Selective Killing of K-ras Mutant Cancer Cells by Novel Small Molecule Inducers of Oxidative Stress

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Selective killing of K-ras mutant cancer cells by small molecule inducers of oxidative stress

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Activating K-RAS mutations are the most frequent oncogenic mutations in human cancer. Numerous downstream signaling pathways have been shown to be deregulated by oncogenic K-ras. However, to date there are still no effective targeted therapies for this genetically defined subset of patients. Here we report the results of a small molecule, synthetic lethal screen using mouse embryonic fibroblasts derived from a mouse model harboring a conditional oncogenic K-rasG12D allele. Among the >50,000 compounds screened, we identified a class of drugs with selective activity against oncogenic K-ras-expressing cells. The most potent member of this class, lanperisone, acts by inducing nonapoptotic cell death in a cell cycle- and translation-independent manner. The mechanism of cell killing involves the induction of reactive oxygen species that are inefficiently scavenged in K-ras mutant cells, leading to oxidative stress and cell death. In mice, treatment with lanperisone suppresses the growth of K-ras-driven tumors without overt toxicity. Our findings establish the specific antitumor activity of lanperisone and reveal oxidative stress pathways as potential targets in Ras-mediated malignancies.

Targeting canonical Ras signaling pathways is complicated by the fact that these pathways are also essential for the proliferation and survival of normal cell types. To target oncogenic Ras selectivity, several different strategies have been taken. One of the initial strategies involved inhibition of Ras farnesylation, a posttranslational modification required for localization of Ras to the plasma membrane. Preclinical studies of farnesyltransferase inhibitors (FTIs) in transgenic mouse models overexpressing oncogenic Ras showed potent antitumor activity (10). However, in patients with solid tumors, FTIs have shown little if any clinical activity, likely due to redundancies in this pathway (11, 12).

More recent approaches to targeting oncogenic Ras have used synthetic lethal screening to identify novel anticaner agents capable of selectively killing tumor cells harboring a specific mutation. In the case of oncogenic K-ras, several compounds have already been identified, including sulfynil cystine derivative (SC-D) (13), erastin (for eradicator of Ras and ST-expressing cells) (14), and oncrasin-1 (for oncogenic Ras tumor-inhibiting compound 1) (15). Though erastin has been shown to induce mitochondrial dysfunction (16), and oncrasin may require protein kinase C iota (PKCi) for activity (15), the precise mechanisms by which any of these compounds selectively kill K-ras mutant cells remain uncertain. Recently, synthetic lethal screens using shRNAs targeting the kinome have identified other potential target pathways in K-ras mutant cell lines, including the noncanonical Isk kinase TBK1 involved in NF-κB signaling (17), the mitotic kinase PLK1 (18), and the serine/threonine protein kinase STK33 of unknown biological function (19).

Despite these promising developments, there are still no effective therapeutic agents or regimens in the clinic for patients with K-ras mutant tumors. Here we report the results of a synthetic lethal chemical screen using mouse embryonic fibroblasts (MEFs) derived from mice harboring a conditional, oncogenic K-rasG12D allele. We have identified a family of compounds, represented by lanperisone (LP), with selective activity in cells harboring oncogenic K-ras. In contrast to other putative Ras inhibitors, LP, which was originally developed as a muscle relaxant (20), has already been tested in the clinic, and serves as a potential lead in targeting Ras mutant cancers safely and effectively.

Results

Identification of Lanperisone and the Related Compound Lanperisone by Synthetic Lethal Chemical Screening. We performed a high-throughput, synthetic lethal chemical screen to identify small
molecules that selectively kill MEFs expressing oncogenic K-ras (Fig. 1A). We derived K-ras<sup>G12D</sup>-expressing MEFs using two different methods. MEFs from K-ras<sup>LSL-G12D</sup> embryos were treated in vitro with a retroviral, self-excisng Cre recombinase (21). Genomic PCR confirmed complete recombination of the K-ras<sup>LSL-G12D</sup>;Mox2-Cre compound mutants. In these mutant embryos, Cre-mediated recombination occurs in utero and efficiently activates K-ras<sup>G12D</sup> throughout the embryo proper as shown previously (22). Several lines of evidence indicate that MEFs are a valid surrogate cell type in which to model oncogenic K-ras signaling. First, MEFs expressing physiologic levels of oncogenic K-ras<sup>G12D</sup> are partially transformed (21). Second, K-ras<sup>G12D</sup> MEFs do not exhibit augmented activation of canonical Ras signaling pathways (21), similar to the early pulmonary lesions in K-ras<sup>LSL-G12D</sup> mice.

