# Targeting EGFR Induced Oxidative Stress by PARP1 Inhibition in Glioblastoma Therapy

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Targeting EGFR Induced Oxidative Stress by PARP1 Inhibition in Glioblastoma Therapy

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Abstract

Despite the critical role of Epidermal Growth Factor Receptor (EGFR) in glioblastoma pathogenesis [1,2], EGFR targeted therapies have achieved limited clinical efficacy [3]. Here we propose an alternate therapeutic strategy based on the conceptual framework of non-oncogene addiction [4,5]. A directed RNAi screen revealed that glioblastoma cells over-expressing EGFRvIII [6], an oncogenic variant of EGFR, become hyper-dependent on a variety of DNA repair genes. Among these, there was an enrichment of Base Excision Repair (BER) genes required for the repair of Reactive Oxygen Species (ROS)-induced DNA damage, including poly-ADP ribose polymerase 1 (PARP1). Subsequent studies revealed that EGFRvIII over-expression in glioblastoma cells caused increased levels of ROS, DNA strand break accumulation, and genome instability. In a panel of primary glioblastoma lines, sensitivity to PARP1 inhibition correlated with the levels of EGFR activation and oxidative stress. Gene expression analysis indicated that reduced expression of BER genes in glioblastomas with high EGFR expression panel of primary glioblastoma lines, sensitivity to PARP1 inhibition correlated with the levels of EGFR activation and oxidative stress.

Results and Discussion

DNA repair as a form of non-oncogene addiction.

EGFR oncogene, EGFRvIII [6], caused increased requirement for BER genes [9,10,11], we tested whether the expression of a clinically pertinent DNA repair process would be most evident when EGFR activation introduces secondary physiologic changes that stress cellular capacity for survival. Consequently, tumor cells become hyper-dependent on processes required to compensate for these stressful conditions. This phenomenon is termed “non-oncogene addiction” since the compensatory processes required for tumor survival are not oncogenic. As an example, RAS hyper-activation in colon cancer cells results in increased mitotic aberrancy and hyper-dependence on mitotic checkpoint function [5].

In this study, we explore the framework of “non-oncogene addiction” as it relates to oncogenic EGFR activation. As hyper-activation of several EGFR downstream effectors, including RAS and STAT3, elicits increased DNA damage accumulation [9,10,11], we tested whether the expression of a clinically pertinent EGFR oncogene, EGFRvIII [6], caused increased requirement for DNA repair as a form of non-oncogene addiction.

Results and Discussion

EGFRvIII over-expressing U87MG cells exhibited increased reliance on BER genes

Given the mutually compensatory nature of many DNA repair pathways [12,13], we reasoned that hyper-dependency on any particular DNA repair process would be most evident when

Introduction

Historically, cancer therapeutic development has largely been driven by the principle of “oncogene addiction” – that cancer cells require increased activity of selected oncogenes and therefore tumor ablation can be achieved by inhibition of these oncogenes [7]. While “oncogene addiction”-based therapies have achieved notable successes in some cancers [7], their application to glioblastoma has yielded little efficacy. For instance, while EGFR mutations or copy number alterations are found in nearly 50% of all glioblastomas [1,2], EGFR inhibition has yet to yield significant improvements in clinical outcome [3]. The ineffectiveness of such targeted therapy is explained in part by mutations in downstream signaling molecules [3] and redundant signaling from multiple co-activated receptor tyrosine kinases [8]. In this context, it is evident that meaningful therapy will require co-extinction of multiple oncogenes.

Emerging literature suggests an alternative strategy to the multi-target approach [4,5]. These studies reveal that oncogene activation introduces secondary physiologic changes that stress cellular capacity for survival. Consequently, tumor cells become


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† These authors contributed equally to this work.
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EGFR, ROS and PARP1 Inhibition
EGFRvIII over-expressing glioblastoma cells exhibited increased levels of ROS, DNA damage, and genomic instability

The increased reliance on BER in EGFRvIII over-expressing cells suggests elevated ROS levels in these cells. To test this hypothesis, U87MG and U87MG-EGFRvIII lines were assayed by the ROS-sensitive fluorophore DCF-DA [10]. U87MG-EGFRvIII cells exhibited markedly increased DCF-DA fluorescence, suggesting elevated ROS accumulation (Fig. 2A). To exclude DCF-DA dye related artifacts, the results were reproduced using two other assays for ROS including 8-oxoguanine and dihydrothiophenium fluorescence (Fig. S2). The increased DCF-DA fluorescence in the U87MG-EGFRvIII cell line was abolished by treatment with the EGFR inhibitor Erlotinib or EGFR siRNA (Fig. S3).

