAGGAATTCGAC (5′→3′ primer)
TGTGGATGAAGTAGCGGTC (3′→5′ primer)
TGGAGAAACTGGGCAAGGCCATCGATCA (probe)

ii) nrdJ:
TCGATTCGTCGATCTCCAAGAC (5′→3′ primer)
ATTGAAGCGGAAGGTGGTGC (3′→5′ primer)
ACCGACTACCCGTTCGAGGACTTCAAGGACA (probe)

iii) rpoD:
ACGTCCTCAGCGGCTATATC (5′→3′ primer)
CTTCTTCCTCGTCGTCCTTC (3′→5′ primer)
CAACCTGAAGGACGATTCCGCCGACTCGAAA (probe)

The comparative cycle threshold (ΔΔCt) method (7) was used to determine the fold-change relative to the transcript level at the point of the first addition of RNR inhibitor, or DMSO for control samples.

Bacterial Cultivation for Measurement of dNTP Pools. Overnight cultures of *P. aeruginosa* O1 in minimal media were diluted 1:15 into fresh minimal media and cultivated at 37 °C with shaking (200 rpm) in conical flasks. When the OD_{600} reached 0.35, RNR inhibitors dissolved in DMSO were added to give subbactericidal concentrations (streptonigrin, 20 μM; toluidine blue, 100 μM). DMSO was only added to control samples. After addition of inhibitor/DMSO, cultivation was continued for 30 min, after which the cultures were centrifuged at 20,000 × g for 4 min at 4 °C to harvest the bacterial cells.

Extraction and Purification of dNTPs. Nucleotide extraction was performed similar to the method of Rabinowitz and Kimball (8). After harvest, the supernatant was carefully removed, the bacterial cell pellet was suspended in 65% acidic acetonitrile (65 volume units of acetonitrile, 35 volume units of water, and 100 mM formic acid), and 50 nmol of dITP was added as an internal standard and to monitor losses in subsequent steps. The bacterial suspension was incubated on ice for 30 min and thoroughly mixed by intense vortexing for 15 s every 10th minute. After incubation, cell debris was removed by centrifugation at 30,000 × g for 20 min at 4 °C. The resulting pellet was discarded, and the supernatant was evaporated to dryness in a freeze dryer. The freeze-dried material was dissolved in 500 μL AmBic buffer [50 mM ammonium carbonate/bicarbonate (pH 8.9) and 15 mM $MgCl_2$] and loaded onto a boronate affinity gel column (Affi-Gel Boronate Gel; BioRad) preequilibrated with AmBic buffer. dNTPs were eluted from the column with 1.5 mL of AmBic buffer (2, 3), and the eluate (2 mL) was freeze-dried and then redissolved in 500 μL of HPLC buffer (next section).

HPLC-Based Quantification of dNTPs. dNTPs were separated by isocratic elution from a PolyWax LP column (200 × 4.6 mm, 5 μm, 300 Å) with 0.6 M potassium phosphate (pH 4.85) containing 2.5% (vol/vol) acetonitrile at a flow rate of 0.75 mL/min, similar to the method of Håkansson et al. (9). Quantification was based on peak area measurements (UV detection at 254 nm), related to the internal standard (dITP), and adjusted for absorbance differences between nucleotides. The deduced nucleotide levels for the different treatments were compared by a two-way ANOVA, with the different dNTPs defining blocks, in conjunction with the Tukey–Kramer test.

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
2. Shewach DS (1992) Quantitation of deoxyribonucleoside 5′-triphosphates by a sequential boronate and anion-exchange high-pressure liquid chromatographic procedure. *Anal Biochem* 206:178–182.
3. Hofer A, Ekanem JT, Thelander L (1998) Allosteric regulation of *Trypanosoma brucei* ribonucleotide reductase studied in vitro and in vivo. *J Biol Chem* 273:34098–34104.
4. Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. *Tech Bull Regist Med Technol* 36:49–52.
5. Andrews JM (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 48(Suppl 1):5–16.
6. Savli H, et al. (2003) Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* 52:403–408.
7. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF (2006) High-throughput real-time quantitative reverse transcription PCR. *Curr Protoc Mol Biol* 73:15.8.1–15.8.28.
8. Rabinowitz JD, Kimball E (2007) Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*. *Anal Chem* 79:6167–6173.
9. Håkansson P, Hofer A, Thelander L (2006) Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* 281: 7834–7841.

