

Title Page

**ENHANCED DEGRADATION OF DIHYDROFOLATE REDUCTASE THROUGH INHIBITION
OF NAD KINASE BY NICOTINAMIDE ANALOGUES**

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Running Title Page

Running Title: Degradation of DHFR by thionicotinamide analogues

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EGFP, enhanced green fluorescent protein; wt, wild type; FBS, fetal bovine serum; dFBS, dialyzed FBS; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; HT, hypoxanthine, glycine.

Abstract

Dihydrofolate reductase (DHFR), because of its essential role in DNA synthesis, has been targeted for the treatment of a wide variety of human diseases including cancer, autoimmune and infectious diseases. Methotrexate (MTX), a tight binding inhibitor of DHFR is one of the most widely used drugs in cancer treatment, and is especially effective in the treatment of acute lymphocytic leukemia, non-Hodgkin's lymphoma, and osteosarcoma. Limitations to its use in cancer include natural resistance and acquired resistance due to decreased cellular uptake and decreased retention due to impaired polyglutamylate formation and toxicity at higher doses. Here we describe a novel mechanism to induce DHFR degradation through cofactor depletion in neoplastic cells by inhibition of NAD kinase, the only enzyme responsible for generating NADP, which is rapidly converted to NADPH by dehydrogenases/reductases. We identified an inhibitor of NAD kinase, thionicotinamide adenine dinucleotide phosphate (NADPS) that led to accelerated degradation of DHFR and to inhibition of cancer cell growth. Importantly, combination treatment of NADPS with MTX displayed significant synergy in a metastatic colon cancer cell line and was effective in a MTX-transport resistant leukemic cell line. We suggest that NAD kinase is a valid target for further inhibitor development for cancer treatment.

Introduction

Dihydrofolate reductase (5, 6, 7, 8-tetrahydrofolate NADP oxidoreductase, EC 1.5.1.3), catalyzes the reduction of dihydrofolate (FH₂) to tetrahydrofolate (FH₄) using NADPH as a cofactor. The binding sites of FH₂ and NADPH are at two different but connected regions of DHFR. They comprise a long channel in the active site of the enzyme positioning the two ligands in close proximity to enable hydride transfer from NADPH to FH₂, generating FH₄. Tetrahydrofolate is converted to 10-formyl and 5-10 methylene FH₄, essential cofactors in the synthesis of purines, thymidylate, and certain amino acids (Abali et al., 2008). Inhibition of DHFR results in a depletion of the reduced folate pool, inhibition of RNA and DNA synthesis, and cell death. For this reason, DHFR has been a critically important therapeutic drug target. DHFR inhibitors targeting the FH₂ binding site have been used in the treatment of cancer, autoimmune diseases, bacterial and fungal infections. However, DHFR inhibitors targeting the FH₂ binding site have limitations due primarily to intrinsic and acquired resistance mechanisms (Cheok and Evans, 2006; Gorlick et al., 1996; Rego-Perez et al., 2008), including decreased uptake and, less commonly, due to gene amplification or changes in polyglutamylation. Thus a search for new strategies that bypass these resistance mechanisms is warranted.

Strategies have been developed to reduce cellular levels of proteins that are involved in cell proliferation and cancer growth. Geldanamycin and its analogues such as 17-allylamino-17-demethoxygeldanamycin induce the degradation of several proteins. These proteins such as mutant p53, bcr-abl and Her-2 are involved in cancer proliferation through complexing with the protein chaperone, heat shock protein 90. For example, geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/β-catenin signaling in HER2-overexpressing human breast cancer cells (Slack et al., 2001). However degradation of these proteins by geldanamycin is not very selective and leads to hepatotoxicity (Latif et al., 2001). Another strategy reported is the induction of enzyme degradation through cofactor depletion in neoplastic cells. Methionine synthase, a vitamin B12 dependent enzyme, is an important enzyme in DNA synthesis.

Inactivation of vitamin B12 by nitrous oxide, by rendering methionine synthase inactive, triggers apoptosis and results in inhibition of proliferation in a variety of cancer cell lines (Livak and Schmittgen, 2001). Recently 5-aza-deoxycytidine has been shown to induce selective degradation of DNA methyltransferase 1 by the proteasomal pathway (Ghoshal et al., 2005).

Ligand-binding-induced conformational changes in proteins affect the thermodynamic stability of proteins (Ainavarapu et al., 2005). In fact, DHFR is stabilized by either its cofactor NADPH, or its potent inhibitor, Methotrexate (MTX), thus slowing down its degradation (Xiang, 2002). We hypothesized that the steady state level of DHFR may be decreased by NAD(P) analogues by either acting as competitive inhibitors with NADPH for DHFR, or by reducing NADPH pools available for proper folding of DHFR, leading to increased degradation.

Recent studies demonstrated that the flux of anabolic pathways such as nucleotide synthesis and fatty acid synthesis, which requires NADPH is increased due to genetic alterations in HIF-1, Akt and myc signaling pathways in cancer cells (Tong et al., 2009). Furthermore NADPH is also important to maintain the reduced pools of glutathione (GSH). While GSH is important for the removal of reactive oxygen species which are elevated in cancers, GSH levels are also increased in many cancers leading to resistance to chemo- or radio therapy (Estrela et al., 2006). We hypothesized that reducing NADPH levels may be important not only for the aforementioned pathways that are dysregulated in cancers but also for regulation of DHFR.

There are several enzymes that reduce NADP to NADPH, but only one enzyme, NAD kinase which generates NADP from NAD. Therefore, we initiated studies to identify inhibitors of NAD kinase that lead to decreased levels of NADPH which might cause accelerated degradation of DHFR. Herein we identify two compounds, thionicotinamide adenine dinucleotide (NADS) and thionicotinamide adenine

dinucleotide phosphate (NADPS) with potent cytotoxicity against cancer cells by decreasing the level of DHFR due to its inhibition of NAD kinase, which is necessary for NADPH generation. While it has been previously shown in vitro that NADPH protects DHFR from degradation in cell free extracts (Ainavarapu et al., 2005; Perkins et al., 1967; Wallace and Robert Matthews, 2002), the studies presented here for the first time show for the first time that NADPH also protects DHFR from degradation in a cellular context.

Materials and Methods

Cell culture and materials

RPMI-1640 medium, Dulbecco's Modified Eagle Medium (DMEM) medium, fetal bovine serum (FBS), dialyzed FBS (dFBS); G418 Sulfate (aka Geneticin), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). F-12 medium without glycine, hypoxanthine and thymidine was obtained from the Media Core facility at Sloan-Kettering Cancer Institute, New York, NY). Human mesenchymal stem cells (MSC) were purchased from Lonza (Cologne, Germany). NAD and NADP analogues (Table 1) and MTX were purchased from Sigma-Aldrich (St. Louis, MO).

C85 (a human colon cancer cell line) and CCRF-CEM (a human T cell acute lymphoblastic leukemia cell line) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The parental CCRF-CEM cell line and two drug resistant sublines derived from the parental line were used in this study. While both of the sublines are resistant to methotrexate, CCRF-CEM/R resistance is due to 5-fold increase in DHFR due to gene amplification, CCRF-CEM/T is resistant to classical antifolates such MTX and D1694 (aka raltitrexed, Tomudex®) due to impaired transport (Mini et al., 1985). DG44-(DHFR-EGFP) and DG44-(DHFR-S118A-EGFP) cell lines are Chinese hamster ovary cell lines with no DHFR expression, but contain stably transfected DHFR-EGFP or DHFR-S118A-EGFP fusion construct (Skacel et al., 2005) and were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and antibiotics.

siRNA transfection

Double stranded RNA complementary to NADK mRNA sequence 5'-GAG CGT CCT TGT CAT CAA GAA-3' was generated by Integrated DNA Technologies Inc. (Coralville, IA). The sequence of the sense strand of the siRNA duplex targeting NADK is 5' CUC GCA GGA ACA GUA GUU CUU 3'. C85 cells were seeded in a 6-well plate at a density of 6×10^5 cells/well in RPMI-1640 medium containing 10% FBS

and no antibiotics the day prior to transfection. Cells (50-60% confluency) were transfected with 100 pmol siRNA using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were harvested 48 hours after transfection for protein analysis.

Western blot analysis

Total protein extracted from cell lines was resolved on a SDS-PAGE as described earlier (Hsieh et al., 2009). Protein concentration was determined using Bradford protein assay according to the manufacturer's guidelines (Bio-Rad Laboratories In., Hercules, CA). Proteins were resolved by 15% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories In., Hercules, CA). After blocking the membrane with 5% nonfat dry milk prepared in phosphate- buffered saline (PBS, pH 7.4) containing 0.1% Tween 20, the membrane was incubated with the desired primary antibody at the suggested dilution by the manufacture's protocol for 2 hrs at room temperature or overnight at 4 °C. The membrane was then incubated with peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence kit (Pierce ECL Western Blotting Substrate, Rockford, IL) according to the manufacturer's instructions. Anti-IMDPH and anti-ALDH1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- α -tubulin and anti-GAPDH antibodies, purchased from Sigma-Aldrich (St. Louis, MO), were used for loading controls. Anti-GFP antibody (Roche Diagnostics Corporation, Indianapolis, IN) was used to detect DHFR expression in DG44-(DHFR-EGFP) and DG44-(DHFR-S118A-EGFP) cell lines. The rabbit polyclonal antibody to human DHFR was custom-produced by Research Genetics/Invitrogen (Carlsbad, CA).

Cytotoxicity assay

Two methods were used for the determination of cytotoxicity of antifolates and NAD(P) analogues. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was performed according to the Cell Titer 96 Aqueous One Solution protocol (Promega,

Madison, WI). Three thousand cells/well were plated in 96-well plates in RPMI-1640 media supplemented with 10% dialyzed fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin or in F-12 media lacking hypoxanthine, glycine and thymidine supplemented with 10% dialyzed fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. After cells were incubated with the NAD analogues for 96 hrs, the MTS assay was performed according to the manufacturer's instructions. The absorbance at 490 nm was measured using a microplate reader. The percent of viable cells was determined by SOFTmax Pro 2.6.1 software (Molecular Devices Corp., Sunnyvale, CA).

Cytotoxicity of NADS and NADPS was also determined using the trypan blue exclusion assay. Briefly, suspension cells with or without drug treatment were collected and cell viability was determined using the Vi-CELL™ Series Cell Viability Analyzer (Beckman Coulter, Carlsbad, CA).

The cytotoxicity data were further analyzed using GraphPad Prism 4 software (GraphPad Software Inc., CA). The 50% inhibitory concentration (ED50; the drug concentration required to obtain 50% cell kill compared to control) was determined using the non-linear regression curve fit of the graphs drawn by GraphPad Prism 4 software. All experimental points were set up in replicate wells, and all experiments were repeated at least three times.