Unrepaired ROS induced DNA damages are often converted to DNA double-strand breaks (DSBs) [21]. We thus determined whether the increased ROS in U87MG-EGFRvIII cells culminate in DSB accumulation [12]. We found increased levels of γ-H2AX and phospho-Chk2, two biomarkers for DSB accumulation [22], in the U87MG-EGFRvIII line relative to the parental U87MG cells (Fig. 2B). A 2–3 fold increase in the levels of γ-H2AX and p-Chk2 were also seen in xenograft tumors derived from U87MG-EGFRvIII cells relative to those derived from U87MG, confirming our results in vivo. We further confirmed our observation by γ-H2AX immunohistochemical staining of tumors derived from U87MG-EGFRvIII and U87MG-EGFRKD (EGFR III Kinase Dead) cells. The U87MG-EGFRKD cells express a mutant derivative of U87MG-EGFRvIII where the kinase activity was inactivated by a point mutation in the ATP-binding site [23]. Consistently, the U87MG-EGFRvIII tumors harbored increased number of cells with nuclear γ-H2AX staining relative to the U87MG-EGFRKD tumors (Fig. 2C) [24]. To ensure that these observations were not unique to the U87MG cells, we recapitulated these results using an U373MG line harboring a tet-repressible EGFRvIII construct (Fig. S4).

DNA damage and strand breaks induced by excessive ROS accumulation are harbinger of genomic instability [21,25]. Thus, we sought to determine whether EGFRvIII induced ROS predisposes accumulation of chromosomal aberrations. Sorted diploid U87MG and U87MG-EGFRvIII were serially passaged. The U87MG cells remained diploid after nine passages. However, the U87MG-EGFRvIII cells exhibited progressive clonal changes (see increased >4N staining cells, Fig. 2D). Together, our results suggest that EGFR hyper-activation causes increased ROS accumulation, with resultant genomic instability.

EGFR over-expression correlated with ROS accumulation and sensitivity to PARP1 inhibition

We wished to confirm our findings in clinical specimens by assessing whether high 8-hydroxyguanosine (8-OG) IHC staining, a well known marker for ROS levels [26], correlates with EGFR immunostaining. Using a commercially available glioblastoma microarray (US Biomax, Rockville, MD), we observed that EGFR staining tends to parallel 8-OG staining (i.e. high EGFR staining...
Figure 2. EGFR hyperactivation increases oxidative stress and DNA damage accumulation in glioma cells. (A) EGFRvIII expression is associated with increased DCF-DA fluorescence. A representative experiment (top). The mean and standard deviation of DCF-DA fluorescence derived from three independent experiments (bottom). (B) EGFRvIII expression is associated with increased γ-H2AX and p-Chk2 accumulation in tissue culture (T.C.) and xenograft models. Intensities of γ-H2AX and p-Chk2 bands were quantified and normalized to the intensity of the RAN loading control (bottom). The experiment was repeated three times with consistent results. A representative experiment is shown. (C) IHC of mouse xenografts confirmed that EGFRvIII expressing U87MG cells exhibit increased γ-H2AX accumulation. Scale bar, 100 μm (∼400), 40 μm (∼1,000). Percent of cells with γ-H2AX homogeneous (large arrow) or punctate nuclear staining (thin arrow, left panel) were quantified (right panel). (D) EGFRvIII expression in U87MG is associated with progressive ploidy alterations.

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glioblastomas tend to exhibit high 8-OH staining; Fig. 3A, p<0.05. The same correlation was seen in low-grade gliomas (Fig. S5).

