Pharmacological inhibition of the protein kinase MRK/ZAK radiosensitizes medulloblastoma

Daniel Markowitz1##, Caitlin Powell1##, Nhan L. Tran2, Michael E. Berens2, Timothy C. Ryken3, Magimairajan Vanan4, Lisa Rosen5, Mingzu He6, Shan Sun6, Marc Symons1**, Yousef Al-Abed6** and Rosamaria Ruggieri1**

1Center of Oncology and Cell Biology, Feinstein Institute, Manhasset, NY; 2Translational Genomics Research Institute, Phoenix, AZ; 3Department of Neurosurgery, Kansas University Medical Center, Kansas City, KS, USA; 4Section of Pediatric Hematology/Oncology/BMT, University of Manitoba, Winnipeg, Canada, 5Biostatistic Unit, Feinstein Institute, Manhasset, NY, 6Center for Molecular Innovation, Feinstein Institute, Manhasset, NY.

# Equal first author contribution
** Equal senior author contribution

Running title: Radiosensitization of medulloblastoma by inhibition of MRK

Keywords: radiosensitization, medulloblastoma, MRK, ZAK, irreversible inhibitor, brain tumor

This study was supported by the following fund sources: Swim Across America (SAA) Foundation and Project to Cure (PTC) Foundation to M. Symons, the Bradley Foundation to R. Ruggieri and The Ben & Catherine Ivy Foundation to M.E. Berens.

Corresponding Authors: Rosamaria Ruggieri, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA. Phone: 516-562-3410; Fax: 519-562-1022; E-mail: mruuggier@northwell.edu; Marc Symons, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, USA. Phone: 516-562-1193; Fax: 519-562-1022; E-mail: msymons@northwell.edu.

The following authors, Yousef Al- Abed, Marc Symons and Rosamaria Ruggieri, have submitted a US patent application: Treatment of solid tumors by inhibiting MRK/ZAK (#50425/507) 2015. This application, however, does not alter the authors' adherence to all the Molecular Cancer Therapeutics policies on sharing data and materials.

Word count: 4,559; Total number of figures: 6 (plus 4 supplementary figures).
Abstract

Medulloblastoma is a cerebellar tumor and the most common pediatric brain malignancy. Radiation therapy is part of the standard care for this tumor, but its effectiveness is accompanied by significant neurocognitive sequelae due to the deleterious effects of radiation on the developing brain. We have previously shown that the protein kinase MRK/ZAK protects tumor cells from radiation-induced cell death by regulating cell cycle arrest after ionizing radiation. Here, we show that siRNA-mediated MRK depletion sensitizes medulloblastoma primary cells to radiation. We have, therefore, designed and tested a specific small molecule inhibitor of MRK, M443, which binds to MRK in an irreversible fashion and inhibits its activity. We found that M443 strongly radio-sensitizes UW228 medulloblastoma cells as well as UI226 patient-derived primary cells, while it does not affect the response to radiation of normal brain cells. M443 also inhibits radiation-induced activation of both p38 and Chk2, two proteins that act downstream of MRK and are involved in DNA damage-induced cell cycle arrest. Importantly, in an animal model of medulloblastoma that employs orthotopic implantation of primary patient-derived UI226 cells in nude mice, M443 in combination with radiation achieved a synergistic increase in survival. We hypothesize that combining radiotherapy with M443 will allow us to lower the radiation dose while maintaining therapeutic efficacy, thereby minimizing radiation-induced side effects.
Introduction

Radiotherapy is commonly used as part of cancer treatment and it is important in the management of 40% of patients who are cured of cancer (1). The therapeutic benefits of radiotherapy are, however, accompanied by late toxicity that severely affects quality of life, especially in pediatric patients. Specifically, in the treatment of medulloblastoma, the most common malignant pediatric brain tumor, radiation, together with high-dose chemotherapy, has improved 5-year survival rates in patients (2). However, it also causes serious deleterious effects that include neurocognitive deficits, developmental problems and secondary malignancies in the majority of survivors (3-6). Identifying new approaches that would allow reduction of the total radiation dose in these treatments without compromising therapeutic efficacy is critical for improving tumor management and quality of life. Radiosensitizers may offer this opportunity by increasing the efficacy of radiation therapy while reducing effects on the normal developing brain.