DHFR half-life studies

To determine the half life of DHFR in the absence or presence of NADPS, pulse-chase experiments were performed as described (Noe et al., 2003) with minor modifications. Briefly, two million CCRF-CEM/R cells were seeded on a 10 cm² dish. Cells were incubated in methionine-free RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% dialyzed FBS for 1 hr and then labeled with 500 µCi/plate of L-[³⁵S]methionine-cysteine Express Cell Labeling Mix (PerkinElmer Life Sciences, Boston, MA) for 1 hr. Labeled cells were harvested and washed with PBS twice. Chase medium (RPMI-1640

medium supplemented 10% FBS and freshly prepared 2 mM methionine (Sigma, St. Louis, MO) in the presence or absence of 10 μ M NADPS were added to cells at different time points except for the control sample. Cells were lysed (150 mM NaCl, 5 mM EDTA, 50 mM HEPES, pH 7.5, and 1.0% Triton X-100) and DHFR was immunoprecipitated with anti-DHFR antibody and protein G Sepharose 4 Fast Flow (GE Healthcare, NJ) at 4 °C overnight. Beads were washed with lysis buffer three times and eluted with SDS/PAGE sample buffer followed by electrophoresis. Gels were exposed to X-ray film and analyzed by densitometry with Image J software (NIH, National Institute of Health, USA).

Clonogenic assay

Two hundred cells per well of C85 cells were plated into 6-well culture plates. The following day, the medium was removed and replaced with fresh RPMI-1640 medium supplemented with 10% dialyzed FBS in the presence or absence of NADPS or MTX or the combination of the two drugs. After 2 weeks of incubation, the plates were washed with PBS (pH 7.4) and fixed with crystal violet solution (0.4% formaldehyde, 0.2% crystal violet) and colonies were counted.

Analysis of drug combination studies

Exponentially growing C85 or CCRF-CEM cells were plated in 6-well dishes at a density of 200 cells per well. At 24 hr after the incubation, the cells were exposed to serial dilutions of MTX, NADPS or both for 2 weeks. After incubation, the cells were stained and viable colonies were counted. After calculating % inhibition, drug interactions were analyzed by the Calcsyn program of Biosoft (Cambridge, UK) which determines synergy or antagonism using the multiple drug effect equation (Chou and Talalay, 1984). Outcomes of the drug interactions were determined by the combination index (CI) values calculated by Calcsyn. If CI is greater than 1, the combination is antagonistic, CI =1, additive and CI less than 1, synergistic.

Cell cycle analysis

C85 cells were incubated with or without NADPS or MTX for 24 hr. Cells were collected, washed with cold PBS twice, and then fixed with 70% ethanol overnight at -20 °C. Cells were centrifuged at 1000xg for 5 minutes, and washed with PBS once, and then stained with 50 µg/ml Propidium Iodide (PI) Staining Solution (BD biosciences, CA) in the dark for 15 minutes. Distribution of the cells in the various phases of the cell cycle was determined with a FACScan flow cytometer (Becton Dickinson, CA). In each sample, 10,000 gated events were acquired. The cell-cycle phases were analyzed using CELLQuest software (Becton Dickinson, CA).

Measurement of DHFR activity

The specific activity of DHFR from lysates of hamster and the variants of human DHFR-EGFP was determined as described (Ercikan-Abali et al., 1996).

NAD kinase activity and inhibition studies

Pure NAD kinase was purchased from Enzo Life Sciences (Farmingdale, NY). Effect of NADPS on NAD kinase activity was measured spectrophotometrically with a coupled assay that converts the NADP generated by NAD kinase into NADPH using glucose-6-phosphate dehydrogenase (G6PDH) (Lerner et al., 2001). Briefly, NAD kinase activity was determined by measuring the increase in absorbance at 340 nm caused by the reduction of NADP to NADPH by G6PDH. The enzyme reaction for inhibition of NAD kinase by NADPS contains 2 mM NAD, 2 µg of human NAD Kinase, 1 U G6PDH, and 1.5 mM or 3 mM NADPS, 10 mM ATP, 10 mM MgCl₂ and 50 mM Tris-HCl (pH 7.8) in a final volume of 1 ml. Controls were without NAD or NADK or NADPS or reaction mix alone. The appearance of NADPH was measured at 340nm using a Beckman spectrophotometer.

Quantitative-real time reverse transcription polymerase chain reaction (Q-RT-PCR)

Quantitative reverse transcription-PCR was used to analyze DHFR mRNA levels in the presence and absence of 40 μ M NADPS for 24 hr in C85 cells. DHFR and β -actin mRNAs were examined by quantitative real time reverse transcription-PCR. Following total RNA purification using TRIzol (Invitrogen), the integrity and the concentration of the RNA was determined using a NanoDrop spectrophotometer and by 1% agarose gel. Quantitative PCR was performed with 50 ng of purified RNA TaqMan One Step RT PCR according to manufacturer's protocol (Applied Biosystem, Foster City, CA) on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with TaqMan probe based gene expression assays for DHFR and β -actin (Applied Biosystems, Foster City CA). Gene expression levels of DHFR were normalized to β -actin. The reaction and cycling conditions for both genes were: 48° for 40 min; Step 2, 95° for 10 min, followed by 40 cycles of 95° for 15 s, 60° for 1 min. Reactions were terminated with incubation at 4° for 10 minutes. Each sample was run using three different wells and each experiment was repeated for three times. The expression of DHFR in NADPS or MTX treated cells relative to untreated control was calculated according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) using β -actin as reference gene.

Computational Methods

The models of Human NADK were built from the x-ray crystal structure of human NAD kinase (3PFN.pdb) (Wang et al., 2010). The Jackal prefix program was used to account for missing residues in the structure (Xiang, 2002). The models of human NAD kinase complexed to NAD^+ and NADP^+ were built from least squares fit of the protein backbone atoms of each model with 1Z0Z.pdb (*Arch. fulgidus* complex with NAD^+) and 1Z0U.pdb (*Arch. fulgidus* complex with NADP^+)(Liu et al., 2005). The STAMP structural alignment tool in the MultiSeq program in VMD was used to align the structures (Humphrey et al., 1996; Roberts et al., 2006; Russell and Barton, 1992). We used the UCSF Chimera

Matchmaker and viewing program to map residue conservation to the cartoon plot of the human structure (Meng et al., 2006).

Dynamics: Each model was refined by energy minimization and relaxed using molecular dynamics using the Amber 11 suite of biomolecular simulation programs (Case et al., 2005). The Amber99SB force-field was used in all molecular mechanics calculations (Hornak et al., 2006). Parameters for NAD⁺, NADP⁺, NADPS⁺ were computed using the Amber Antechamber accessory program with AM1-BCC partial atomic charges (Wang et al., 2006). Each model was energy minimized in vacuo using 500 steps of steepest descents followed by 2000 steps of conjugate gradient minimization with a non-bonded forces cutoff of 12.0 Å. The resulting model was solvated in a periodic octahedral box of TIP3P water and the net formal charge neutralized by addition of the appropriate number of Na⁺ ions. A short range cutoff of 9.0 Å was used and the PME method (Darden et al., 1993; Essmann et al., 1995) was used to account for long range electrostatic interactions. Each model was energy minimized keeping the protein atoms restrained using 750 steps of steepest descent followed by 750 steps of conjugate gradient minimization. The restrained minimization was followed by unrestrained minimization of the system using 1000 steps of steepest descents followed by 1500 steps of conjugate gradient. A solvent equilibration dynamics run was performed keeping the protein atom positions restrained, using a Langevin thermostat with $\gamma = 1.0$ ps⁻¹, random seed generation, and temperature equal to 300 K with no pressure coupling (an NVT ensemble) for a period of 100 ps. A 2 fs time step was used and the shake constraint was used on all bonds to hydrogen. The solvent equilibration step was followed by an unrestrained production dynamics run using pressure coupling with a Berendsen barostat (Berendsen et al., 1984) set at 1 atm and temperature set at 300 K. The production dynamics was run for a period of 2 ns. The final snapshot of each trajectory was stripped of water and ions and refined using energy minimization with the generalized Born implicit solvation method (Hawkins et al., 1995; Hawkins et al., 1996) used to account for solvation. A 16.0 Å nonbonded forces cutoff was used for generalized Born minimization using OBC model I

(Onufriev et al., 2000; Onufriev et al., 2004) with 500 steps of steepest descents followed by 1000 steps of conjugate gradient minimization.

MM/GBSA calculations: The generalized Born OBC I model (Onufriev et al., 2000; Onufriev et al., 2004) with bondi2 radii was used with an external dielectric constant of 78.5 and an internal dielectric constant of 4.0 per the optimum MM/GBSA method (Hou et al., 2011) for proteins with polar binding sites (Wang et al., 2006).

Results

An NAD analogue, NADPS selectively kills cancer cells, while sparing normal cells

Thirteen NAD/NADP analogues were screened for cytotoxicity using the MTS assay in DG44-(DHFR-EGFP) cells. These cells are the DHFR-deficient Chinese hamster ovary cell line stably transfected with a DHFR-EGFP fusion construct driven by a CMV promoter. Detection of the basal levels of DHFR in most of the cell lines is hard to detect, therefore these cells were chosen for initial studies since the transfected DHFR fusion protein with EGFP makes detection much easier. Moreover, DG44 cells with or without DHFR expression were used to verify that the cytotoxicity of the inhibitors is directly related to the expression of DHFR. To assess cytotoxicity of the analogues, Ham's F-12 media was used, as this media has 28-fold less nicotinamide than the RPMI-1640 media (8.2 μM vs. 0.295 μM), levels more comparable to human serum. Two compounds, thionicotinamide adenine dinucleotide (NADS) and thionicotinamide adenine dinucleotide phosphate (NADPS) had potent cytotoxic effects when tested against the DG44-(DHFR-EGFP) cells. The ED₅₀ for both compounds was 1 μM for DG44-(DHFR-EGFP) cells (Fig. 1A). The fact that both compounds were equally effective suggested that NADS was converted rapidly to NADPS by NAD kinase in cells. The other NAD(P) analogues tested (Table 1) had EC₅₀ values greater than 200 μM (see the inset in Fig. 1A).

To investigate whether the cytotoxicity observed with these analogues was cell specific, two different cell lines; a metastatic human colon cancer cell line (C85) and a T-cell lymphoblastic leukemia cells (CCRF-CEM) were treated with NADS and NADPS. Unlike DG44 cells, C85 and CCRF-CEM cells did not grow well in Ham's F-12 media, therefore RPMI-1640 media supplemented with 10% dialyzed FBS was used to grow these cells. In contrast to DG44(DHFR-EGFP) cells grown in Ham's F-12 medium, the ED₅₀ for NADPS and NADS was 40 μM in C85 cells and CCRF-CEM cells grown in RPMI-1640 (Fig. 1B). In order to demonstrate that the higher ED₅₀s for these two cell lines were due to the higher concentration of nicotinamide in RPMI-1640, DG44-(DHFR-EGFP) cells were also grown in RPMI-1640, and the

cytotoxicity of NADS and NADPS were determined. As expected, the ED50s for both compounds were about 10-fold higher in RPMI-1640 than Ham's F-12 media (Fig. 1C) indicating that the lower toxicity of these analogues was due to higher non-physiologic concentrations of nicotinamide, the precursor of NAD(P) in RPMI-1640 medium.

We also tested whether NADPS exhibits selective antitumor effect. Normal human cells, namely mesenchymal stem cells were treated with NADPS. Seventy five thousand C85 cells and mesenchymal stem cells were plated into 24-well tissue culture plates and the following day, the medium was removed and replaced with fresh medium containing NADPS. After 96 hours of incubation, cells viability was determined. At ED50 and ED75 for NADPS in C85 cells, NADPS was only mildly cytotoxic to human mesenchymal stem cells (Fig. 1D). Thus, we identified an NAD analogue, NADPS which exhibited a potent and selective antitumor effect.