We further tested the correlation between EGFR hyperactivity, ROS, and sensitivity to PARP1 using a panel of primary glioblastoma cell lines. These lines were classified by DCF-DA fluorescence intensity (Fig. 3A). The two lines with the highest DCF-DA fluorescence exhibited the highest degree of EGFR activation, as measured by levels of phospho-EGFR (Tyr 1173), phospho-Stat3, and phospho-ERK1/2 [27] (Fig. 3B). Interestingly, these lines were sensitive to PARP1 inhibition without IR. The two lines with moderate DCF-DA fluorescence exhibited IR sensitization upon PARP1 inhibition. Such sensitization was not observed in the lines with low DCF-DA fluorescence (Fig. 3C). RT-PCR of the EGFR transcript from the two high EGFR/DCF-DA lines revealed that neither harbored the EGFRvIII variant (Fig. S6). Together, these results suggest that EGFR hyperactivation in the absence of EGFRvIII is sufficient to induce ROS accumulation and sensitivity to PARP1 inhibition.

BER gene expression inversely correlated with survival in patients afflicted with glioblastoma with high EGFR expression

Since BER activity can be transcriptionally regulated [28], we wished to determine whether EGFR hyperactive glioblastomas increased transcription of BER genes in response to increased oxidative stress. Using The Cancer Genome Atlas (TCGA) glioblastoma database [1,29], tumors were stratified into groups based on EGFR transcript level. Because most of the BER genes functionally overlap [12,28], we developed a BER score (see Methods) to assess global BER transcription as a proxy for overall BER activity. The BER score did not appear to correlate with EGFR transcript level (Fig. 4A). Given these findings, we reasoned that tumors with high EGFR expression (high ROS) and low BER gene expression (diminished compensatory mechanism) should exhibit reduced survival fitness and therefore associate with favorable patient survival. Indeed, patients with glioblastomas exhibiting high EGFR expression and low BER score exhibited improved survival (Fig. 4B). For the patient group with high EGFR expressing glioblastomas, median survival times with low and high BER scores were 14.5 and 9.2 months, respectively (log rank p = 0.01). Strikingly, such a survival benefit was not observed in the low EGFR group (log rank p = 0.99, Fig. 4C). Among the seven TCGA patients with EGFRvIII expressing tumors, median survival times with low and high BER scores were 22.6 and 8.9 months, respectively (log rank p = 0.008, Fig. 4D), further supporting the importance of BER in EGFR hyperactive glioblastomas.

Our results indicate that ROS and DSB accumulation in EGFR hyperactive glioblastoma cells is mitigated by BER and lay the conceptual framework for the application of PARP1/BER inhibition beyond the subset of tumors deficient in HR [18,19,20]. To the extent that BER genes have yet been implicated in glioblastoma oncogenesis, the framework constitutes a form of “non-oncogene addiction.” This addiction occurs in the absence of exogenous DNA damage. That is, the EGFR hyperactive glioblastomas accumulated increased ROS/DSBs and were hypersensitive to BER inhibition at baseline. IR induced ROS/DSBs magnified this form of non-oncogene addiction by “overloading” the already taxed BER pathway. The efficacy of IR in glioblastoma patients can, in part, be rationalized by this paradigm. A corollary of this paradigm is that therapeutic insights can be derived by mapping the intersection between the various forms of non-oncogene addiction and the molecular effects of existing glioblastoma therapies.

The work further provides several insights into the clinical translation of the “non-oncogene addiction” framework. First and foremost, the effects of therapies designed based on the principles of “oncogene addiction” and of “non-oncogene addiction” are inherently antagonistic. In our study, EGFR inhibition leads to reduction of ROS, obviating the need for BER. In this context, combination of PARP1 and EGFR inhibition would not be desirable. Additionally, there appears to be sufficient variability in the pathways of oncogenesis [30], such that not every EGFR hyperactive glioblastoma develops increased BER capacity. The discrepancy between oncogenic stress and stress support pathways in select tumors offers an opportunity to maximize therapeutic efficacy. We propose the use of the transcriptome-based BER score and EGFR status as a means of capitalizing on this opportunity in clinical trial design involving BER inhibitors. Finally, our screen results suggest that a much larger set of non-oncogenes serves to support EGFRvIII induced oncogenic stress. Indeed, EGFR activation has been shown to modulate potential oncogenic stress support pathways including DSB repair [31] and apoptosis [32]. Understanding the physiologic interactions between the various stress support pathways should afford the development of synergistic drug combinations [4] ultimately required for meaningful therapeutic efficacy.

