Novel Cancer Therapeutics with Allosteric Modulation of the Mitochondrial C-Raf–DAPK Complex by Raf Inhibitor Combination Therapy

Yi-Ta Tsai1,2, Mei-Jen Chuang2, Shou-Hung Tang2, Sheng-Tang Wu2, Yu-Chi Chen3, Guang-Huan Sun1,2,4, Pei-Wen Hsiao5, Shih-Ming Huang5, Hwei-Jen Lee6, Cheng-Ping Yu7,7, Jar-Yi Ho4,7, Hui-Kuan Lin6,8,9, Ming-Rong Chen3,4, Chung-Chih Lin2, Sun-Yran Chang2,10, Victor C. Lin5, Dah-Shyong Yu1,2,4, and Tai-Lung Cha1,2,4,6

Abstract

Mitochondria are the powerhouses of cells. Mitochondrial C-Raf is a potential cancer therapeutic target, as it regulates mitochondrial function and is localized to the mitochondria by its N-terminal domain. However, Raf inhibitor monotherapy can induce S338 phosphorylation of C-Raf (pC-RafS338) and impede therapy. This study identified the interaction of C-Raf with S308 phosphorylated DAPK (pDAPKs308), which together became colocalized in the mitochondria to facilitate mitochondrial remodeling. Combined use of the Raf inhibitors sorafenib and GW5074 had synergistic anticancer effects in vitro and in vivo, but targeted mitochondrial function, rather than the canonical Raf signaling pathway. C-Raf depletion in knockout MEF<sup>C-Raf<sup>−/−</sup></sup> or siRNA knockdown ACHN renal cancer cells abrogated the cytotoxicity of combination therapy. Crystal structure simulation showed that GW5074 bound to C-Raf and induced a C-Raf conformational change that enhanced sorafenib-binding affinity. In the presence of pDAPKs308, this drug–target interaction compromised the mitochondrial targeting effect of the N-terminal domain of C-Raf, which induced two-hit damages to cancer cells. First, combination therapy facilitated pC-Raf S338 and pDAPKs308 translocation from mitochondria to cytoplasm, leading to mitochondrial dysfunction and reactive oxygen species (ROS) generation. Second, ROS facilitated PP2A-mediated dephosphorylation of pDAPKs308 to DAPK. PP2A then dissociated from the C-Raf–DAPK complex and induced profound cancer cell death. Increased pDAPKs308 modification was also observed in renal cancer tissues, which correlated with poor disease-free survival and poor overall survival in renal cancer patients. Besides mediating the anticancer effect, pDAPKs308 may serve as a predictive biomarker for Raf inhibitors combination therapy, suggesting an ideal preclinical model that is worthy of clinical translation. Cancer Res; 75(17); 1–15. ©2015 AACR.

Introduction

A better understanding of the molecular mechanisms of cancer causation and progression has led to the development of targeted therapeutics, which is a step toward personalized clinical and precision medicine. Besides the canonical Ras–Raf–MEK–ERK signaling pathway, Raf proteins also participate in the regulation of mitochondrial function (1). In vascular endothelial cells, fibroblast growth factor (FGF) induces C-Raf mitochondrial translocation through the phosphorylation of Ser338 (S338) of C-Raf (pC-Raf<sup>S338</sup>; ref. 2). C-Raf binds directly to the mitochondria through its N-terminal domain to regulate mitochondrial shape and cellular distribution (3). Such morphologic alterations in the mitochondrial network are involved in cell migration and Ca<sup>2+</sup> metabolism, which contribute to the oncogenic processes in cancer cells (4–6). The overexpression of Bcl-2 results in the recruitment of C-Raf into the mitochondria for cell survival (1). Mitochondrial targeting of C-Raf confers prosurvival properties that prevent dimerization and phosphorylation of MST2 (7) and induce the phosphorylation and inactivation of BAD (1), respectively. Furthermore, both C-Raf and B-Raf can induce some of the metabolic features mediated by the mitochondria, irrespective of the canonical Raf signaling pathway (7–9).