The effect of NAD/NADP analogues on DHFR activity and expression

ThioNADP(H) has been used as an inactive cofactor for NADPH in experiments using chicken, mouse, *E. coli* and *L. casei* and it has been shown to bind to the cofactor binding site (Dunn et al., 1978; McTigue et al., 1993; Smith and Burchall, 1983; Thillet et al., 1990). However, when we tested the effect of NADPS on DHFR enzyme activity, only slight inhibition of human DHFR was observed even with 180 μ M NADS or NADPS (data not shown). We considered that the cytotoxicity by NADS and NADPS in the cell lines tested is due to a decrease in DHFR rather than inhibition of enzyme activity.

DHFR is stabilized in vitro by NADPH, FH₂, and MTX in vitro (Ainavarapu et al., 2005; Johnston et al., 1995; Perkins et al., 1967; Salvador et al., 2000). Therefore, a decline in NADPH levels due to NADS and NADPS might lead to DHFR instability and degradation. We used DG44-(DHFR-EGFP) to determine if DHFR levels are affected by NADS. After a 48 hr treatment with 1 and 10 μ M NADS,

DHFR fusion protein levels decreased as measured by Western blot analysis (Fig. 2A). We further tested the effect of NADS and NADPS on DHFR levels in the CCRF-CEM and CCRF-CEM/R cell lines. The basal level of DHFR is significantly higher in CCRF-CEM/R cell line than in the parental CCRF-CEM cells, therefore while DHFR levels decreased in CCRF-CEM/R cells; they were almost undetectable in the parental CCRF-CEM cell line when treated with NADS and NADPS (Fig 2B).

Does NAD(P)S primarily target DHFR?

NADP and NADPH play multiple roles in cells and are involved in redox reactions, signal transduction, DNA repair, and post-translational protein modifications (Bogan and Brenner, 2008). Although cell toxicity experiments demonstrated that cells overexpressing DHFR were less sensitive to NADPS (Fig. 3A), DHFR might not be the only target for these NAD/NADP analogues. Therefore the effect of NADPS on other dehydrogenases was examined using C85 cells. Three different enzymes were selected for study. Two of which are NAD-dependent dehydrogenases: GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and IMPDH (inosine-5'-monophosphate dehydrogenase) and the other is NAD(P)-dependent dehydrogenase: ALDH1a (aldehyde dehydrogenase). IMPDH is the rate limiting enzyme in the *de novo* purine biosynthesis and NAD analogues have been used to inhibit the activity of IMPDH. For example, tiazofurin, an NAD analogue is an orphan-drug for the treatment of chronic myelogenous leukemia (Malek et al., 2004). NAD analogue, N6-Naphthalenemethyl-2'-methoxybenzamido-beta-NAD⁺, is used to inhibit trypanosomal GAPDH. ALDH1a is a significant contributor to the reduction of NADP to NADPH. Protein levels of these three dehydrogenases were determined at different time intervals after treatment with 10 μ M NADPS (Fig. 3A). Although DHFR levels were significantly decreased even with 6 hr of treatment with NADPS, the protein levels of the other three dehydrogenases (ALDH1a, GAPDH and IMPDH) remained the same.

Next, we determined whether the cytotoxicity observed with NADP analogues was due to specifically targeting DHFR. We compared the cytotoxicity of NADPS in cells overexpressing DHFR and in cells not expressing DHFR to cells with normal levels of DHFR. We hypothesized that the cells overexpressing DHFR should be resistant to NADPS. In the first experiment, CCRF-CEM, a human T-cell lymphoblastic leukemia cell line, and a resistant subline (CCRF-CEM/R), due to a 5-fold increase in DHFR, were treated with NADPS. The EC₅₀ of NADPS was 2-fold higher in the resistant cell line (CCRF-CEM/R) than in the parental CCRF-CEM cell line (Fig. 3B). While the difference in EC₅₀s between the resistant and sensitive cell line is not striking, this result was not unexpected, since 5% of activity of the total basal levels of DHFR is sufficient for cell survival (Chattopadhyay et al., 2007) and as shown in Fig 2B, while NADPS treatment of CCRF-CEM/R cells leads to significant reduction in DHFR expression, these cells still had residual DHFR activity.

In the second experiment, both parental DG44 cells which have no DHFR activity and DG44-(DHFR-EGFP) cells stably transfected with wild type DHFR were treated with NADPS. Parental DG44 cells were grown in RPMI 1640 media which was supplemented with hypoxanthine and thymidine to allow these cells to grow in the absence of DHFR. If there is another target of NADPS, hypoxanthine and thymidine supplementation of these DHFR-null cells should not block the activity of NADPS, but these cells were resistant to NADPS treatment; there was more than 50% survival even at 200 μ M NADPS (Red line in Fig. 3C). However DHFR-transfected cells were sensitive to NADPS treatment with an EC₅₀ of 30 μ M (Black line in Fig. 3C) and the supplementation with hypoxanthine and thymidine led to an EC₅₀ of 130 μ M which is 4-fold higher than NADPS treatment alone (Blue line in Fig. 3C).

Taken together, these experiments indicated that the cytotoxicity of NADPS was mainly due to decreased levels of DHFR. However since DHFR-null cells were not completely resistant to NADPS treatment and the DG44-(DHFR-EGFP) were not rescued by NADPS cytotoxicity completely when the media was

supplemented with thymidine and hypoxanthine, there may be other targets of NADPS which contribute to the cytotoxicity observed.

The effect of NADPS on DHFR transcription

To investigate the possible mechanism of decreased DHFR protein levels we first determined whether it is due to the G1/S cell cycle arrest with NADPS treatment. DHFR activity has been shown to increase during S-Phase as it is required for DNA synthesis (Abali et al., 2008). Since MTX blocks DNA synthesis and arrests cell growth in early S phase, we reasoned that the decrease in DHFR caused by NADPS might also block cells at the G1/S phase. C85 cells were incubated with NADPS for 24 hr and then stained with propidium iodide for DNA content analysis. Compared to the no-treatment control, the percent of cells in the G1 phase increased to 11% at 10 μ M and 44% at 40 μ M NADPS concentrations (Fig 4 A-C). MTX was used as a positive control to show a G1/S phase block which showed very similar results albeit at lower concentration (Fig. 4D). Removal of NADPS from the cell culture after 24 h reversed the G1/S block (Fig. 4E-G).

Although expression of DHFR is induced dramatically at the G1/S boundary after growth stimulation, *dhfr* mRNA is present throughout the cell cycle suggesting that there is continuous transcription of DHFR at low levels throughout the cycle (Slansky and Farnham, 1996). Therefore while MTX treatment leads to G1/S arrest, DHFR mRNA levels remain similar before and after MTX treatment (Sowers et al., 2003). We investigated whether the same is true with NADPS treatment under the same conditions as the cell cycle experiments described above. CCRF-CEM cells were treated either with 10 μ M NADPS or 10 nM MTX for 24 hr. After harvesting the cells, quantitative real time reverse transcription-PCR was performed to compare DHFR mRNA levels between the drug-treated and untreated cells. There was no difference in the DHFR mRNA levels after either MTX treatment or NADPS treatment as compared to

untreated cells (Fig. 4H) confirming that the decreased DHFR protein levels after NADPS treatment cannot be explained by transcriptional down regulation of DHFR mRNA through G1/S block .

The effect of NAD(P)S on MTX-mediated translational upregulation of DHFR

Administration of MTX and other antifolates increases the steady-state DHFR levels through a translation-dependent mechanism (Chu et al., 1993; Ercikan-Abali et al., 1997; Hsieh et al., 2009; Skacel et al., 2005; Tai et al., 2004a; Tai et al., 2004b) and we proposed that DHFR mRNA is bound to the NADPH binding site of DHFR protein. Upon MTX administration, DHFR mRNA is released, thereby increasing DHFR translation (Hsieh et al., 2009). As NADS and NADPS significantly decreased DHFR levels, we tested whether these compounds would interfere with MTX-mediated DHFR translational upregulation (Fig. 5). After treating CCRF-CEM cells with 10 μ M NADS or NADPS alone for 24 hr and 48 hr, or simultaneously combined with 10 nM MTX for 24 hr and 48 hr, DHFR levels were determined using Western blotting. The first lane in both panel A and B are untreated cells. After treating the CCRF-CEM cells with MTX (lanes 2 and 3) for 24 and 48 hrs, DHFR levels were increased, however there was no detectable levels of DHFR after NADS or NADPS treatment (lanes 4 and 5 in panel A and B). When the CCRF-CEM cells were treated simultaneously with NAD analogues and MTX, translational upregulation of DHFR by MTX was not hindered by the NAD analogues NADS or NADPS (lanes 6 and 7 in Fig. 5A and 5B).

The effect of NADPS on DHFR Degradation

We further investigated whether proteasome inhibitors, such as MG132, could inhibit degradation of DHFR by NADPS. Due to high toxicity of MG132 in C85 cell, MCF-7 cells, (human breast adenocarcinoma cell line) were treated with 0.5 μ M MG132 in the presence of NADPS alone for 48 hr. (Fig. 5C) To verify that MG-132 was active, cyclin D1, which is known to be regulated by proteasomal degradation was used as a positive control and GADPH was used as a negative control (Wang et al.,

2007). MG132 prevented cyclin D1 degradation but did not affect DHFR levels either in the presence or absence of NADPS. These results are in accordance with the published data. Noe et al also demonstrated that treatment with the proteasome inhibitor MG132 did not affect the levels of DHFR (Noe et al., 2003). These findings provide evidence that the decreased DHFR protein levels after NADPS treatment is not due to inhibition of ubiquitin-mediated degradation.

Decreased half-life of DHFR due to NAD Kinase inhibition by NADPS

Since the decreased DHFR levels by NADPS could not be explained by any of the mechanisms described so far, we hypothesized that the decreased steady-state levels of DHFR protein may be due to the decreased levels of the cofactor NADPH. DHFR is stabilized by its ligands such as NADPH and MTX, thus inhibiting its degradation (Xiang, 2002). NAD kinase which catalyzes phosphorylation of NAD to NADP is the only enzyme in mammals that maintains the NADP pool (Pollak et al., 2007). Therefore we tested whether NADPS inhibits the activity of NAD kinase. NAD kinase activity was determined spectrophotometrically using a coupled assay which generates NADP using NADK. NADP in turn acts as a substrate for glucose-6-phosphate dehydrogenase (Lerner et al., 2001). The activity of NAD kinase in the presence of 1.5 mM and 3.0 mM NADPS was reduced by 55% and 69% (Fig. 6a). We also determined the effect of NADS on NAD kinase. NADS unlike NADPS supported the activity of NAD kinase in the absence of NAD suggesting that it is a substrate for NADK kinase. However, higher concentrations of NADS led to accumulation of NADPS inhibiting the reaction (data not shown). These results are in accordance with the rest of the results since the outcome of treating the cells with either NADS and NADPS were identical.

To further demonstrate that the decrease in cellular DHFR levels in the presence of NADPS is indeed mediated by decreased NADPH levels through inhibition of NADK, we determined the effects of the knockdown of NADK expression on DHFR levels. C85 cells were transiently transfected with siRNA

targeting NADK in C85 cells and NADK and DHFR levels were determined using Western blotting. Compared to untreated cells, cells transfected with siRNAs specific for NADK exhibited significant decreases in DHFR protein levels (Fig. 6B) similar to the levels seen in cells treated with NADPS. These results provide further evidence of the role of NADPH levels in stabilizing DHFR levels.