Materials and Methods

Cell culture and reagents

The U87MG, U87MG-EGFRvIII, U87MG-EGFRKD, and U87/3MG tet-EGFRvIII cell lines were obtained from Dr. Webster K. Cavenee and propagated as reported [6,23]. Primary glioblastoma cell lines were derived from fresh surgical specimens after obtaining written informed consent under Institutional Review Board-approved Dana-Farber/Harvard Cancer Center Protocol 07-231. These were passaged as described for U87MG; passage 2–3 lines were used. NU1025 (Sigma Aldrich, St. Louis, MO), 3-aminoindomethamide (3-AB, Sigma), 4-amino-1,8-naphthalimide (4-ANI, Calbiochem, Gibbstown, NJ) and doxycycline (Sigma) were dissolved in DMSO. N-acetylcycteine (NAC, Sigma) and hydrogen peroxide (H2O2, Sigma) were dissolved in culture media.

Reactive Oxygen Species (ROS) and chromosomal instability assays

Levels of ROS in U87MG and U87MG-EGFRvIII cells were assessed using the OxyDNA Assay Kit (Calbiochem), DCF-DA (Invitrogen, Carlsbad, CA), and dihydroethidium (Invitrogen) according to manufacturers’ instructions. To assay chromosomal instability,
sorted diploid populations of U87MG and U87MG-EGFRvIII were passaged every 3 days, and cell cycle distributions were analyzed by flow cytometry after propidium iodide staining [33].

siRNA library screen and validation

Cells were transfected with 20 nM of siRNA oligonucleotides using HiPerfect transfection reagent (QIAGEN, Valencia, CA).

Figure 3. Clinical correlation between EGFR expression, oxidative stress and sensitivity to PARP inhibition. (A) EGFR expression correlated with 8-hydroxyguanosine (8-OG) staining in a glioblastoma microarray. Glioblastomas were stratified into high or low EGFR staining groups. Within each group, the percent of cases with high 8-OG staining is shown in white; low 8-OG staining in gray (right). Representative pictures of high and low staining patterns of EGFR and 8-OG are shown (left). Scale bar, 200 mm (×200), 50 mm (×600). (B) Correlation between DCF-DA fluorescence and EGFR. Primary glioblastoma lines were grouped based on DCF-DA staining (left), p-EGFR, p-Stat3, and p-ERK1/2 levels were assessed (right). (C) The level of oxidative stress/EGFR activation correlated with sensitivity to PARP1 inhibition.

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Twenty-four hours after transfection, cells were irradiated with 5 Gy IR. Viability was assessed 72 hours after irradiation using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI). The experiment was performed twice for each cell line on independent days to allow for statistical analysis. The corrected viability for cells transfected with each siRNA was calculated as a percentage of the mean viability of the control wells containing GFP or scrambled siRNAs (Qiagen DNA repair subset v2.0) for each 96-well plate. The corrected viability of the irradiated cells was then subtracted from the corrected viability of the non-irradiated cells to calculate the relative viability after irradiation for each respective gene target. The mean of these values (referred to as the mean percent viability after irradiation) was calculated (averaging the two distinct siRNAs directed against the same target gene in each of the two experiments) and termed the radiation effect. The radiation effect derived from the U87MG-EGFRvIII line was then subtracted from that derived from the U87MG line. This value was expressed as a percent of the radiation effect for EGFRvIII and reported as the EGFRvIII IR index. The siRNA targets are ranked based on this index [33].

**Immunoblotting and immunohistochemistry**

Western blotting and IHC were performed using standard techniques as previously described [6,33]; see Methods S1 for details.

**siRNA transfection**

The initial siRNA screen was performed using the Qiagen DNA repair subset v2.0 siRNA library. Subsequent PARP1 confirmation studies were performed using an independent siRNA directed against PARP1 (ON-TARGET plus J-0065605-0005, Dharmacon, Denver CO). Qiagen Hs_EGFR_12 (5’-CAGGACTGGATATTCTGAAA-3’) was used for EGFR silencing. The control siRNA was obtained from Qiagen (AllStars Negative Control and anti-GFP: 5’-AACACTTGTCTTTCTC-3’). The siRNA transfections were done by using either RNAiMax (Invitrogen) or HiPerfect reagent (Qiagen). In brief, $1 \times 10^5$ cells were plated on 6-well plates the day before transfection, then 20 nM of siRNA was transfected according to manufacturer’s protocol for 24 hours prior to subsequent manipulations.