The protein kinase MRK (mixed lineage kinase related kinase), also known as ZAK (sterile alpha motif and leucine zipper containing kinase AZK) (referred to as MRK in this study) (7), belongs to the family of mitogen activated protein kinase kinase kinase (MAPKKK) and has close homology to the MLK proteins (7-10). MRK has two splice variants, MRKα and MRKβ, which share some functions, but differ in other regards. In particular, while MRKα overexpression promotes tumor growth (11), MRKβ has been shown to have an important role in the response to DNA damage and in the S and G2/M checkpoints (12). At the mRNA level, the two MRK splice forms are ubiquitously expressed (7, 8), although the MRKβ mRNA is more abundant than the MRKα mRNA. Recently, however, the MRKα mRNA has been shown to be abnormally spliced in gastric tumors as well as in colorectal, bladder and breast cancers with consequent overexpression of the respective protein (13). MRKβ is activated by stress (9,14-16), including ionizing radiation (IR) (7,12,17) upon which it contributes to activation of Chk2 and p38 proteins, which leads to
IR-induced cell cycle arrest. MRK silencing caused both failure to arrest cell cycle progression in response to IR and increased killing by radiation (12).

In this study we examine the role of MRK as a radiosensitization target in medulloblastoma. In addition, we describe a novel specific small molecule that we have designed to inhibit MRK activity and show that it works as an effective radiosensitizer in an orthotopic xenograft model of medulloblastoma.

**Materials and Methods**

**Cell lines and Transfections**

The human medulloblastoma cell line UW228 (18) was kindly provided in 2007 by Dr. James Rutka, Hospital for Sick Children, Toronto, Canada, and it was authenticated by PCR. Cells were cultured in DMEM containing 10% FBS, 1 mM glutamine and 1% penicillin/streptomycin. UMDI cells are a derivative of the osteosarcoma cell line U2-OS, described in Korkina et al (19). In UMDI cells, expressing plasmid pC4-Fv1E-MRK, MRK can be activated by induced homodimerization with the drug AP20187 (Clontech). The UMDI cells are cultured in McCoy media supplemented with 10% FBS, 0.6 μg/ml puromycin, 50 I.U./mL penicillin and 50 μg/mL streptomycin, and 2 mM L-glutamine. The K-562 and 4T1 cell lines were purchased from ATCC and cultured according to distributor’s protocol. The human NSC were purchased from Invitrogen (Thermo-Fisher Scientific) and cultured in StemPro media (Life Technologies), as directed by the manufacturer. Human astrocytes were purchased from Gibco (Life Technologies) and cultured in Gibco astrocyte medium.

The MRK and control luciferase siRNAs sequences were previously described (12) and transfected at a concentration of 10 nM using DharmaFect #1 (Dharmacon) as recommended by the manufacturer.
Human primary tumor cells and medulloblastoma xenograft model

The primary UI226 medulloblastoma cells are patient-derived xenografts that were established by Dr. Timothy Ryken, University of Kansas Medical Center. UI226 were largely propagated as flank cultures in nude mice and cultured in StemPro media (Life Technologies) for less than three weeks before intracranial injections.

All animal work was carried out in accordance with NIH guidelines and was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute in Manhasset, NY. Medulloblastoma cells (5.0 \times 10^5 UI226 in 5 \mu l of StemPro medium) were injected over 5 minutes into the cerebellum of 4-weeks old athymic female mice (Taconic), using a stereotactic frame and the following coordinates: 2 mm to the right and 1 mm posterior to the lambda, using a sterile dental drill. The Hamilton 10 \mu l syringe loaded with cells was lowered 3 mm under the surface of the brain and then lifted for 0.5 mm to create a pocket for the cells. Three weeks after tumor cell implantation and approximately 2 weeks before the animals became moribund, treatment was started by implantation of an osmotic pump (Alzet, Durect) filled with either 0.05 mg/ml solution of M443 or vehicle [0.01% DMSO in PBS] and implanted in a subcutaneous pocket on the dorsal flank of the animal. A catheter with attached cannula delivered the drug intracranially and directly into the tumor over a period of 2 weeks, at a steady rate of 0.25 \mu l/h. Irradiation of the mice head was initiated 2 days after pump implantation and conducted over 2 days (two fractions of 3Gy each). It was performed using a biological irradiator (RS2000, Rad Source Technologies), while the rest of the body was shielded.

Western Blot Analysis and Antibodies

Cells were processed for western blotting as previously described (19). The MRK monoclonal antibody, 4-23, was previously described (7). The MRK phospho-specific monoclonal antibody, A12, was generated
against the following di-phospho MRK peptide: FHNHpTTHMpqSLVGFP. The β-tubulin antibody was from Millipore, the Chk2, P-Chk2, c-Abl and P-cAbl antibodies were from Cell Signaling. The Hu-HLA antibody was from Abcam. The P-MPM2 antibody was purchased from Millipore.