The inhibition of NAD kinase by NADPS may provide an explanation for the lowered NADPH levels found after treatment of cells with NADPS, therefore we considered whether the lower levels of DHFR in the presence of NADPS was due to the accelerated degradation of DHFR because of low levels of its cofactor, NADPH. A pulse-chase labeling experiment was performed to determine the half-life of DHFR protein in the presence and absence of NADPS. We chose CCRF-CEM/R cells for this study as they have higher levels of DHFR due to gene amplification, making it easier to detect radiolabeled DHFR protein levels. The half life of DHFR was reduced 42% from 11.3 hr to 6.6 hr upon treatment with 10 μ M NADPS (Fig. 6C).

This experiment unequivocally demonstrated that the decrease in levels of DHFR is due neither to transcriptional nor translational inhibition but due to accelerated degradation of DHFR protein upon NADPS treatment as transcriptional or translational inhibition of DHFR would not affect the DHFR half-life.

Modeling studies of NADPS⁺ and NADP⁺ binding to NAD Kinase

In order to better understand the binding of NADPS⁺ to NAD kinase we developed models of NAD⁺, NADP⁺ bound to human structure as described in Materials and Methods in order to better understand mechanism and explore potential for development of novel therapeutics based on inhibition of the kinase. The models produced for NAD⁺ and NADP⁺ complexes with NAD kinase were used to study NADPS⁺ via simple replacement of the nicotinamide amide oxygen with sulfur to study the impact of this

replacement on NAD binding to NAD kinase. The model of the human NAD kinase complex with NAD⁺ is shown in Fig. 7A and B. The nicotinamide ring in NAD⁺ leads to π - π stacking with the aromatic side chain of Y327. The 2' and 3' of the nicotinamide ribose sugar are anchored by hydrogen bonds to E285. The pyrophosphate linker between the sugars is stabilized by electrostatic interaction with R375 of chain B. The 2' and 3' hydroxyl groups of the adenine ribose sugar are in proximity to residue D184. The adenine ring is stabilized by hydrogen bonds to side chains of T325 (chain A) and H351 (chain B). Finally, the nicotinamide CONH2 N-H forms a strong hydrogen bond to the backbone C=O of C349 of chain B. All of these residues are well conserved.

In the model of NADP⁺ product in complex with NAD kinase (Fig. 7C), we see the impact the phosphorylated 2'-hydroxyl of the adenine ribose ring has on bound conformation relative to the substrate NAD⁺. The 2'-phosphate benefits from hydrogen bond stabilization from H351 of chain B. The pyrophosphate linker remains stabilized by R375 of chain B as in the case of NAD⁺; however, the adenine ring N6 nitrogen is now in proximity to hydrogen bond with residue N284 of chain A. The nicotinamide ring is canted further away from the plane of the tyrosine (Y327) aromatic ring. The glutamate residue (E285) serves a key role in stabilizing the 2' and 3' hydroxyls of the nicotinamide ribose sugar. The MM/GBSA results (Table 2) comparison for NAD⁺ binding compared to NADP⁺ indicates a loss in electrostatic energy stabilization at the expense of solvation. This might be a driving force for expulsion of the NADP⁺ product out of the binding site of the enzyme.

The NADPS⁺ bound NAD kinase (Fig. 7D) benefits from hydrogen bonds of the NADPS⁺ adenine ring with the side chain of R237 in addition to hydrogen bond with N284. The nicotinamide ring is now almost perpendicular in orientation to the side chain of Y327. The R375 and H351 residues of chain B stabilize the pyrophosphate linker. Residue E285 of chain A remains in proximity to stabilize the 2' and 3' hydroxyls of the nicotinamide ribose sugar. The MM/GBSA data in Table 2 indicate NADPS⁺ enjoys a

more favorable solvation energy in the lower dielectric environment of the NAD kinase binding site compared to NADP⁺ most likely due to the more hydrophobic and less polar nature of the thioamide in NADPS⁺ compared to the amide in NADP⁺. This lower dielectric environment in the region of the nicotinamide within 10 Å is bordered by A329, A330, H351 and L353. The NADPS⁺ complex with NAD kinase has a much more favorable van der Waals energy in comparison to NADP⁺. The NADPS⁺ binding enthalpy is about 13.8 kcal/mol more favorable than NADP⁺.

Protection of DHFR by MTX from degradation after NADPS treatment

MTX has been shown to stabilize DHFR in vitro and in particular in ternary complex when DHFR is bound to NADPH/NADP (Nakano et al., 1994), however it has not been previously shown in a cellular context. Demonstration of stabilization of DHFR by MTX in cells has been difficult because DHFR is always bound to NADPH. Since NADPS decreases the half-life of DHFR and hence the steady state levels of DHFR, we tested whether we can reverse this phenomenon with MTX. We used DG44-(DHFRS118A-EGFP) cells stably transfected with a variant of DHFR with a point mutation, S118A. This variant of DHFR is not upregulated by MTX, but kinetic properties are very similar to the wild type. Therefore it allowed us to avoid interference with degradation (Hsieh et al., 2009; Skacel et al., 2005). DG44-(DHFRS118A-EGFP) cells were treated with NADPS alone or simultaneously with NADPS and MTX. While NADPS by itself decreased DHFR levels of the S118A mutant, the addition of MTX protected DHFR levels from degradation (Fig. 8A). DG44 wt DHFR-EGFP cells were used as a control to ensure that NADPS did not inhibit MTX-induced DHFR levels, as observed in CCRF-CEM cells (Fig. 8B). Thus, MTX indeed stabilizes DHFR in a cellular context and protects the enzyme from degradation due to cofactor depletion.

Combination studies with NADPS and MTX

MTX and MTX polyglutamates tight-binding competitive inhibitors of DHFR, and at slightly acidic pH values are pseudo-irreversible inhibitors. Lowering DHFR levels in the cell effectively decreases the concentration of MTX required to kill the cancer cells and reduces the toxicity of MTX to normal cells, therefore we hypothesized that the addition of NADPS prior to MTX administration would enhance the cytotoxicity of MTX as NADPS lowered DHFR levels. The effect of the combination of NADPS and MTX compared to each drug alone was determined using C85 cells. Ten μM NADPS was added to these cells for 4 or 6 hr to decrease DHFR levels, followed by the addition of 1 and 10 nM MTX for 24 or 48 hr. DHFR levels were significantly decreased upon sequential combination with NADPS and MTX in C85 cells (Fig. 9A). To determine if the combination of MTX and NADPS was synergistic, several concentrations of a fixed ratio of 1000:1 (NADPS to MTX) were used using a clonogenic assay in C85 cells as the readout. Starting with 40 μM NADPS and 40 nM MTX, as the drug concentrations were increased, the % survival of the C85 cells dramatically decreased in combination treatment in comparison to either drug alone (Fig. 9B). The Chou-Talalay method for drug combination was used to determine combination index (CI) values for NADPS and MTX combination treatment. Combination index is a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations (Chou and Talalay, 1984). Figure 9C illustrates that at effective doses (ED) that kill 25, 50, 70 and 90 percent of the cells, the combination index value was less than 1 indicating that NADPS treatment followed by MTX resulted in a significant synergy.

The effect of NADPS against MTX-transport resistant cells

Impaired MTX accumulation, due to either decreased uptake or lack of retention because of poor polyglutamate formation, is the major factor limiting MTX effectiveness (Gorlick et al., 1996). However, in the clinic, an increase in MTX concentration is not an option to overcome resistance to MTX due to increased toxicity of MTX at high concentrations. NADPS, by lowering DHFR levels would be predicted to be effective in tumors with intrinsic or acquired resistance to MTX. Therefore, we treated the CCRF-

CEM /T cell line, resistant to classical antifolates such MTX and D1694 (aka raltitrexed, Tomudex®) due to impaired transport (Mini et al., 1985) and compared the results to the parent CCRF-CEM cells with NADPS. Trimetrexate (TMTX), a DHFR inhibitor is lipid soluble and passively diffuses into cells was used to demonstrate that both transport-resistant and sensitive cells would respond to TMTX treatment (Fig. 10 A and B, black line). Both cell lines were also treated with either MTX or D1694, the latter an antifolate which targets thymidylate synthase primarily. While the parent cells were sensitive to both of these classical antifolates (Fig. 10 A, green and blue lines), CCRF-CEM/T cells were extremely resistant to MTX (Fig. 10 B, green line) and D1694 (aka raltitrexed and Tomudex) (Fig. 10 B, blue line), but not TMTX. CCRF-CEM/T cells were also sensitive to NADPS treatment, albeit 9-fold less sensitive compared to the parental CCRF-CEM cells (Fig. 10 A and B, red line). A moderate increase in resistance to TMTX and NADPS could be due to the slower growth rate of the CCRF-CEM/T cells than the parental CCRF-CEM cells.

Discussion

Modifying protein stability is a valid therapeutic target, as seen with the success of the proteasome inhibitors such as Bortezomib, which is currently used for the treatment of multiple myeloma and mantle cell lymphoma. However, there is not an approved drug that accelerates degradation of proteins important for cell proliferation. Geldanamycin and its analogues such as 17-allylamino-17-demethoxygeldanamycin inhibit the ATPase activity of heat shock protein Hsp90 leading to improper folding and activity of client proteins. Often these misfolded proteins become the target of ubiquitin-dependent degradation (Miyata, 2005). However degradation of these proteins by geldanamycin is not very selective and leads to hepatotoxicity (Latif et al., 2001). Recently Phase III trials with tanespimycin, a less toxic analogue of geldanamycin for the treatment of multiple myeloma, were terminated due to lack of efficacy. In this study we evaluated whether cofactor depletion, specifically NADPH may also be an effective strategy to target cancer cells. NADPH pools are maintained in cells mainly through the hexose monophosphate pathway reducing NADP to NADPH, the demand for NADPH in cancer cells is increased due to increased metabolic flux of nucleotide and fatty acid synthesis (Vander Heiden et al., 2009). In humans, the only enzyme that is capable of synthesizing NADP from NAD is NAD kinase, therefore we initiated efforts to identify NAD(P)H analogues that may decrease the NADPH concentration in cells rendering DHFR unstable and increasing its degradation.