Using an ATP-based cell viability assay (CellTiter-Glo or CTG), we screened over 50,000 compounds and identified two with potent and selective activity against K-ras mutant cells compared with wild-type controls (Fig. 1B and Fig. S2). We focused on a piperidine derivative, tolperisone, which is an orally available, centrally acting muscle relaxant used to treat painful muscle spasms. Tolperisone exhibited significant differential activity in both the cell viability assay (Fig. 1C) as well as in a second, independent assay based on BrdU incorporation (Fig. 1D). To explore structure-function relationships, we synthesized or obtained from commercial sources >10 derivatives of tolperisone (Fig. 1E and Fig. S3). Among tolperisone and its derivatives, LP showed the most potent and selective activity against K-ras<sup>G12D</sup> MEFs, with an IC<sub>50</sub> value in the CTG viability assay of 4 μM (Fig. 1F). We also created compound mutant MEFs carrying the K-ras<sup>LSL-G12D</sup> allele in a p53-null background. K-ras was activated using the same retroviral, self-excisng Cre recombinase described above. As shown in Fig. 1G, K-ras<sup>G12D</sup>p53<sup>−/−</sup> MEFs were similarly sensitive to LP-induced killing as K-ras<sup>G12D</sup> MEFs, indicating that the death was p53 independent. Both K-ras<sup>G12D</sup> and K-ras<sup>G12D,p53<sup>−/−</sup></sup> MEFs were used in subsequent experiments and showed similar results.

**LP-Mediated Induction of Cell Death in K-ras Mutant Cells.** To determine the cellular basis for the decreased viability of LP-treated, K-ras mutant MEFs, we analyzed cells after short-term (6 h) exposure to LP or vehicle (DMSO). In contrast to DMSO-treated K-ras mutant MEFs, LP-treated cells showed striking morphologic changes (Fig. 2A), with the vast majority of cells becoming spherical and beginning to detach from the plate. Wild-type MEFs exposed to LP or DMSO showed minimal changes in morphology (Fig. S4A). We next performed FACS analyses on both wild-type and K-ras mutant MEFs treated with LP or DMSO for 6 h. Consistent with the morphologic observations, LP treatment of K-ras mutant, but not wild-type MEFs, induced a large population of cells with sub-2N DNA content (Fig. 2B). FACS analysis for BrdU incorporation demonstrated normal cell cycle progression of LP-treated cells (Fig. 2C). These results suggest that LP selectively kills K-ras<sup>G12D</sup> MEFs in a cell cycle-independent fashion.

To examine the mechanism of LP-induced cell death, we first stained cells with propidium iodide (PI) for FACS analysis. Compared with controls, K-ras mutant MEFs treated with LP for 6 h showed a significantly higher percentage of dying, PI+ cells (Fig. 2D). In separate FACS-based TUNEL-labeling experiments, LP consistently induced higher levels of TUNEL positivity in K-ras mutant compared with wild-type MEFs (Fig. S4B). To investigate whether LP induces death through apoptosis, caspase activation and the effect of the pan caspase inhibitor zVAD on LP-induced cell death were determined. Cleaved caspase 3 was variably detected by immunoblotting and flow cytometry after LP treatment of MEFs, but pretreatment with zVAD did not inhibit LP-induced death (Fig. S4C). Taken together, these results suggest that the cell death induced by LP involves DNA...
mediated cell killing, we examined the consequences of activating or inhibiting HIF in the setting of LP treatment. To activate HIF, we exposed MEFs to varying concentrations of the prolyl hydroxylase inhibitor DMOG or to hypoxia (0% O₂) before treatment with LP. Neither DMOG nor hypoxia significantly enhanced cell killing by LP (Fig. S5B). Similarly, overexpression of either wild-type HIF-1 or HIF-2, or a mutant HIF-1 or HIF-2 in which the oxygen degradation domain (ODD) is deleted, did not enhance LP-mediated cell killing (Fig. S5C). Finally, overexpression of a dominant-negative form of HIF-1 and HIF-2 did not reduce LP-induced cell death (Fig. S5C). These findings suggest that though HIFs are induced by LP treatment, they do not play a functional role in the selective killing of K-ras mutant cells by LP.