**Cell viability and Colony Formation Assay**

Two days after transfection with siRNAs, medulloblastoma cells were seeded in a 96 well plate and the following day, they were exposed to different doses of radiation. Cell viability was determined 72 hrs post radiation treatment by MTT (Sigma) absorbance at 595 nm. For cells that were treated with M443, 6 hours after treatment with different concentrations of the drug or vehicle control, they were exposed to radiation and processed for the MTT assay 72 hrs later as above.

Clonogenic survival was determined using a colony formation assay. Five hundred cells were seeded in 6 cm dishes two days after siRNA transfections. The following day, cells were exposed to the different doses of radiation using a biological irradiator (RS2000, Rad Source Technologies) and cultured for 7 days with media changes every other day. Subsequently, cells were fixed in 3.7% formaldehyde in PBS and stained with 0.2% (w/v) sulpharhodamine B (SRB) dye in 1% acetic acid for 20 min. The dishes were washed with 1% glacial acetic acid and allowed to dry. Colonies containing more than 50 cells were counted and the results were used to calculate the surviving fractions. For the treatment with M443 or vehicle control, 24 hrs after seeding, cells were exposed to the drug for 6 hours and subsequently to radiation. Colonies were fixed and processed as described above. The dose enhancement factor (DEF) was calculated from the dose response curves as the ratio between the dose reducing survival to 10% for radiation alone and that for radiation of MRK siRNA-transfected or M443-treated cells.

**M443 synthesis**
M443 was synthesized in seven steps with a total yield of 3%. Methyl 3-amino-4-methylbenzoate (Aldrich CAS :18595-18-1) was reacted with cyanamide to obtain the guanidine M439 at 90% yield; The reaction of compound A (CAS: 858643-92-2) with N,N-dimethylformamide dimethyl acetal yielded M422. By refluxing M422 with M439 in absolute ethanol, M440 was obtained with a yield of 75%. After saponification of ethyl ester with NaOH in hot aqueous ethanol, M441 was obtained and coupled with compound C (CAS-641571-11-1) employing DECP (diethyl cyanophosphate) to provide the amide M442; after deprotection with acid, coupling with acid chlorides completed the synthesis of M443.

**MRK homology model and M443 covalent docking**

The MRK sequence was obtained from UniProt (ID: Q9NYL2). A homology model was produced for MRK by alignment with the cAbl crystal structure (PDB: 3CS9) followed by three-dimensional model building and energy minimization using MOE [Molecular Operating Environment, 2014.0901; Chemical Computing Group Inc., Montreal, QC, Canada]. A database of 10 distinct protein models was generated and showed good overall alignment with the \(\alpha\)-carbon backbone of cAbl template (rms deviation from 0.37 – 0.43 Å). The phi/psi backbone angles and side chain rotamers were evaluated to discard structures with high-energy, disallowed bonds and the best model was selected for the following studies.

The MRK homology model obtained from the previous step was subjected in Maestro (v. 10.2, Schrödinger, LLC, 2015). M443 structure was constructed in 2D Sketcher and subsequently prepared by LigPrep module (version 3.4, Schrödinger, LLC, 2015) in Maestro. The CovDock docking program (20) within the Schrodinger suite was employed for M443 and MRK covalent docking. Cysteine 22 on MRK was targeted as the reactive residue to undergo Michael addition with compound M443. The ranking of poses by affinity score calculates the average of the pre-reacted and post-reacted Glide score for the given pose (20). The top ranking CovDock affinity pose was chosen as the final pose of M443.
Immunofluorescence