To that effect, we identified two NAD analogues, thioNAD (NADS) and thioNADP (NADPS) which inhibit NAD kinase causing increased degradation of DHFR. These studies encourage development of additional novel analogues targeted to NAD kinase. The IC₅₀ of both thionicotinamide adenine dinucleotides NADS and NADPS were very similar (10-40 μ M) in RPMI-1640 media and were inversely dependent on the concentration of nicotinamide present within the media. Ham's F-12 medium (0.295 μ M) has 28-fold less nicotinamide than RPMI-1640 (8.2 μ M), associated with 10-fold decrease in the IC₅₀ of both NAD analogues. The concentration of nicotinamide in Ham's F-12 media is very similar to

the mammalian plasma concentration, suggesting that these two analogues would be more potent in vivo (O'Reilly and Niven, 2003). We excluded the following mechanisms for the cytotoxicity of NADS and NADPS: These analogues neither inhibit the activity of DHFR nor repressed the transcription of DHFR. They also did not interfere with the MTX-induced translational regulation of DHFR. Pulse-chase experiment clearly demonstrated that the decrease in the steady state levels of DHFR was due to accelerated degradation of DHFR in the presence of NADPS. We propose that a plausible mechanism for the degradation of DHFR by these nicotinamide analogues is due to lowered levels of NADPH, as a consequence of inhibition of NAD kinase. We recently demonstrated that benzamide riboside, a nicotinamide analog, reduces cellular NADP and NADPH levels by inhibiting NADK. In accordance with our findings here, NADK inhibition led to low levels of NADPH and DHFR levels were significantly decreased (Roussel et al., 2012). Due to the similar absorbance and fluorescence of NADP and NADPS, we were unable to quantify the cellular NADP(H) pools in this study. To understand the binding of NADPS with NAD kinase, we performed a computer modeling study of the human NAD Kinase with NADPS. Predictions pertaining to NADPS+ demonstrate a more stable complex with NAD kinase compared to NADP+. The NADPS+ binding enthalpy is about 13.8 kcal/mol more favorable than NADP+ due to more stable van der Waals interactions with the enzyme and solvation energy. The binding enthalpies indicate that when NAD+ is phosphorylated its product NADP+ results in a less stable complex leading to its expulsion from the enzyme hence driving the process forward. However, in the case of NADS+ we see that formation of its phosphorylated product leads to a complex that is equally stable for the product NADPS+ which is more stable than the NADP+/kinase complex. Disruption of DHFR folding occurs when DHFR is not bound to NADPH. DHFR folding mechanisms involve formation of three ensembles. The first ensemble has considerable secondary structure with exposed hydrophobic core. The second ensemble is obligatory and begins to resemble the native state, but is unable to bind either MTX or NADPH. The interconversion between the second ensemble and the third is slower in human DHFR than *E.coli* or *L.casei* DHFR. It is possible that NADPS, by lowering NADPH

levels, may be interfering with the interconversion of the second ensemble to third ensemble which is the native state (Abali et al., 2008) . Our results support this hypothesis as synergy in combination studies was obtained only if the cells were treated with NADPS analogues first.

Earlier studies have shown that the level of DHFR determines whether cells would be sensitive to MTX treatment, and one mechanism of acquired resistance to MTX is an increase in DHFR level due to amplification of the DHFR gene (Alt et al., 1978; Banerjee et al., 2002). As the thioNAD analogues significantly lowered DHFR levels, we anticipated that NADPS would potentiate MTX cytotoxicity. Combination studies with the C85 cells, a metastatic colon cancer cell line demonstrated significant synergy when the cells were first treated with NADPS followed by MTX. Combination index (CI) values were lower than 1 at all effective dose values (ED). Synergy is increased with a CI to 0.6 at 75% and 90 % effective dose. Our data raise the hope that the resistance to MTX, one of the most widely used cancer chemotherapeutic agent could be blocked with synergistic combinations of NADPS and MTX. We plan to continue our studies with pre-clinical studies.

Limited screening of other dehydrogenases indicated that NADPS lowered DHFR levels alone. However NADPS may lower the steady state levels of other NADP or NADPH requiring enzymes. Other targets are most likely the enzymes that participated in anabolic pathways which are shown to be activated in cancer cells due to dysregulation of upstream signaling pathways such as HIF-1, AMPK and mTOR (Tong et al., 2009), therefore it may be desirable to target several of these enzymes at once. In summary, our results demonstrate that by inhibiting NAD kinase, steady state levels of DHFR are lowered. Given that NAD kinase is the only enzyme responsible for generating NADPH, this enzyme is a valid target for further inhibitor development and DHFR may be a marker for demonstrating the activity of inhibitors targeting NAD kinase in cancer cells.

Authorship Contributions

Participated in research design: Hsieh, Tedeschi, Banerjee, Bertino, E. Ercikan Abali

Conducted experiments: Hsieh, Lawal, Kerrigan, Johnson Farley, Tedeschi, Lee

Performed data analysis: Hsieh, Tedeschi, Kerrigan, Banerjee, Bertino, E. Ercikan Abali

Wrote or contributed to the writing of the manuscript: Kerrigan, Scotto, Banerjee, Tedeschi, Bertino and

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Footnotes

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Legends for figures

Figure 1. Screening of NAD/NADP analogues for cytotoxicity. Panel A: Two thousand DG44-(DGFR-EGFP) cells per well were plated and grown in Ham's F12 medium supplemented with 10% dialyzed FBS (dFBS) containing no glycine, hypoxanthine or thymidine. Concentrations of drugs tested ranged from 200 μ M concentration. After incubation with NAD/NADP analogues for 96 hrs, the viability of the transfectants was assessed using the MTS proliferation assay (see Materials and Methods for detail). **Panel B:** NADS and NADPS were tested in C85 and CCRF-CEM cells in RPMI-1640 medium for 96 hrs. While cell toxicity in C85 cells was determined using MTS assay, trypan blue exclusion assay was used to determine cell viability in the presence of NADS and NADPS in CCRF-CEM cells. **Panel C:** Cytotoxicity of NADS and NADPS was tested for DG44-(DHFR-EGFP) cells in RPMI-1640 and Ham's F12 using the MTS assay. **Panel D:** Seventy five thousand C85 cells and mesenchymal stem cells (MSC) were plated into 24-well tissue culture plates in triplicate in RPMI-1640 (C85) or DMEM (mesenchymal stem cells) supplemented with 10% FBS. The following day, the medium was removed and replaced with fresh medium containing NADPS. After 96 hours of incubation, the cell viability was determined. The percent cell survival was determined by Softmax Pro software and GraphPad Prism 4 software was used to determine ED50 values using a sigmoidal dose-response curve fit.

Figure 2. NADS or NADPS treatment decreased steady-state levels of DHFR. Panel A: Western blot analysis of total lysates from DG44-(DHFR-EGFP) cells was performed after treatment with 0, 1 and 10 μ M NADS for 48 hrs. DHFR-EGFP fusion protein was detected using an antibody against EGFP. **Panel B:** CCRF-CEM and CCRF-CEM/R cells were exposed to 0, 1 and 10 μ M NADS and NADPS for 0, 24 and 48 hrs. The DHFR levels were detected using an anti-DHFR antibody. Equal loading was determined with GAPDH or α -tubulin, respectively.

Figure 3. Cytotoxic effect of NADPS is primarily due to targeting DHFR. Panel A: Western blot analysis of DHFR and other dehydrogenases (Aldehyde dehydrogenase (ALDH1a), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and IMPDH (inosine-5'-monophosphate dehydrogenase). Protein levels were determined using antibodies specific to each dehydrogenase after treating C85 cells with 10 μ M NADP-S for 0, 4, 6, 15 and 24 hrs. α -tubulin was used as a loading control. **Panel B:** Trypan blue exclusion assay was used to determine cell viability of CCRF-CEM/R cells, containing a fivefold increase of DHFR levels and CCRF-CEM parental cells after NADPS treatment for 96 hrs. The cells were grown in RPMI-1640 medium supplemented with 10% dialyzed FBS. After harvesting cells, cell viability was determined using the Vi-CELL™ Series Cell Viability Analyzer. **Panel C:** MTS assay as described in the Materials and Methods was performed to determine cytotoxic effect of NADPS in parental DG44 cells, a hamster ovary cell line with no DHFR expression and in DG44- (DHFR-EGFP), a subline of DG44 cells stably transfected with a DHFR-EGFP fusion construct. Parental DG44 cells were grown in RPMI 1640 media which was supplemented with hypoxanthine and thymidine to allow these cells to grow in the absence of DHFR (■, red). DG44-(DHFR-EGFP) cells were grown in RPMI 1640 media with (▲, blue) and without (▼, black) hypoxanthine and thymidine supplementation. Two thousand cells per well were plated and cells were incubated with NADPS for 96 hrs. The percent cell survival was determined by Softmax Pro software and GraphPad Prism 4 software was used to determine ED50 values using a sigmoidal dose-response curve fit.

Figure 4. Transcriptional down regulation of DHFR mRNA through G1/S block cannot explain the decrease in DHFR levels of NADPS treated cells. Panels A-C: Non-synchronized C85 cells were treated with 0, 10 and 40 μ M NADPS for 24 hrs and processed for propidium iodide labeling and flow cytometry. **Panel D:** Non-synchronized C85 cells were treated with 10 nM MTX for 24 hrs and processed for PI-labeling and flow cytometry. **Panels E-G:** Recovery G1 block by removing NADPS in C85 cells. Non-synchronized C85 cells were treated with 0 or 10 μ M NADPS for 48hrs (E and F). G. C85 cells were

incubated with 10 μ M NADPS for 24 hrs and then replaced with new medium in the absence of NADPS for 24 hrs. Cells were harvested and processed for flow cytometry. **Panel H:** After treating CCRF-CEM cells with 10 μ M NADPS and 10 nM MTX for 24 hrs, total cytoplasmic RNA was extracted for quantitative RT-PCR analysis. DHFR mRNA levels were normalized to β -actin mRNA levels. The expression in the untreated sample was set to 1. The y-axis is the mean of the fold changes in DHFR mRNA expression level; bars, standard deviation. The results were averaged from three independent experiments and each experiment was performed in triplicates.

Figure 5. A decreased DHFR level by NAD/NADP analogues is neither due to inhibition of MTX-mediated translational regulation of DHFR protein nor inhibition of ubiquitin proteasome pathway. DHFR protein levels were determined by Western blotting in CCRF-CEM cells. **Panel A:** CCRF-CEM cells were treated with 10 nM MTX alone, 10 μ M NADPS alone, and simultaneously with 10 nM MTX and 10 μ M NADPS for 24 and 48 hrs. **Panel B:** CCRF-CEM cells were treated with 10 nM MTX alone, 10 μ M NAD alone, and simultaneously with 10 nM MTX and 10 μ M NAD for 24 and 48 hrs. DHFR protein was detected by polyclonal anti-DHFR antibodies and GAPDH was used as a loading control (A and B). Quantification of DHFR level was shown at the bottom of gels and measured by Image J program (provided by NIH). **Panel C:** MCF-7 cells treated with 0.5 μ M MG132, a proteasome inhibitor, in the presence and absence of 10 μ M NADPS for 48 hrs. GAPDH was used as the loading control and Cyclin D1 was used as positive control for the activity of MG132. MCF-7 cells were also treated with MTX in the presence and absence of NADPS and MG132.

Figure 6. NAD Kinase is inhibited by NADPS leading to decreased half-life of DHFR. Panel A: Inhibition of NAD kinase was determined spectrophotometrically by measuring the increase in absorbance at 340 nm caused by the reduction of NADP to NADPH by G6PDH using a coupled assay

(see details in Materials and Methods). **Panel B:** Effect of NADK suppression on DHFR protein levels were determined by Western blotting in C85 cells after transfection for 48 hrs with siRNA targeting NADK. DHFR protein levels were compared to untreated cells and also to NADPS-treated cells. **Panel C:** The half life of DHFR was measured by a ^{35}S methionine radiolabeled pulse-chase experiment. CCRF-CEM/R cells were treated with 10 μM NADP-S and at the indicated times samples were subjected to immunoprecipitation with an anti-DHFR antibody, followed by SDS-PAGE and autoradiography. The lower panel shows the quantification of results in the presence and absence of NADPS. Values shown are the means \pm SD for three independent pulse-chase experiments. For statistical analysis Student's t-test was used; paired comparisons of cellular DHFR levels between NADPS treated and untreated were made. P-values were less than 0.05 both at 16 and 23 hrs suggesting that there was statistical difference in DHFR levels between treated and untreated cells.