**Viability assays**

Cells were seeded into 10 cm plates and treated for 24 hours with various siRNAs or PARP inhibitors, and then irradiated with ionizing radiation (IR). Cells were trypsinized 24 hours after irradiation and plated in serial dilution, 10–14 days after irradiation, cells were fixed and stained with crystal violet (0.1%) and numbers of colonies were counted. All experiments were performed in triplicates and repeated at least twice. For doxycycline experiments, cells were treated with doxycycline for 72 hours prior to subsequent manipulation.
RT-PCR analysis of EGFRvIII
Total RNA was isolated from primary glioblastoma cultures, U87MG and U87MG-EGFRvIII cell lines (2×10⁶ cells) using the RNaseeasy Mini kit (Qiagen, Valencia, CA). Standard RT-PCR was done with primers designed to flank the deletion of exons 2 to 7 (5'-ATGGCAACCTTCGGGACG-3' and 5'-ATTCCGTTACACCTTGGCGGC-3'). Final reaction concentration = 100 nM. Reverse transcription was done at 50°C for 30 minutes followed by enzyme inactivation at 95°C for 15 minutes. This was followed by 35 cycles of (94°C; 1 min; 55°C; 1 min; 72°C; 1 min) and a final extension of 72°C for 10 minutes. PCR products were visualized on a 1% agarose gel containing ethidium bromide.

Biostatistical analysis
Glioblastoma gene expression and correlating clinical data were obtained from The Cancer Genome Atlas (http://www.broadinstitute.org/~gadgetz/TCGA/TCGA_206_median.txt) [1,29]. Tumors were categorized as showing high (greater than mean) vs. low EGFR expression. An exhaustive literature search revealed 24 genes implicated in BER (PARP1, PARP2, XRCC1, LIG3, POLB, UNG, SMUG1, MBD4, TDG, DUT, OGG1, NTHL1, NEIL1, NEIL2, NEIL3, NUDT1, MUTFH, APEX1, APEX2, PNKP, MGMT, ALKBH2, ALKBH3 and MPG) [12]. For each tumor, one point was added for each BER gene showing greater than mean expression. The sum of these points was termed the BER score. A high BER score was defined as one greater than 12. Kaplan-Meier plots were generated using JMP (SAS Institute, Cary, NC). Seven tumors were previously identified via SNP microarray (US Biomax) was stained for EGFR and 8-OG (see Supplemental Materials and Methods). Samples were stratified for high or low 8-OG staining cases shown in gray. High 8-OG staining cases shown in gray. A low-grade gliomas (8-OG) staining in low-grade gliomas. A low-grade gliomas expressing EGFRvIII transcript were quantified and normalized to the intensity of the RAN loading control (bar graph in the bottom panel).

Supporting Information
Methods S1 Supplemental Materials and Methods. Found at: doi:10.1371/journal.pone.0010767.s001 (0.05 MB DOC)

Figure S1 PARP1 silencing preferentially radiosensitized EGFRvIII hyperactive glioblastoma cells. Two independent siRNAs against PARP1 preferentially sensitized U87MG-EGFRvIII cells to IR (left). The efficiency of knock down is shown in the right column. The siRNAs were taken from the Qiagen DNA repair subset v2.0. Cells were transfected with 20 nM of siRNA oligonucleotides using HiPerfect transfection reagent (QIAGEN, Valencia, CA). Twenty-four hours after transfection, cells were irradiated with 2 Gy IR. Viability was assessed by colony survival as described in methods. In parallel, cells were seeded into a 10 cm plate and treated as described above. RNA extraction was performed at 72 hours after irradiation. The Qiagen QuantiTect SYBR Green RT-PCR Kit was used to quantify the efficiency of gene silencing as per manufacturer’s instructions. Each experiment was repeated twice. Results from a representative experiment is shown. Found at: doi:10.1371/journal.pone.0010767.s002 (0.12 MB TIF)