UW228 cells were seeded at 70,000 cells/well on coverslips in a 24-well plate. The following day, cells were treated with M443 or vehicle and 3 hr later exposed to 6 Gy of radiation. Cells were subsequently fixed at different time points and processed for immunofluorescence as previously described (21) with the P-MPM2 antibody (Millipore) and counterstained with DAPI. Images were collected using a Zeiss axiovert 200M inverted microscope, equipped with a 20X objective (0.3 NA) and a Zeiss AxiocamMR camera running on Axiovision software.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the mean of each outcome between groups of interest and either radiation dose or time. The interaction term between group and dose/time was also examined. If a significant difference between means was found, then multiple comparisons between pairs were computed. The Tukey-Kramer method was used to adjust for multiple comparisons. Results were considered significant at a significance level of p<0.05. Analyses were conducted using SAS version 9.4 (SAS Institute, Inc., Cary, NC). The Kaplan-Meier estimate and a log-rank (Mantel-Cox) test were used to generate survival curves with the GraphPad Prism 6.0 software. To determine whether there was a synergistic effect between M443 and radiation treatment (IR), the null hypothesis of an M443 x IR interaction was tested in a Cox model applied to a 2x2 factorial design (M443 [no/yes] x IR [no/yes]). Results were considered significant at a significance level of p<0.05. Analyses were conducted using SAS version 9.3 (SAS Institute, Inc.).

Results

Down regulation of MRK radiosensitizes medulloblastoma cells
We have previously shown that MRK is activated by IR and controls the cellular response to DNA damage in osteosarcoma and colorectal cancer cells (7,12). To test if MRK has a similar role in medulloblastoma cells, we asked if down regulation of MRK expression can sensitize these cells to killing by radiation. UW228 medulloblastoma cells were transfected with control (luciferase) or specific MRK siRNAs and, subsequently tested for viability after exposure to different doses of IR with the MTT and clonogenic assays. Figure 1A shows that MRK depletion decreased cell viability after IR by 33% of control at 3 Gy. Similarly, the clonogenic assay showed a significant decrease in survival with a dose enhancement factor (DEF) of 1.6 at 10% viability (Fig. 1B). In both assays, down regulation of MRK expression alone did not significantly affect viability by more than 5% (R. Ruggieri, unpublished observations). Analysis of the signaling elements known to be activated downstream of MRK (12) demonstrated a significant attenuation of IR-induced Chk2 phosphorylation at 1 and 2 hours after IR, and effective reduction in p38 phosphorylation (Fig. 1C). The lack of complete Chk2 inhibition following MRK down regulation could be explained by the fact that Chk2 is known to be activated by multiple upstream factors that include DNA-dependent protein kinase and Polo-like kinase-3 (22). Thus, MRK influences the DNA damage response in medulloblastoma cells and MRK down regulation sensitizes medulloblastoma cells to radiation.

Design of an irreversible inhibitor of MRK

To identify MRK-specific small molecule inhibitors, we selected a number of known kinase inhibitors that had been tested against the kinome and found to bind to MRK (23). We assessed their ability to inhibit MRK kinase activity using a cell-based assay that we previously described (19). Briefly, we made use of the M28 osteosarcoma cell derivative (UMDI) that expresses a recombinant form of MRK, which can be activated by homodimerization with the drug AP20187. Figure 2A shows that pre-incubation of these cells with the different drugs led to various levels of MRK inhibition. Among the inhibitors, we selected
nilotinib, which had been previously shown by Manley et al to bind with very high affinity to MRK (24). This study also included a model of the MRK active site bound to nilotinib, based on the corresponding structure of nilotinib bound to its original target c-Abl. We noticed that the pyridine ring in nilotinib was located in close proximity to cysteine 22 in MRK, suggesting the possibility of designing a nilotinib derivative that can covalently interact with MRK. Importantly, c-Abl has a leucine at the corresponding position. In addition, none of the known off-targets of nilotinib (Fig. 2B) and none of the MRK family members or related kinases (Fig. 2C) have a cysteine at this position. Thus, in an effort to increase the specificity of nilotinib for inhibiting MRK, we decided to derivatize nilotinib by modifying the pyridine ring to a piperidine ring with the addition of an αβ unsaturated amide to create compound M443 (see Materials and Methods, Fig. 2D-G and Supplementary Fig. S1).

We anticipated that M443 would bind covalently to MRK via cys 22 and irreversibly inhibit MRK activity (Fig. 2D and 2E). To examine whether M443 indeed inhibits MRK in an irreversible manner, we pretreated UMDI cells with M443 for different times. After extensive washes, we treated cells with the homodimerizing AP compound to activate MRK. We first noted that the extent of MRK inhibition by M443 was time-dependent and not reversed by washing out the inhibitor (Fig. 3A). As MRK inhibition was optimal after 6 hour of exposure to M443 (Fig. 3A), we chose this time point to compare M443 with nilotinib. Figure 3B shows that in contrast to M443, inhibition by nilotinib could be totally reversed after washing out the drug. These results provide strong evidence that M443 is an irreversible inhibitor of MRK.