Figure 7. NADPS+ has a more favorable binding to NAD Kinase compared to NADP+. Panel A: Human NAD Kinase (cartoon) complexed with NAD^+ (space-filling model). Chain A is colored deep blue and chain B is colored forest green (last snapshot from 2 ns of dynamics in TIP3P water box). Built from 3PFN.pdb (Human) and 1Z0Z.pdb (*Arch. fulgidus*). **Panel B:** Close-up of binding region with NAD^+ and key interacting residues depicted as stick models where NAD^+ (ball and stick model) carbon atoms are colored gray and chain A protein residue carbon atoms are colored navy blue with chain B residue carbon atoms colored teal. **Panel C:** Close-up of binding region with NADP^+ and key interacting residues depicted as stick models where NADP^+ (ball and stick model) carbon atoms are colored gray and chain A protein residue carbon atoms are colored navy blue with chain B residue carbon atoms colored teal. **Panel D:** Close-up of binding region with NADPS^+ and key interacting residues depicted as stick models where NADPS^+ (ball and stick model) carbon atoms are colored gray and chain A protein residue carbon atoms are colored navy blue with chain B residue carbon atoms colored teal.

Figure 8. MTX protects cells from DHFR degradation by NADPS. DHFR protein levels were determined by Western blotting in DG44-(DHFR-EGFP) cells. **Panels A and B:** DG44 DHFR-EGFP cells or DG44 DHFRS118A-EGFP cells were treated with 10 nM MTX alone, 10 μ M NADPS alone, and the simultaneous addition of 10 nM MTX and 10 μ M NADPS for 24 and 48 hrs. DHFR-EGFP and DHFRS118A-EGFP protein were detected by a monoclonal anti-EGFP antibody. GAPDH was used as a loading control. Quantification of DHFR levels (shown at the bottom of gels) was measured by the Image J program (provided by NIH).

Figure 9. NADPS has a synergistic cytotoxic effect with MTX. DHFR levels were determined by Western blotting after sequential addition of NADPS and MTX. C85 (**Panel A**) and CEM-CCRF (**Panel B**) cells were treated with 10 nM MTX alone for 24 or 48 hrs pre-treated with 10 μ M NADPS for 4 or 6 hrs and followed by 10 nM MTX for 24 or 48 hrs. DHFR protein was detected by an anti-DHFR antibody and GAPDH was used as a loading control. **Panel C:** A clonogenic assay was performed to determine the cytotoxic effect of the combination of NADPS and MTX in C85 cells. Values were given as means \pm SD for three independent experiments. **Panel D:** Combination index plots for C85 cells exposed to NADPS and MTX (see details in Materials and Methods).

Figure 10. NADPS is still effective in MTX-transport resistant cells. Parental CCRF-CEM (**Panel A**) and CCRF-CEM/T cells, resistant to classical antifolates such as MTX and D1694 (aka Raltitrexed, Tomudex®) due to impaired transport (Mini et al., 1985) (**Panel B**) were treated with MTX, D1694 and the lipophilic antifolate trimetrexate (TMTX). The cells were grown in RPMI-1640 medium supplemented with 10% dialyzed FBS. After incubation with NADPS for 96 hrs, cells were collected and viability was determined using Trypan blue exclusion assay with the Vi-CELL™ Series Cell Viability Analyzer. The percent cell survival was determined by Softmax Pro software and sigmoidal dose-response curve fit of the graphs drawn by GraphPad Prism 4 software was used to determine ED50

values. While the concentrations of NADPS used in the experiment was micromolar, the antifolates concentrations were nanomolar.

Table 1. Thirteen NAD/NADP analogues were screened for targeting NAPDH site in DHFR protein.

	Name
A1046	3-Acetylpyridine adenine dinucleotide phosphate
A5137	3-Acetylpyridine adenine dinucleotide
A55403	3-Aminofluoranthene
N7256	Nicotinamide hypoxanthine dinucleotide phosphate
N5881	β -Nicotinamide adenine dinucleotide 3'-phosphate
N6756	Nicotinamide hypoxanthine dinucleotide
A7266	3-Aminopyridine adenine dinucleotide
A5298	3-Acetylpyridine hypoxanthine dinucleotide
N4256	Nicotinic acid adenine dinucleotide
N5257	β -Nicotinamide adenine dinucleotide 2':3'-cyclic monophosphate
P2171	3-Pyridinealdehyde adenine dinucleotide
T7375	Thionicotinamide adenine dinucleotide
T551	Thionicotinamide adenine dinucleotidephosphate

Table 2. MM-GBSA results in kcal/mol based on 100 evenly spaced snapshots from last 1 ns of 2 ns production run. See computational methods section for calculation details.

Compd	E_{vdw}	E_{elec}	E_{GB}	E_{surf}	ΔE_{gas}	ΔE_{solv}	ΔH_{bind}
NAD+	-66.2 ± 4.5	-51.9 ± 7.3	51.1 ± 5.9	-8.4 ± 0.2	-118.0 ± 7.0	42.8 ± 5.9	-75.3 ± 3.5
NADP+	-54.0 ± 4.4	-14.3 ± 10.5	22.7 ± 9.4	-8.2 ± 0.3	-68.3 ± 10.6	14.5 ± 9.4	-53.8 ± 3.0
NADS+	-56.1 ± 4.7	-57.7 ± 5.6	55.5 ± 4.6	-8.6 ± 0.2	-113.8 ± 6.9	46.9 ± 4.5	-66.9 ± 4.1
NADPS+	-70.0 ± 4.1	10.3 ± 7.6	1.1 ± 6.9	-9.1 ± 0.2	-59.7 ± 8.0	-7.9 ± 6.9	-67.6 ± 3.7

$$\epsilon(\text{ext}) = 78.5; \epsilon(\text{int}) = 4.0$$

$$\Delta E_{gas} = E_{vdw} + E_{elec}$$

$$\Delta E_{solv} = E_{GB} + E_{surf}$$

$$\Delta H_{bind} = \Delta E_{gas} + \Delta E_{solv}$$

Figure 1

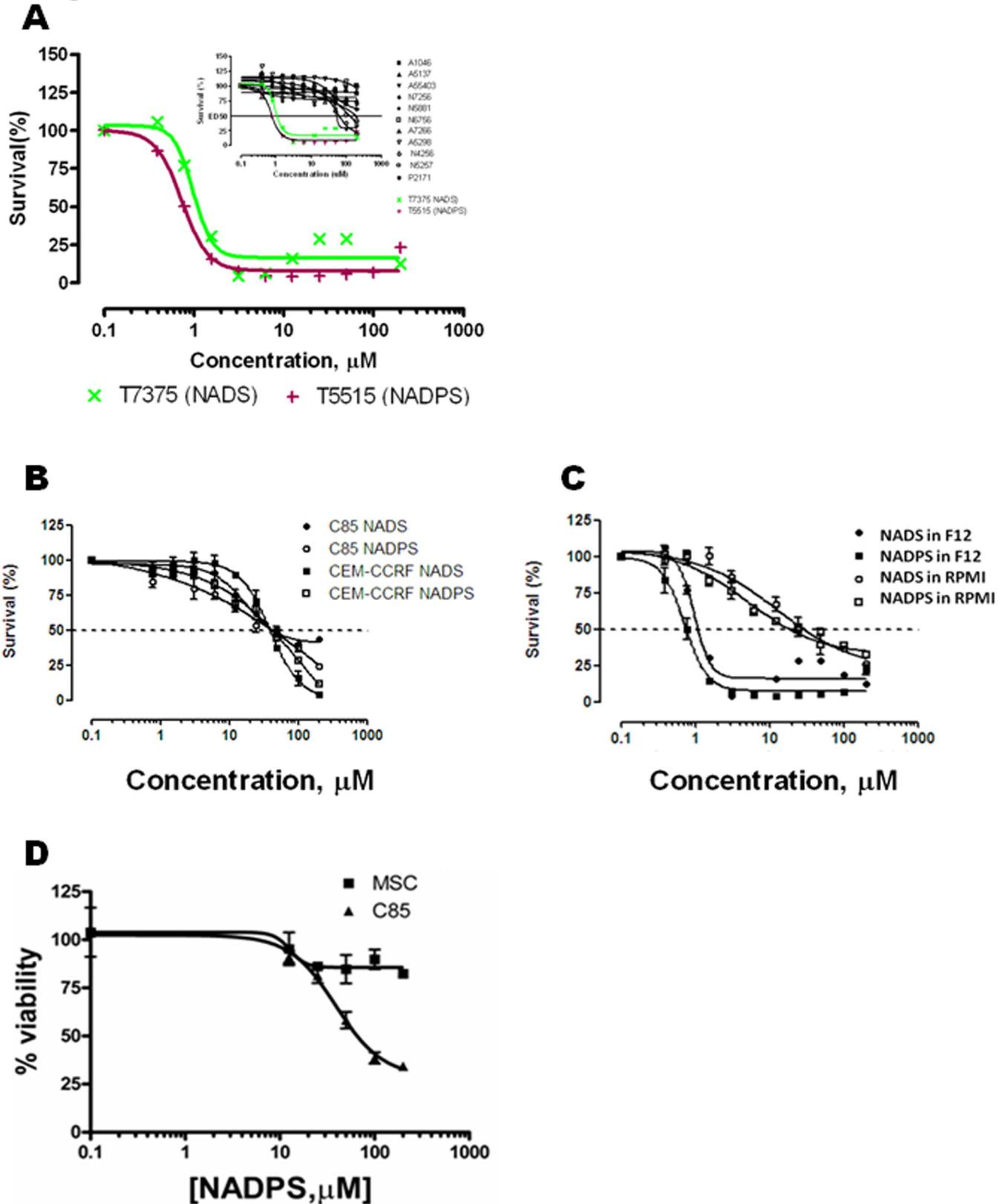
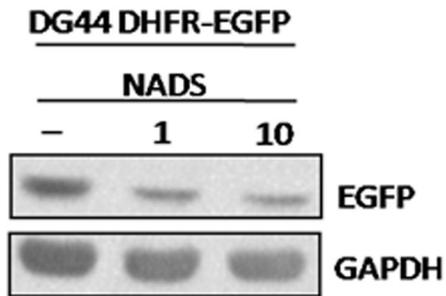