**Enhanced Induction of Reactive Oxygen Species by LP in K-ras Mutant Cells.** By GSEA, the LP gene expression signatures also show similarity to those associated with oxidative stress pathways (Table S1). In addition, we queried the Connectivity Map (CMap; http://www.broadinstitute.org/cmap/) to identify known drugs that induced similar gene expression changes as did LP (23). This query yielded numerous drugs that showed strong positive (i.e., highly correlated with the LP signature) or negative (anti-correlated with the LP signature) connectivities with LP treatment. Among the strong positive connectivities were multiple drugs of different mechanism but all known to induce oxidative stress, including parthenolide (a sesquiterpene lactone), 15-ß-prostaglandin J2 (an endogenous anti-inflammatory signaling molecule), lomustine (an alkylating agent), and hsp90 inhibitors such as geldanamycin (Fig. S6). Of note, these connectivities were similar in both wild-type and K-ras mutant MEFs.

To test whether the mechanism of LP-induced cell killing does indeed involve oxidative cell death, we first measured the levels of reactive oxygen species (ROS) in wild-type and K-ras mutant MEFs. Both control and LP-treated MEFs were stained with the redox-sensitive dye DCF-DA, followed by FACS analysis to quantify intracellular ROS levels. After 6 h of exposure to LP, K-ras mutant MEFs displayed a significant surge in ROS levels compared with LP-treated wild-type MEFs (Fig. 3A). Approximately 30–40% of K-ras mutant cells showed high ROS levels, compared with 5% or less of wild-type controls (Fig. 3A and B). Significant ROS levels were detected as early as 1 h after LP treatment in K-ras mutant MEFs, whereas wild-type MEFs treated with LP showed a more gradual accumulation of intracellular ROS over time (Fig. 3C).

To assess the functional importance of ROS induction in LP-treated K-ras mutant MEFs, we pretreated cells with a variety of different ROS scavengers, including deferoxamine (DFO), butylated hydroxyanisole (BHA), and the antioxidant trolox, a vitamin E analog. As shown in Fig. 3D, all of the ROS scavengers tested completely abolished cell killing by LP. FACS confirmed the viability of cells pretreated with ROS scavengers, and also demonstrated a variable reduction in intracellular ROS levels, depending on the ROS scavenger used (Fig. 3D and Fig. S7A). Consistent with these results, hydrogen peroxide (H₂O₂), a cell-permeable ROS, synergized with LP in killing K-rasG12D-expressing MEFs (Fig. S7B). In addition, cobalt chloride, which is known to compete with iron for cellular transport and for binding sites on a variety of proteins, did not significantly diminish ROS production, but potentely blocked LP-induced cell death (Fig. 3E and Fig. S8). Furthermore, LP-induced cell death was also inhibited by pretreatment with the MEK1/2 inhibitor U0126 but not by the protein synthesis inhibitor cycloheximide (Fig. 3F and Fig. S8). Taken together, these results demonstrate that the selective killing of K-ras mutant cells by LP is mediated by the induction of ROS, and is both iron- and Ras/MAPK-dependent.
Antitumor Activity of LP in Vivo.
To test whether LP has activity against K-ras mutant tumors in vivo, we used compound mutant MEFs carrying the oncogenic K-ras allele in a p53-null background. Unlike K-ras<sup>G12D</sup> MEFs, K-ras<sup>G12D</sup>;p53<sup>−/−</sup> MEFs form s.c. tumors when injected into nude mice. Mice with established tumors were treated with either water or with LP at 40 mg/kg twice daily by oral gavage. The mice that received LP tolerated treatment well, with no overt toxicities and no statistically significant change in body weight (Fig. 4A). After 7 d, tumors were measured, dissected, and weighed. Control and LP-treated mice both showed similar tumor sizes at the start of treatment (Fig. 4B). However, compared with tumors from control animals, tumors from LP-treated mice were 24% smaller based on estimated tumor volumes (P = 0.16), and 37% smaller based on actual tumor weights (P < 0.03; Fig. 4C).