**Specificity of M443**

To assess the specificity of M443, we compared the degree to which nilotinib and M443 inhibit the activity of BCR-Abl, the original target of nilotinib. To this end, we used the chronic myelogenous leukemia cell line K-562, which expresses the constitutively active BCR-Abl protein. Figure 3C shows that
while both drugs inhibit MRK activity, nilotinib and M443 clearly diverge in their effect on BCR-Abl: nilotinib efficiently inhibits BCR-Abl, whereas M443 does not. Thus, the nilotinib derivative M443 selectively inhibits MRK.

**M443 effectively inhibits MRK downstream signals in primary medulloblastoma cells and prevents cell cycle arrest induced by IR**

To examine whether M443 inhibits signaling downstream of MRK, we tested both the UW228 cell line as well as the UI226 primary patient-derived medulloblastoma cells for their response to IR in the presence and absence of M443. Figure 4A shows that MRK activation by IR is maximal at 30 minutes after exposure to radiation. Therefore, for subsequent analysis, this time point was used. In both cell cultures the IR-stimulated activation of MRK, Chk2 and p38 were greatly inhibited by 500 nM M443. As Chk2 activity is important for the S and G2/M checkpoints, we also examined the effect of M443 on cell cycle arrest after IR. To this end, we measured the mitotic index in UW228 cells after treatment with M443 and exposure to IR. Cells were seeded on coverslips, pretreated with 500 nM M443 or vehicle, exposed to 6 Gy of IR, fixed at different times after IR and processed for immunofluorescence with the MPM2 phospho-specific antibody that specifically stains mitotic cells. Figure 4B shows that, in contrast to control cells, the M443-treated cells failed to arrest after IR and maintained a similar mitotic index as the non irradiated cells. Thus, inhibition of MRK leads to inhibition of Chk2 and failure to arrest in the cell cycle in response to IR-induced DNA damage.

**M443 radiosensitizes medulloblastoma cells**

As a consequence of failed cell cycle arrest in M443-treated medulloblastoma cells, we would expect to see increased sensitivity to IR. To confirm this, we used both UI226 and UW228 cells. As the UI226 medulloblastoma primary cells do not form colonies when plated sparsely, we tested colony formation by the UW228 cell line and used the MTT assay for the UI226 cells. Figure 5 shows that M443 effectively
radiosensitizes both cell cultures to radiation. The clonogenic assay in UW228 cells showed a DEF at 10% viability of 1.6, which is the same observed upon silencing of MRK in these cells (Fig. 1B). A similar response was observed with the parental compound, nilotinib (Fig. 5B). The MTT assay showed that M443 in conjunction with IR caused a 48% reduction in viability of the primary medulloblastoma cells in comparison to IR alone. Interestingly, these cells responded maximally already to 125 nM of M443, the lowest dose used, indicating that the IC$_{50}$ for the radiosensitization effect is lower than 125 nM. Similar results were obtained with a second medulloblastoma primary line (R. Ruggieri, unpublished observations). Importantly, M443 did not show any toxicity in the absence of radiation and failed to radiosensitize normal neuronal stem cells and astrocytes (Supplementary Fig. S2). Thus, these data indicate that M443 can be used to radiosensitize medulloblastoma tumors without compromising the surrounding parenchyma.

To test the extent to which M443 radiosensitizes tumors other than medulloblastoma, we tested breast cancer 4T1 cells for their response to the drug in conjunction with IR. Supplementary Fig. S3 shows that M443 can radiosensitize breast cancer cells as well.

**M443 synergizes with IR to extend survival in an orthotopic medulloblastoma xenograft model**

To examine whether M443 can act as a radiosensitizer in a mouse model of medulloblastoma, we used the primary UI226 cells to generate an orthotopic medulloblastoma tumor in nude mice. Previous work had established that approximately 3 weeks after tumor cell implantation, mice become symptomatic within a relatively narrow time frame, just over a week, indicating similar tumor growth rates among the animals. Two weeks before mice became moribund, they were randomized into four groups: control, treated with M443 alone, treated with 6 Gy of IR (2x 3Gy over two days) and with the combination of M443 and IR. M443 was delivered directly into the tumor via an osmotic pump and two days later, mice were exposed to IR.
Control mice survived with a median of 32 days after tumor cell implantation. Treatment with M443 alone added 5.5 days to this survival, whereas the chosen low dose of radiation did not significantly increase survival (median of 1 day additional survival over that of control). In contrast, the combination of M443 and IR extended survival with a median of 16 days longer than control. Statistical analysis of the data demonstrated a synergistic effect of the two combined treatments (Fig. 6A). Supporting the safety data shown in Supplementary Fig. S2, treatment with M443 did not affect the animal weight, as the weight loss observed was observed in all groups just a few days before the animals became moribund (Supplementary Fig. S4).