Figure 2

A



B

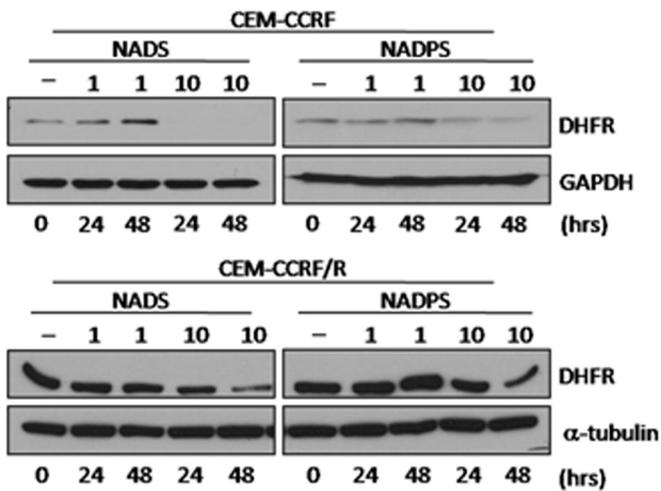


Figure 3

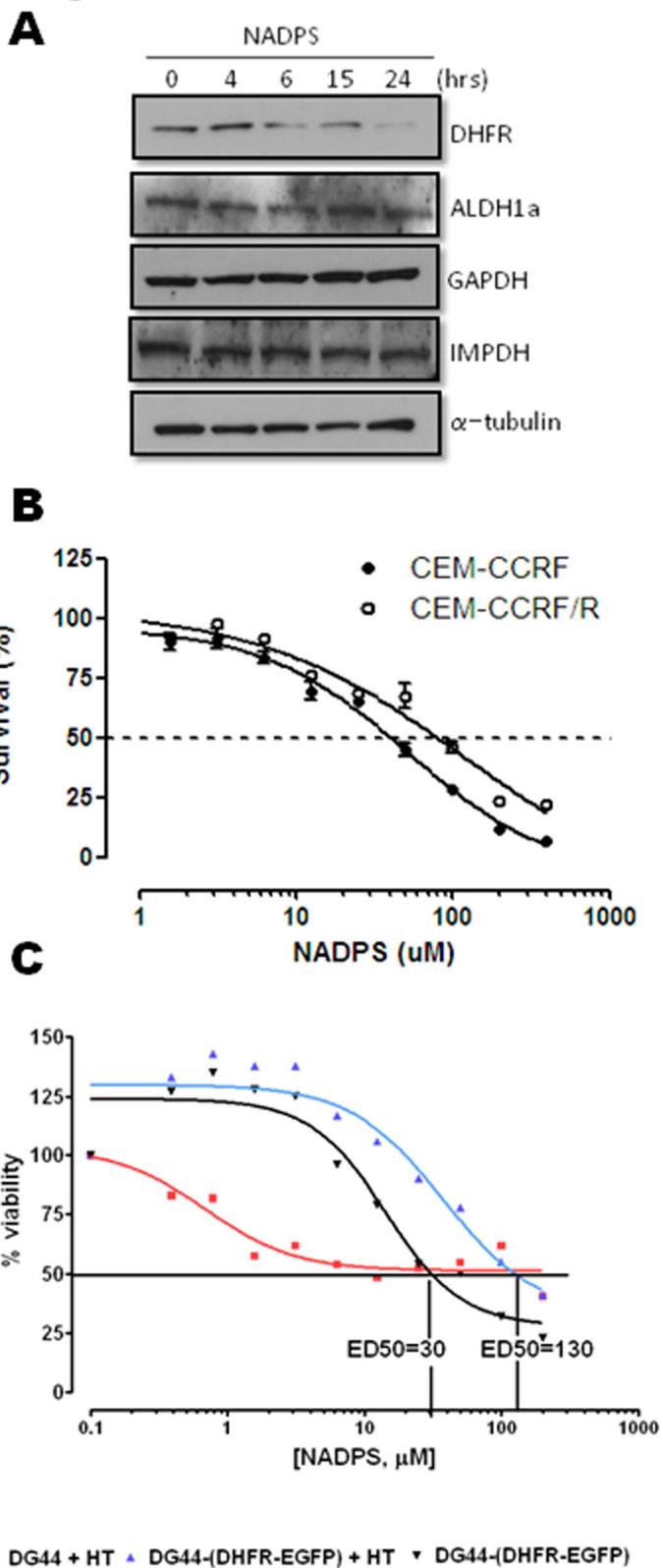


Figure 4

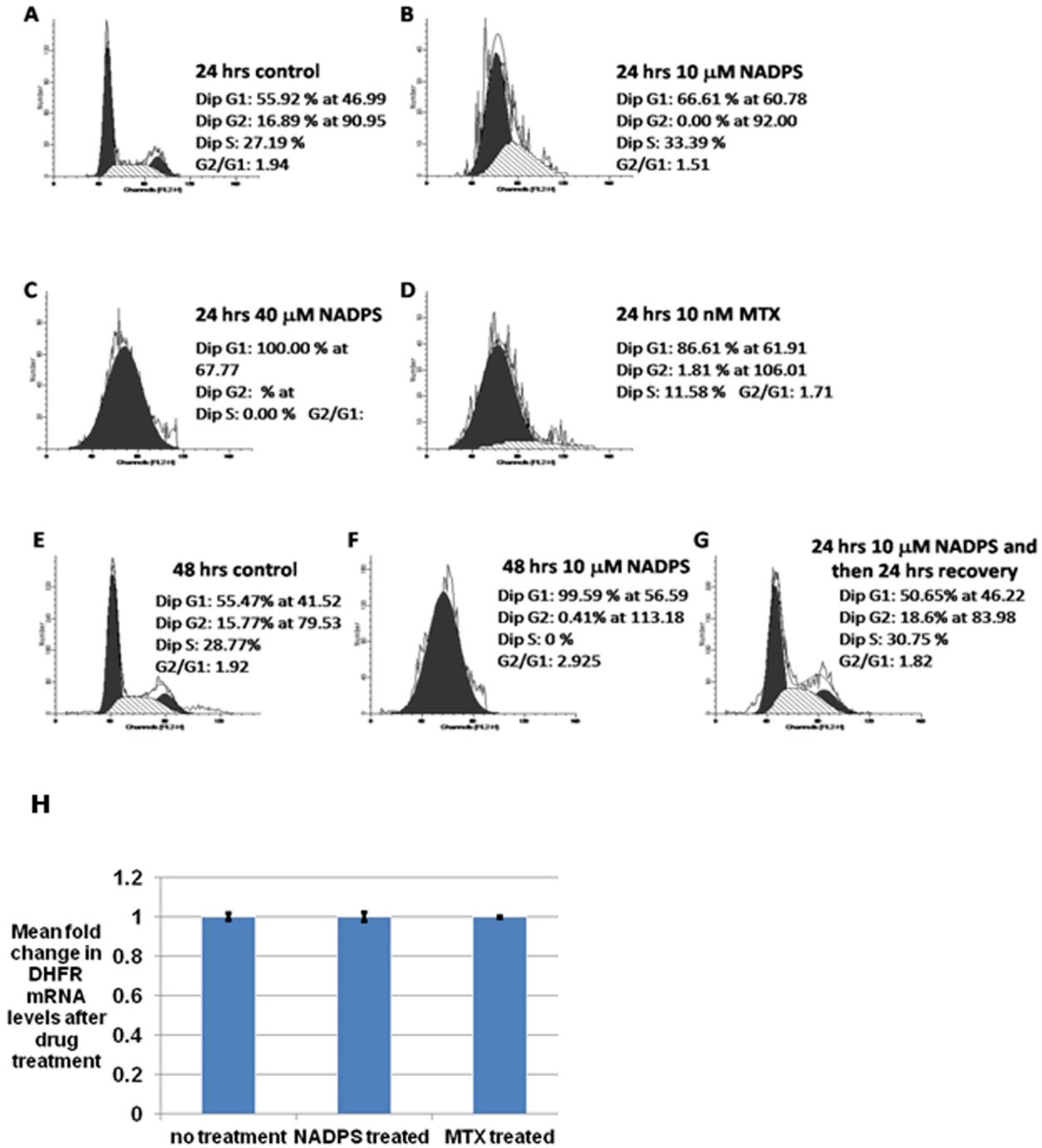
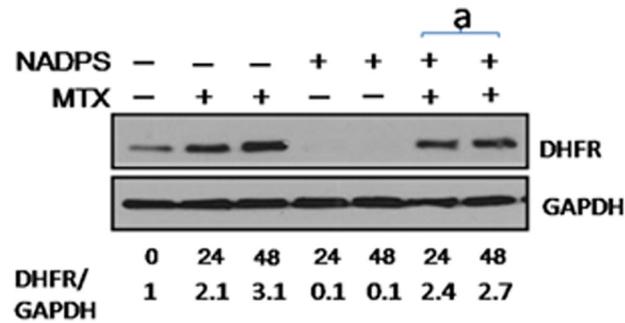


Figure 5

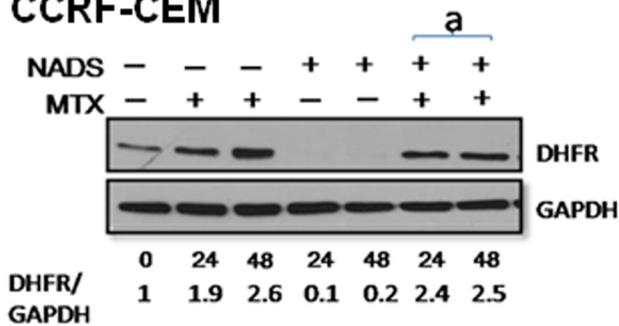
A

CCRF-CEM



B

CCRF-CEM



C

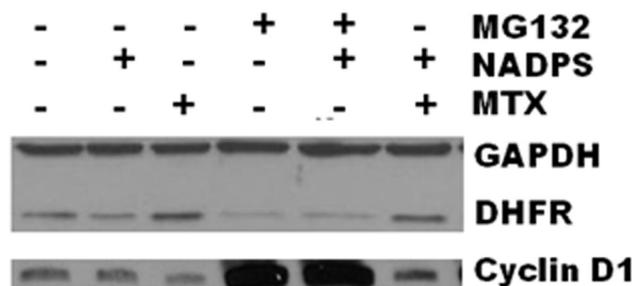


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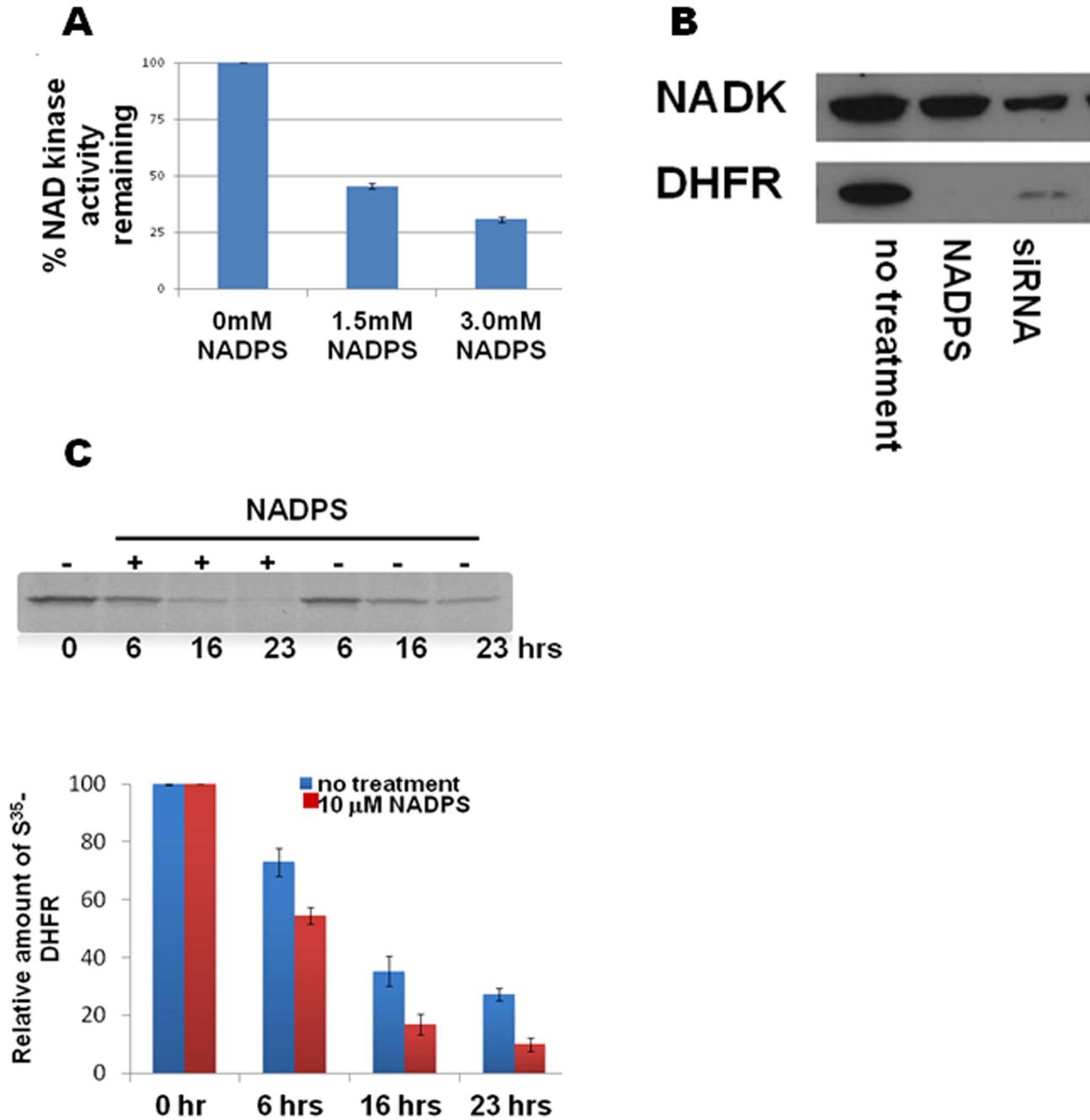