Figure S2 EGFRvIII over-expression induced increased ROS accumulation. (A) Levels of 8-oxoguanine, the most common form of ROS induced DNA damage, are increased in U87MG-EGFRvIII cells relative to U87MG cells. U87MG and U87MG-EGFRvIII cells were harvested, washed with PBS, and fixed with ice-cold 70% ethanol. The cells were then washed with PBS and incubated with FITC-conjugated 8-oxoguanine probe (1:100) for 60 min. Fluorescence was measured by FACS. (B) EGFRvIII over-expression in U87MG is associated with increased ROS accumulation as gauged by dihydroethidium fluorescence. U87MG and U87MG-EGFRvIII cells were harvested, washed with PBS, and incubated with dihydroethidium (5 mM) for 15 min. As a positive control, U87MG cells were also treated with 0.03% hydrogen peroxide (H2O2) for 30 min before harvest. Levels of dihydroethidium fluorescence were measured by FACS. Each bar depicts mean dihydroethidium fluorescence intensity ± SEM (normalized to unstained control) derived from triplicates. Found at: doi:10.1371/journal.pone.0010767.s003 (0.47 MB TIF)

Figure S3 EGFR inhibition or silencing decreased ROS level in U87MG-EGFRvIII cells. (A) EGFR inhibition by erlotinib in U87MG-EGFRvIII cells decreased ROS levels. U87MG-EGFRvIII cells were treated with DMSO or erlotinib (10 nM) or N-acetylcysteine (NAC, Sigma) as a positive control for 48 hours. Cells were harvested, washed with PBS, and incubated with DCF-DA (5 mM) for 15 min. Cells were then washed with PBS and analyzed by FACS. (B) EGFR silencing reduces ROS levels in U87MG-EGFRvIII cells. U87MG-EGFRvIII cells were transfected with siRNA against EGFR or a control siRNA. Cells were cultured for another 72 hours, DCF-DA fluorescence was measured by FACS (left panel). Efficiency of EGFRvIII silencing is shown in the right panel. Found at: doi:10.1371/journal.pone.0010767.s004 (0.48 MB TIF)

Figure S4 EGFRvIII expression is associated with DNA damage accumulation in U373MG cells. U373MG cells harboring a tet-repressible EGFRvIII construct [1] were treated with doxycycline (1 mM) or vehicle for 72 hours. Cell lysates were prepared and levels of γ-H2AX and p-Chk2 were analyzed (top panel). Intensity of γ-H2AX and p-Chk2 bands were quantified and normalized to the intensity of the RAN loading control (bar graph in the bottom panel).

Found at: doi:10.1371/journal.pone.0010767.s005 (0.38 MB TIF)

Figure S5 EGFR expression correlates with 8-hydroxyguanine (8-OG) staining in low-grade gliomas. A low-grade gliomas microarray (US Biomax) was stained for EGFR and 8-OG (see Supplemental Materials and Methods). Samples were stratified into high or low EGFR staining groups. Within each group, the percent of cases with high 8-OG staining is shown in white; low 8-OG staining cases shown in gray. High EGFR staining glioblastomas tend to exhibit high 8-OG staining (p<0.05 by Student’s t-test).

Found at: doi:10.1371/journal.pone.0010767.s006 (0.18 MB TIF)

Figure S6 Primary glioblastoma lines used did not harbor EGFRvIII transcript. EGFR transcripts in primary glioblastoma lines were examined by RT-PCR. Ethidium bromide stained gel showing EGFR PCR products from two primary glioblastoma lines, GBM1 and GBM2. mRNAs isolated from U87MG (expressing EGFR) and U87MG-EGFRvIII were used as controls.

Found at: doi:10.1371/journal.pone.0010767.s007 (0.31 MB TIF)

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Author Contributions
Conceived and designed the experiments: MN DK RDK ADD CCC. Performed the experiments: MN DK RDK KN POZ DK KH CCC. Analyzed the data: MN DK RDK KN POZ DK KH CCC. Contributed reagents/materials/analysis tools: JS SK FBF LC RAD WKC ADD CCC. Wrote the paper: MN DK CCC.
References