To confirm in vivo target inhibition by M443, we tested MRK levels and activity in brains from treated and untreated mice 18 hours after exposure to the drug. For this assay, the brains were harvested and their cerebella were divided into four sections that were then examined by western blotting with phospho-specific and total MRK antibodies. The tumor-containing sections of the cerebellum were identified by the human anti-HLA antibody, because, despite performing the tumor cell injections in a standard fashion, it is difficult to predict the invasion and growth path of the tumor. Figure 6B shows that the tumor-containing fraction had elevated levels of both total and active MRK (lane RB in the vehicle-treated brain). In contrast, the tumor-containing fraction from the M443-treated brain showed total loss of MRK activity. Interestingly, the MRK protein levels were also reduced in the treated fractions. This result is consistent with our previous observation that sustained inhibition of MRK leads to its instability (R. Ruggieri, unpublished observation). Interestingly, the fractions containing normal brain, which in the control brain showed some level of MRK protein, in the treated brain also had lost MRK, suggesting that diffusion of M443 across the whole cerebellum inhibited normal MRK as well.
Taken together, our study demonstrates that MRK is a suitable target for radiosensitization of medulloblastoma tumors and likely additional types of tumors.

**Discussion**

In this study, we have identified the protein kinase MRK/ZAK as a promising novel target for radiosensitization in medulloblastoma and designed a small molecule inhibitor M443 that irreversibly blocks its activity. Importantly, administration of M443 *in vivo* effectively synergizes with radiation in a mouse model of medulloblastoma, where it increases the survival time by 50%. This result provides a proof of principle that MRK is a target for radiosensitization in medulloblastoma.

We have developed an irreversible inhibitor of MRK, M443, based on the close proximity of the M443 parental compound nilotinib to cysteine 22 in the model structure of the MRK ATP-binding domain (24). The unique presence of this cysteine at this position in MRK, compared to other nilotinib targets and other MRK related proteins, suggested that an irreversible inhibitor of MRK could provide high selectivity towards MRK, which is likely to result in reduced off-target effects of a treatment based on this molecule. Our initial investigation on the effects of M443 on BRC-Abl, the original target of nilotinib, indicates that M443 selectively inhibits MRK, but not BCR-Abl. Although a wider specificity screen is needed, this result suggests good selectivity for this compound.

The past reservations about irreversible inhibitors that were based on safety concerns recently have been mitigated by several clinical successes. In fact, irreversible kinase inhibitors for epidermal growth factor receptor (EGFR), Bruton's tyrosine kinase (BTK), and MEK1 are being tested in the clinic as anticancer agents (25). Importantly, this type of inhibitors is expected to have extended pharmacodynamic duration of inhibition, which could allow for lower circulating doses with less potential side effects. Although the toxicity profile of M443 needs to be assessed after systemic administration *in vivo*, it is interesting to note that we did not observe any apparent toxicity in tumor
cells treated with M443 (R. Ruggieri, unpublished observations), nor in animals based on lack of weight loss following drug administration. In addition, no effect was observed on normal brain cells (Supplementary Fig. S2). We believe that this characteristic of the MRK inhibitor is particularly important in the context of pediatric brain tumors for which safer therapeutic approaches are needed. Future characterization of the pharmacokinetic properties of M443 will establish the extent to which this compound crosses the blood brain barrier.

Analysis of the tumor tissue after convection delivery revealed a clear in vivo inhibition of MRK activity by M443. The phospho-specific MRK antibody should be a very valuable tool to identify patients that might have elevated activation levels of MRK and therefore may be more responsive to a treatment that includes MRK inhibition.