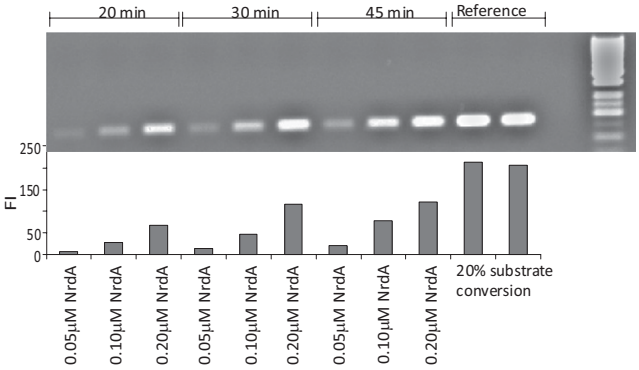

Fig. S1. PCR-based detection of RNR activity. Various concentrations of class I RNR from *Escherichia coli* (0.6 μM NrdB and 0.05, 0.1, or 0.2 μM NrdA) were incubated with 500 μM CDP for 20, 30, or 45 min to form dCDP. NDPK was subsequently added, and reactions were incubated to form dCTP before the PCR. Reference reaction mixtures, with identical constituents as in the test samples but with inactivated enzyme and substrate and product concentrations corresponding to 20% substrate conversion were also prepared. (*Upper*) PCR products (DNA of ~180 bp) were separated by agarose gel electrophoresis [2% (wt/vol) agarose gel with 0.5 μg/mL ethidium bromide for 35 min at 100 V] and visualized by UV light. (*Lower*) PCR products were quantified by measuring the fluorescence intensity (excitation at 485 nm and emission monitored at 520 nm) after mixing with SYBR green (10 μL of PCR sample, 190 μL of 1× SYBR green in TAE buffer).

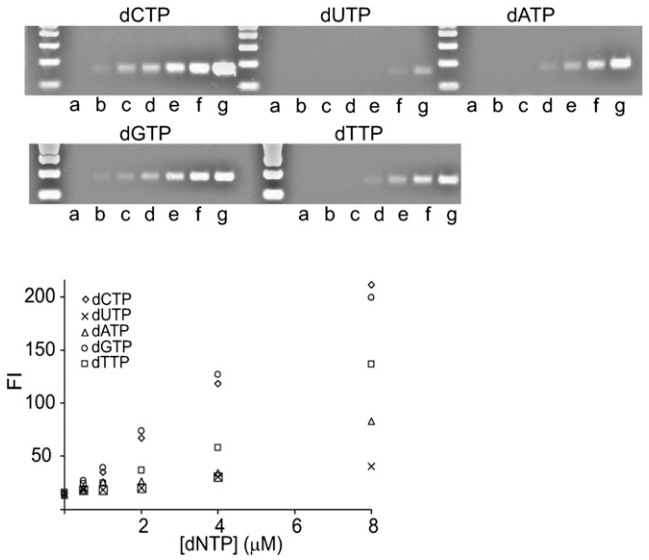

Fig. S2. PCR-based quantification of RNR enzyme activity. (*Upper*) PCR-based assay with three dNTPs in excess (200 μM each) and limiting amounts of either dCTP, dUTP, dATP, dGTP, or dTTP [0 (a), 0.5 (b), 1 (c), 2 (d), 4 (e), 8 (f), and 16 (g) μM] shows that quantifications are linear up to at least 8 μM dNTP for all five assays. DNA (*ca*. 180 bp) from PCRs was separated on an ethidium bromide-containing (0.5 μg/mL) agarose gel [2% (wt/vol)] for 35 min at 100 V. Visualization of DNA was achieved by UV excitation of ethidium bromide-stained DNA. (*Lower*) PCR-based assay with limiting amounts of dCTP or dGTP gave a better response compared with dTTP and dATP, and dUTP gave the lowest response. An aliquot (10 μL) from each PCR was mixed with SYBR green dissolved in TAE buffer (190 μL), and the fluorescence intensity was recorded (excitation set to 485 nm and emission monitored at 520 nm).

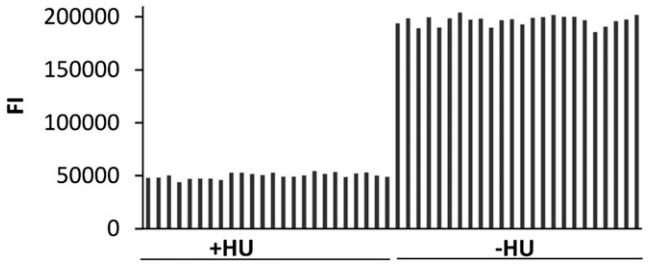

Fig. S3. Representative example of samples for calculation of the Z-factor of the PCR-based assay. The Z-factor was calculated according to the equation $1-(3\sigma_P+3\sigma_N)/|\bar{P}-\bar{N}|$, where σ_P and σ_N are SDs of the positive and negative controls and $\bar{P}$ and $\bar{N}$ are the average of positive and negative controls. The figure shows the recorded fluorescence intensity of PCR samples derived from positive and negative RNR control incubations. RNR reactions were performed with 3.5 mM hydroxyurea (+HU) or without hydroxyurea (–HU). Other reaction conditions were as described in the main text. The Z-factor of the given data is 0.86.