Discussion
Through synthetic lethal chemical screening, we have identified a class of drugs with selective activity against oncogenic K-ras-expressing cells. These drugs have been developed as centrally acting muscle relaxants, and until now have not been studied as potential anticancer agents. Here we show that LP, the most potent member of this family, induces nonapoptotic cell death in a cell cycle- and translation-independent manner. Based on gene expression and biochemical studies, the mechanism of selective cell killing appears to involve the induction of ROS, which may be either overproduced and/or inefficiently scavenged in the setting of oncogenic K-ras activation. High intracellular levels of ROS in turn appear to contribute to the selective, oxidative cell death of K-ras mutant cells.

To date, among the K-ras targeted therapies identified through synthetic lethal chemical screening, LP is structurally distinct. However, in terms of potential mechanism of action, LP seems to mostly closely resemble erastin. Erastin was first discovered in a synthetic lethal chemical screen using engineered human tumor cells derived from primary BJ fibroblasts (14). Like LP, erastin induces nonapoptotic, oxidative cell death in cell lines expressing oncogenic Ras (14, 16). Affinity purification and mass spectrometry led to the identification of mitochondrial voltage-dependent anion channels (VDACs) as putative targets of erastin (16). By binding to VDACs, erastin is believed to alter...
VDAC gating, leading to mitochondrial dysfunction, release of ROS, and oxidative cell death. The mechanistic basis for erastin’s selectivity, however, remains poorly understood, and has been attributed to either up-regulation of VDACs (16) or higher levels of iron (26) in HRAS mutant cells.

Though the molecular target of LP and related family members is unknown, functional studies suggest that this class of compounds may also perturb voltage-gated ion channels. In dorsal root ganglion neurons, the related drug tolperisone inhibits voltage-gated sodium and calcium channels (27). This combined blockade leads to presynaptic inhibition of neurotransmitter release from primary afferent terminals, which in turn depresses the spinal reflex machinery. As a result, tolperisone-type drugs like LP have been developed to treat painful reflex muscle spasms. Whether LP interacts with voltage-gated channels in K-ras mutant cells to cause mitochondrial dysfunction remains to be determined. However, this hypothesis is tautologous in light of the known pharmacology of LP (27), as well as the similarities in LP- and erastin-mediated cell killing. Of note, erastin also exhibited selective lethality for K-ras mutant MEFs relative to wild-type controls (Fig. S9). Additionally, in the CMAP experiments, lanperisone did show moderate connectivity with erastin (Fig. S6c).

The potential role of ROS in promoting and maintaining the oncogenic phenotype of cancer cells is well known. However, ROS and the resulting oxidative stress can also induce cellular senescence and apoptosis in certain contexts, suggesting a role for ROS-generating drugs as anticancer agents. The exact response to ROS depends on the magnitude of ROS induction, the type of ROS produced, and cell type and tissue-specific factors. A number of drugs with known ROS-generating activity have already proved therapeutically useful: arsenic trioxide, for example, is an FDA-approved treatment for patients with relapsed or refractory acute promyelocytic leukemia (APL). Numerous lines of evidence suggest that the antitumor activity of arsenic trioxide in APL is due to the production of ROS (28), possibly via arsenic-induced up-regulation of NADPH oxidase (29). Whether arsenic trioxide is also active in cancer cells harboring mutant K-ras is unknown; however, our findings with LP provide a rationale for pursuing future studies of arsenic trioxide specifically in the setting of oncogenic K-ras activation.

In conclusion, we have identified the tolperisone-like drug LP as a potential targeted therapy for K-ras mutant cancers. By inducing high levels of ROS, LP appears to selectively target K-ras mutant cells by exploiting their inherent vulnerability to oxidative stress. The remarkable mechanistic similarities of LP as well as erastin underscore the potential of ROS-mediated therapies as a novel strategy to treat K-ras mutant tumors. Such mechanisms may also underlie the function of two recently identified, synthetic lethal K-ras targets, TBK1 and STK33 (17, 19). Among all of the candidate K-ras–targeted agents, LP is the only one with human safety and pharmacodynamic data. If preclinical studies of LP continue to show promise, this drug could undergo rapid clinical development and prove efficacious for K-ras mutant tumors, particularly those of mesenchymal origin.