M443 inhibits MRK downstream pathways that include IR-induced activation of Chk2 and the consequent cell cycle arrest. The fact that these effects of MRK silencing were replicated by M443 supports the specificity of these phenotypes and strengthens a role for the MRK-β isoform in the DNA damage response, previously observed in other cell types (12). Both Chk2 and p38 have been shown to control cell cycle arrest after DNA damage by regulating the activity of the CDC25 family of proteins and thus the activity of the CDK proteins (26). The role of p38α in the DNA damage response has, however, been demonstrated only in response to UV-induced damage (27). The activation of p38α by IR in medulloblastoma is clearly dependent on MRK activity and it may point to either a role for p38α in IR-induced DNA damage, as suggested for the response to UV, or to additional roles that MRK may have in response to IR. As we have shown previously, IR-induced MRK activation is dependent on Nbs1 and partially on ATM (17), two proteins involved in the DNA damage response and in cell cycle checkpoint regulation (28,29). In turn, MRK activates Chk2, a checkpoint protein that, together with Chk1, is considered a good target for radiosensitization in cancer and for which inhibitors are actively being
evaluated in the clinic (30,31). Thus, M443 represents an additional novel approach to target the DNA
damage response in cancer.

As the two splice forms of MRK share the same kinase domain, M443 is expected also to inhibit
MRKα. Thus, because MRKα is aberrantly expressed in cancers like gastric, colorectal, bladder and
breast tumors, where it plays a critical role in tumor cell proliferation, M443 also may have therapeutic
effects in these cancers (13). Interestingly, we found that in addition to medulloblastoma cells, breast
cancer cells are also sensitized to radiation following MRK inhibition by M443 (Supplementary Fig. S2). In
addition, we found that siRNA-mediated MRK silencing radiosensitizes several types of cancer cells
including osteosarcoma and glioblastoma cells [(12) and data not shown)]. Thus, inhibition of MRK may
be a useful approach to radiosensitize different types of solid tumors. Future studies will investigate this
possibility.

In summary, we have developed a specific and irreversible inhibitor of MRK that effectively
radiosensitizes medulloblastoma tumors, and potentially other cancers as well. Future development of
this small molecule may provide a new therapeutic approach for radiosensitization in cancer.
Acknowledgements

We would like to acknowledge James Rutka (The Hospital for Sick Children) for kindly providing the UW228 cells. We also thank Amanda Chan (The Feinstein Institute) for her help with microscopy data acquisition.

References


Figure Legends

Figure 1. MRK down regulation radiosensitizes medulloblastoma cells and inhibits downstream signals. UW228 cells were transfected with Luciferase control (Luc) or MRK siRNAs, exposed to the indicated doses of radiation and cell survival was determined by the MTT assay (A) or for the clonogenic assay (B) as described in Materials and Methods. The western blot shows a typical level of MRK silencing. Data are the average ± s.e.m. of three independent experiments. p values for all treatment points were <0.005. (C) Forty eight hours after transfection with the respective siRNAs, cells were exposed to the indicated doses of radiation. Cell lysates were processed for western blotting with the indicated antibodies. Histograms show quantification of phospho-Chk2 signals from 3 independent experiments ± s.e. * p < 0.001. Data represent Chk2 specific activity, i.e. P-Chk2/Chk2 ration. As the values of P-Chk2 in the absence of radiation are very low and in some experiments undetectable by western analysis, the Chk2 specific activity data at the 1 hour time point for the Luciferase siRNA control cells were chosen to normalize the other data.

Figure 2. Design of the inhibitor of MRK, M443. (A) UMDI cells that express homodimerizable MRK were pretreated for 1 hr with 0.5 μM of the indicated inhibitors (top panel) or at the indicated concentration (bottom panel) in the serum-free medium and then subjected to MRK activation with the homodimerizer AP20187 (AP) for 1 hr before harvesting for western blotting with the indicated antibodies. (B) Amino acid sequence alignment of the indicated proteins (known targets of nilotinib) across part of the conserved kinase active site domain. * Unique Cys 22 in MRK. (C) Amino acid sequence alignment of the indicated proteins (MRK homologs). (D) Ribbons representation of the structure of MRK homology model in complex with compound M443 (magenta). (E) Close-up view of the binding site of M443 in comparison of nilotinib (green). M443 is illustrated after forming a covalent
bond with cysteine 22. The nilotinib structure was extracted from c-Abl crystal structure (PDB:3CS9). MRK residues are shown in gray. Non-polar hydrogens are hidden. (F) Nilotinib structure. (G) M443 structure.

**Figure 3.** M443 inhibits MRK in an irreversible and specific fashion. (A) Time course of MRK inhibition by M443. UMDI cells were pre-treated with 500 nM M443 (M) or treated with DMSO for the indicated time. Then, cells were washed 3 times with fresh medium (+), medium was refreshed after 15 minutes twice, and AP was added for 30 minutes before harvesting and processing cells for western blot. (B) UMDI cells were treated as in (A) with 100 nM nilotinib or 500 nM M443 for 6 hours. Histogram shows quantification of data from 3 independent experiments, expressed as mean ± SD. (C) K-562 cells were treated with 500 nM M443, nilotinib or vehicle control for 6 hours and cell lysates were processed for western blotting with the indicated antibodies. GAPDH was used as a loading control. The blot is a representative of 3 independent experiments.