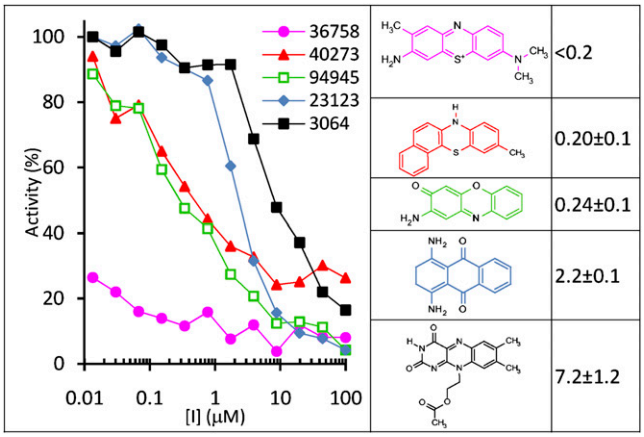

Fig. S4. Anthraquinone-like RNR inhibitors. Five anthraquinone-like compounds showed >90% inhibition of RNR activity in the PCR-based assay. Structures and the associated data are colored identically: the redox indicator toluidine blue (magenta), a methylene blue derivative (red), the resorufin analog questiomycin A (green), crystal violet 47 (blue), and an isoalloxazine/riboflavin derivative (black). The normalized dose–response data (%) of the compounds roughly cluster in three groups with apparent IC_{50} values below 200 nM (NSC36758), close to 200 nM (NSC40273 and NSC94945), and above 2 μM (NSC23123 and NSC3064). Toluidine blue (NSC36758) is the most potent compound and exhibits >70% inhibition at 13 nM (discussed in main text); it also had a bactericidal effect on the growth of *P. aeruginosa* (Table 1). MarvinSketch (version 5.3.8, 2010, www.chemaxon.com) was used for structure drawing.

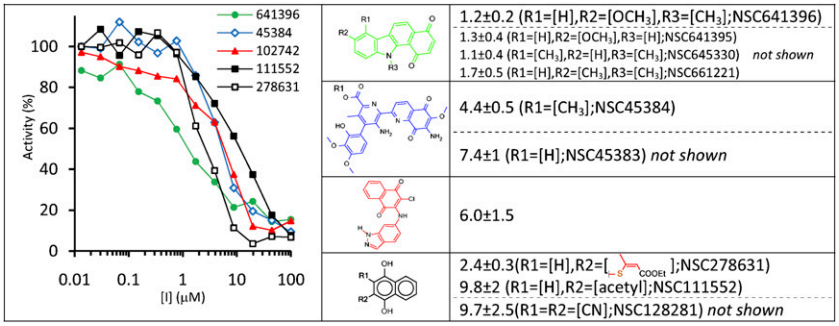

Fig. S5. Naphthoquinone-like RNR inhibitors. Ten naphthoquinone-like compounds showed >90% inhibition of RNR activity in the PCR-based assay. Structures and the associated data are colored identically: four naphthoquinones fused in a four-ring system (green), streptonigrin and esterified streptonigrin (blue), a linked naphthoquinone (red), and three hydronaphthoquinones (black). The normalized dose–response data (%) of the compounds show apparent IC_{50} values between 1.1 μM and 9.8 μM. For the naphthoquinones with fused four-ring systems data, one of four identified compounds is shown because all four compounds elicited very similar responses. For streptonigrin, the effect of the modification was small and only data for one compound are presented. For the hydronaphthoquinones, data for two of three identified compounds are shown (■ and □), which exemplifies the pronounced effect of a substituent in the R2 position. Streptonigrin had bactericidal effect on the growth of *P. aeruginosa* (Table 1). MarvinSketch (version 5.3.8, 2010, www.chemaxon.com) was used for structure drawing.