Figure 7

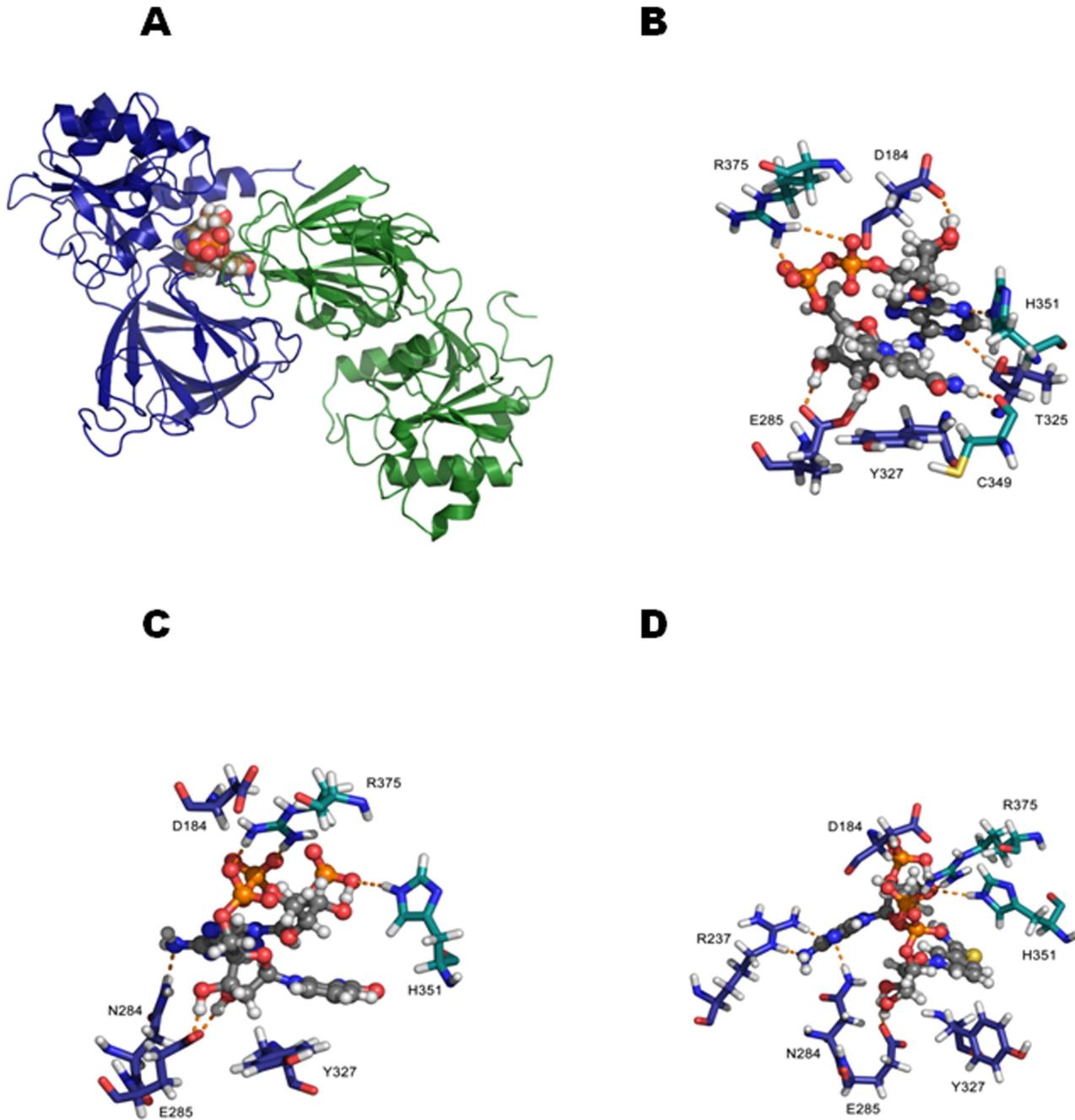
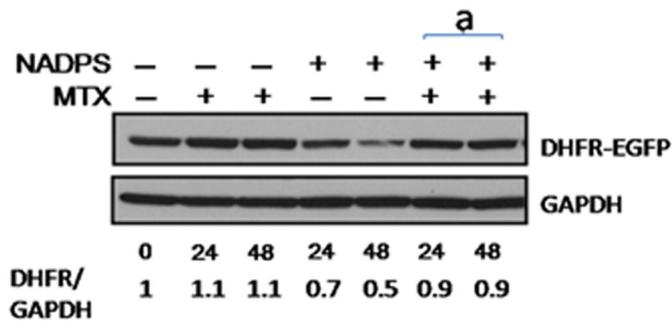


Figure 8

A

DG44-(DHFRS118A-EGFP)



B

DG44-(DHFR-EGFP)

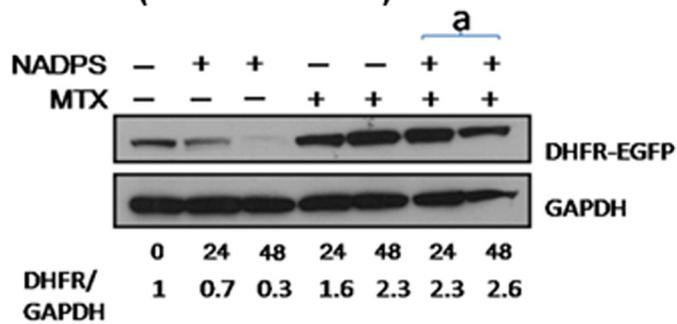
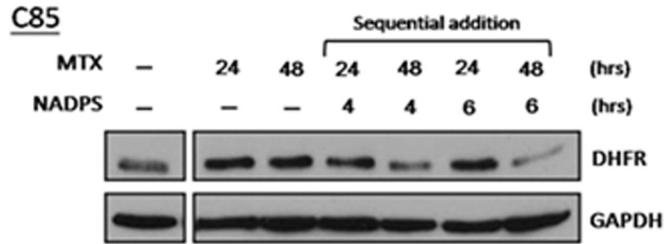
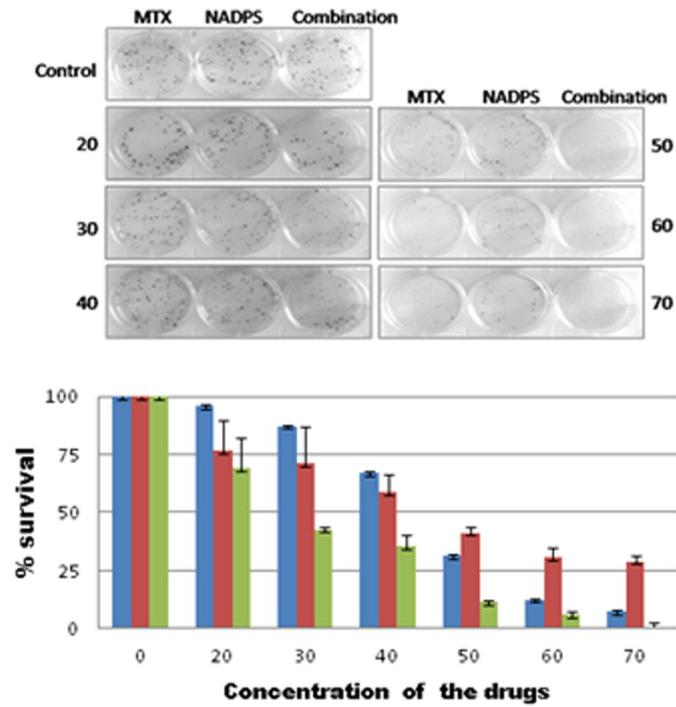


Figure 9

A



B



C

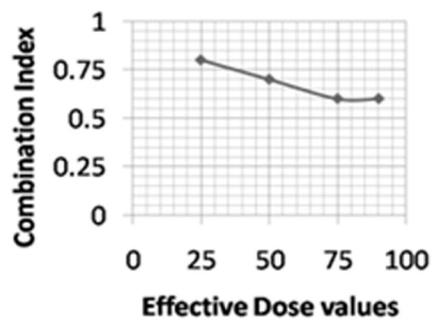
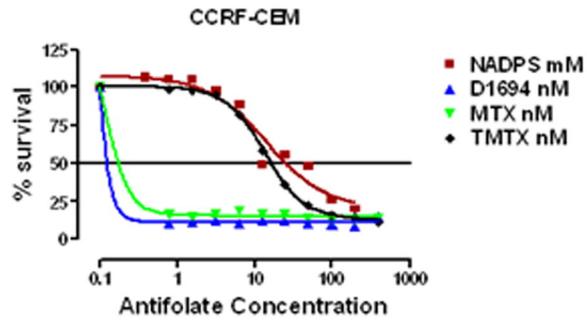


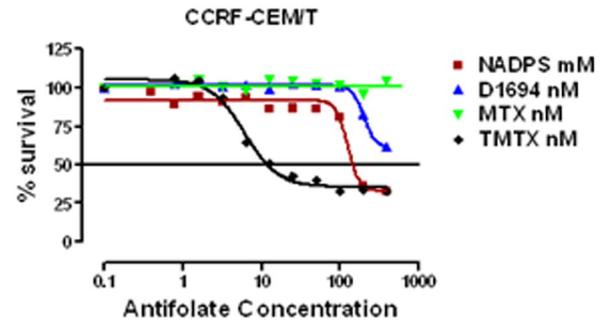
Figure 10

A



	NADPS mM	D1694 nM	MTX nM	TMTX nM
EC50	14.42	0.08508	0.1138	13.85

B



	NADPS mM	D1694 nM	MTX nM	TMTX nM
EC50	128.2	205.8	6.074	6.074