Materials and Methods
Mouse Strains and MEFs. The K-ras<sup>LSL-G12D</sup> strain was interbred to Mox2-Cre mice to enable recombination within the embryo proper and derivation of K-ras<sup>LSL-G12D</sup>–expressing MEFs (22). Recombination of the K-ras<sup>LSL-G12D</sup> allele was also performed in vitro using self-excisng Cre recombinase as described (21). K-ras<sup>LSL-G12D</sup> mice were crossed to p53<sup>+/−</sup> mice to generate K-ras<sup>LSL-G12D</sup>p53<sup>−/−</sup> MEFs. Mice and MEFs were either on a pure 129S5 or a mixed 129Sv/C57BL/6 background. Early passage (P3–4) MEFs were used for all experiments.

Small Molecule Screening. High-throughput small molecule screening was performed at the Institute for Chemical and Cellular Biology, Harvard Medical School. At least three independently derived MEF lines of each genotype (wild-type or K-ras<sup>LSL-G12D</sup>) were plated in triplicate in 384-well format at 1,500 cells/well. Compounds were added robotically the following day to a final concentration of ~10 μM. Compounds (~1,700) were obtained from commercial libraries (ChemDiv, Maybridge, Peakdale, and Bionet), known bioactive collections (National Institute of Neurological Disorders and Stroke, SpecPlus, Broad Institute), natural product libraries (Starr Foundation Extracts and Philippines Plant Extracts), and diversity oriented synthesis (DOS) collections from the Broad Institute. Growth inhibition was determined 48 h after compound addition using a luminescent cell viability assay (CellTiter-Glo; Promega). Viability scores were calculated for each compound within each genotype by normalizing to DMSO controls. Positives were defined as any compounds that reduced the relative viability of mutant vs. wild-type MEFs by 0.3. Positives were selected for secondary screening using a BrdU cytoblot assay as previously described (33), and for dose–response assays.

Proliferation, Cell Cycle, and Apoptosis Assays. BrdU incorporation was determined using the BD BrdU staining kit according to the manufacturer’s protocol (BD Bioscience). Fluorescein TUNEL staining was performed with the In Situ Cell Death Detection Kit according to the manufacturer’s protocol (Roche). The caspase inhibitor Z-VAD-FMK was used at 25 μM (Promega). Cells were analyzed using FacScan or FacsCaliber flow cytometers (BD Bioscience) and analyzed using FlowJo Software (TreeStar).

Gene Expression Profiling and Analysis. Three independently derived MEF lines per genotype were treated with either DMSO or LP (10 μM) for 6 h. RNA was isolated by TRIzol extraction, and labeled cRNA was hybridized to
Affymetrix Mouse Genome 430A 2.0 microarrays according to the manufacturer’s protocol. Expression signatures were calculated using the Comparative Marker Selection module of the GenePattern software suite, using the signal-to-noise statistic and standard settings. GSEA was performed using the GSEA module (25) of GenePattern; P values were determined by random permutation of gene sets. CMAP analysis was performed with the CMAP Web tool found at http://www.broad Institute.org/cmap/ (23). Details of these analyses are available on request.

### Intracellular ROS Measurements

MEFs were harvested, washed with PBS containing 5 mM glucose, and incubated with DCF-DA (10 μM; Molecular Probes) for 15 min. Cells were then washed with PBS and analyzed by FACS. Modulators of ROS were tested at the following working concentrations: DFO 150 μM, Trolox 200 μM, BHA 100 μM, CoCl₂ 0.250 mM, H₂O₂ 400 μM, and U0126 5 μM.


In Vivo Studies. K-rasG12D;p53−/− MEFs (1 × 10⁶) were injected s.c. into the flanks of nude mice. Mice harboring tumors measuring at least 1 cm in longest dimension were treated with either dH₂O or LP (40 mg/kg twice daily, dissolved in dH₂O). All mice were killed after 7 d.

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