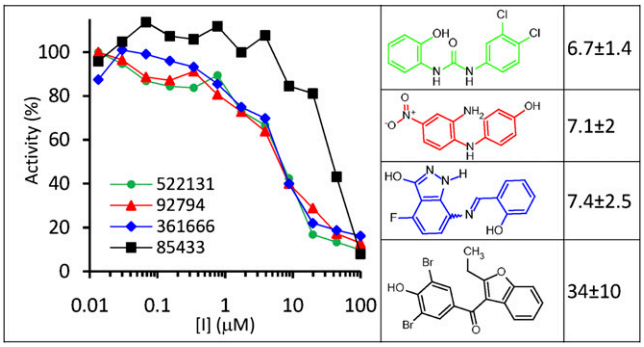

Fig. S6. Phenol-containing RNR inhibitors. Four phenol-containing compounds showed >90% inhibition of RNR activity in the PCR-based assay. Structures and the associated data are colored identically. The normalized dose–response data (%) of the compounds show that three of the compounds have apparent IC_{50} values clustered around 7 μM and one has a value of 34 μM. With respect to the phenol functionality, few ring substituents give a lower IC_{50} value. NSC361666 (blue) had a bactericidal effect on the growth of *P. aeruginosa* (Table 1). MarvinSketch (version 5.3.8, 2010, www.chemaxon.com) was used for structure drawing.

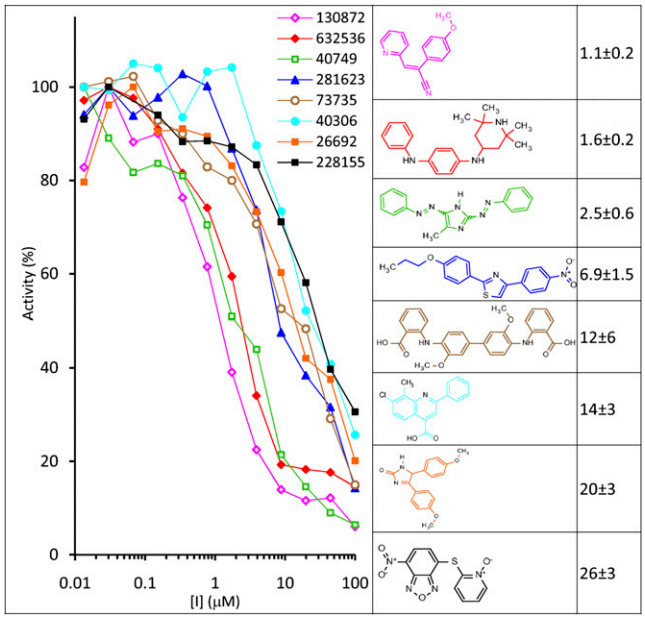

Fig. S7. Functionally diverse RNR inhibitors. Eight chemically diverse compounds showed >90% inhibition of RNR activity in the PCR-based assay. Structures and the associated data are colored identically. The normalized dose–response data (%) of the compounds show that IC_{50} values ranged from 1.1 to 26 μM. Among these, the most potent compound NSC130872 (magenta) has been shown to inhibit cyclin-dependent kinase. NSC228155 (black) had a bactericidal effect on the growth of *P. aeruginosa* (Table 1). MarvinSketch (version 5.3.8, 2010, www.chemaxon.com) was used for structure drawing.

Table S1. Majority of *P. aeruginosa* class I RNR inhibitors are inactive against human cells and in anticancer tests according to PubChem BioAssays

Structural classification of *P. aeruginosa* class I RNR inhibitors*	Results from PubChem BioAssays†		
	BioActivity outcomes		
	Inactive	Inconclusive/unspecified	Total no. of screens
Anthraquinone-like			
36758 (also inhibits *P. aeruginosa* growth, expression of RNR genes, and dNTP pools)	2		2
40273	1		1
94945	1		1
23123	1		1
3064	5		6
Naphthoquinone-like			
641396	73		77
45383 (also inhibits *P. aeruginosa* growth, expression of RNR genes, and dNTP pools)	18	5	101
45384	41		73
102742	1		1
111552	1		1
278631	7	1	8
Fenol-containing			
522131	5		7
92794	6		6
361666 (also inhibits *P. aeruginosa* growth)	7		7
85433	60		66
Diverse			
130872		No data	
632536	77		77
40749		No data	
281623	1		1
73735	80	8	99
40306		No data	
26692	1		1
228155 (also inhibits *P. aeruginosa* growth)	2		2

P. aeruginosa RNR inhibitor compounds characterized in this study are structurally classified and identified by their NSC identification number [with links to PubChem BioAssays (1)].

*Left column also notes compounds that were found to inhibit the growth of *P. aeruginosa* and affected *P. aerugionsa* RNR gene expression and dNTP pools.

†PubChem BioAssay results are primarily based on data from NCI Human Tumor Cell Line Screens (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html) and/or Yeast Anticancer Drug Screens (http://dtp.nci.nih.gov/yacds/index.html).

1. Wang Y, et al. (2012) PubChem's BioAssay Database. *Nucleic Acids Res* 40(Database issue):D400–D412.