**Figure 4.** M443 inhibits MRK downstream signaling and prevents radiation-induced cell cycle arrest. (A) Top panel: time course of MRK activation by IR. UW228 cells were exposed to 3 Gy of IR, harvested at the indicated time points and processed for western blotting with the P-MRK antibody. Tubulin was used as loading control. Bottom panel: UW228 cells and primary UI226 cells were pretreated with 500 nM M443 for 6 hours, then they were exposed to 3 Gy of radiation and 30 minutes later they were harvested and processed for western blot with the indicated antibodies. (B) UW228 cells, seeded on coverslips, were pretreated with 500 nM M443 for 3 hours, exposed to 6 Gy of radiation and then processed for immunofluorescence with the phospho-specific MPM2 antibody as described in Materials and Methods. Mitotic cells from 10 fields per condition were calculated from 3 independent experiments and plotted in the graph below. Bars = 50 μm.
**Figure 5.** M443 radiosensitizes medulloblastoma cells *in vitro*. UW228 cells were pretreated with the indicated doses of M443, (A) or nilotinib (B), exposed to the indicated doses of radiation and then processed for the clonogenic assay as described in Materials and Methods. (C) UI226 primary medulloblastoma cells were pretreated with the indicated doses of M443, exposed to various doses of radiation and processed for the MTT assay 72 hours later.

**Figure 6.** M443 synergizes with radiation to prolong survival in a murine model of medulloblastoma. (A) Kaplan-Meier survival curves demonstrating synergism between M443 and radiation treatment. N= 10-12 mice/group. Time refers to days after IC tumor cell injections. The bar and asterisks above the Kaplan-Meier curves indicate the respective treatment periods (2 weeks of continues exposure to the drug via the osmotic pump and 2 doses of IR indicated by the asterisks). (B) *In vivo* target inhibition and specificity of M443. Eighteen hours post pump implantation brains were harvested and each cerebellum was sectioned in four parts: right top (RT), right bottom (RB), left top (LT) and left bottom (LB). Tissue extracts were processed for western blotting with the indicated antibodies. The hu-HLA antibody identifies the tumor-containing fractions.
Figure 1

A) Cell survival (% of control) vs. Gy for Luc siRNA and MRK siRNA.

B) Colony number (% of control) vs. Gy for Luc siRNA and MRK siRNA.

C) Chk2 specific activity (% of 1 hr activity) vs. Time (h) for Luc siRNA and MRK siRNA.
Figure 3

A

Time: 2 h 4 h 6 h
Wash: - - - + + +

P-MRK -
Tubulin -

B

Wash: - - + -

P-MRK -
MRK -
Tubulin -

C

Wash: - - + -

P-BCR-Abl -
P-MRK -
GAPDH -
Figure 4

A

Time after IR (min): 0 15 30 60 120
P-MRK - Tubulin -

M443: - + - +
IR: - + - +
P-MRK - MRK -
P-Chk2 - CHK2 -
P-p38 - Tubulin -

B

Control M443

MPM2

DAPI

Bright Field

Overlay

MPM2 positive cells (% of total)

Time (hr): (post IR)

Control M443

p<0.0001
Figure 5

A

B

C

Colony Number (% of control)

IR (Gy): 0 2 4 6

0 nM 250 nM 500 nM

p<0.0001

p<0.0001

p<0.0001

Cell viability (% of control)

IR (Gy): 0 3 6

0 nM 125 nM 250 nM 500 nM

p<0.0001

p<0.0001
**Figure 6**

A.

**Drug treatment**  
- Control  
- M443  
- IR  
- IR+M443

**Percent survival**  
- Time (days after tumor cell implantation)

B.

**Treatment**: vehicle, M443  

- P-MRK  
- MRK  
- hu-HLA  
- Tubulin

**Notes**
- Treatment: vehicle, M443 cells
- Drug treatment
- IR treatment
- * *
Molecular Cancer Therapeutics

Pharmacological inhibition of the protein kinase MRK/ZAK radiosensitizes medulloblastoma

Daniel Markowitz, Caitlin Powell, Nhan L. Tran, et al.

Mol Cancer Ther Published OnlineFirst May 20, 2016.