Mitochondria are the powerhouses of cells and play crucial roles in regulating the cell signal transduction that contributes to various anabolic reactions. Thus, they are more important to cancer cells exhibiting profound modifications in their energy metabolism. The unique role of Raf proteins in the mitochondria provides a strong rationale to target mitochondrial Raf proteins in cancer therapeutics (1, 10, 11). Although Raf inhibitors have been
shown to have survival benefits for a variety of cancers, emerging problems include the lack of long-term therapeutic efficacy and rapid development of evasive-resistance in the clinics (12–15). Raf inhibitor monotherapy promotes S338 phosphorylation of C-Raf and tumor progression (13–15). These facts suggest that Raf inhibitor monotherapy may paradoxically activate C-Raf in the mitochondria and impede cancer treatment. Currently, no selective cancer therapeutics that target mitochondrial Raf are available.

Death-associated protein kinase (DAPK), a serine/threonine protein kinase, was identified by a genetic screen for positive mediators of cell death (16). It participates in a number of apoptosis-inducing pathways downstream of interferon-γ, CD95 (Fas), TNFα, and TGFβ (17, 18). Besides regulating INK signaling, DAPK serves as a mitochondrial sensor to induce capase-independent necrotic cell death under oxidative stress (19). However, the detailed molecular mechanisms remain unknown.

The DAPK kinase activity is important for its biologic functions that mediate cell death processes. The auto-phosphorylation of the Ser308 (S308) residue of DAPK inhibits its kinase catalytic activity. Dephosphorylation of S308 of DAPK by PP2A enhances its kinase activity and promotes cell death processes (20, 21). Besides S308 phosphorylation of DAPK, other phosphorylation sites also affect its kinase activity. For instance, ERK and Src kinases phosphorylate S735 and Y491/Y492, to activate and inhibit the DAPK kinase activity, respectively (22–24).

DAPK functions as a tumor-suppressor gene, and its expression is attenuated in many cancer types by promoter hypermethylation (25). Loss of DAPK kinase function, rather than lack of protein expression, is also found in renal cell carcinoma (RCC), lung cancer, and other types of cancer (26–28). Posttranslational modification, such as S308 phosphorylation of DAPK, may be one of the reasons for the loss of tumor-suppressor function in cancers with DAPK expression.

This study determined that C-Raf interacted with pDAPK S308 and directed it to become colocalized to the mitochondria for regulating mitochondrial remodeling. A novel combination therapy, which used the Raf inhibitors sorafenib and GW5074, targeted mitochondrial C-Raf and DAPK complexes instead of the canonical Raf signaling pathway. GW5074 bound to C-Raf and induced a C-Raf conformational change that enhanced sorafenib-binding affinity. In the presence of pDAPK S308, GW5074 and sorafenib bound to C-Raf and further induced a conformational change of the N-terminal domain, which compromised its mitochondrial-targeting effect. This facilitated pDAPK S308 and C-Raf translocation from the mitochondria to the cytoplasm, causing mitochondrial dysfunction and reactive oxygen species (ROS) generation. ROS-facilitated PP2A-mediated dephosphorylation of DAPK at S308 activated DAPK, which then dissociated from C-Raf–DAPK in the cytoplasm, and induced profound cancer cell necroptosis. An animal model of orthotopic spontaneous metastases of RCC was established to mimic clinical situations. This model demonstrated the synergistic anticancer effect of the combination therapy in vivo. This study also identified pDAPK S308 as a mediator and predictive biomarker of sorafenib and GW5074 combination therapy efficacy for cancer treatment.

Materials and Methods

The procedural details are in the Supplementary Experimental Procedures.

### Chemicals and antibodies

The GW5074, PLX7420, L779450, and NAC (N-acetylcysteine) were purchased from Sigma-Aldrich. Sorafenib and zVAD-fmk (carbobenzoxy-valyl-alamyl-aspartyl-[O-methyl]-fluoromethylketo)were purchased from Santa Cruz Biotechnology. Cantharidin and okadaic acid were purchased from Merck Millipore (KGaA).

The following antibodies were purchased from Cell Signaling Technology: Akt (#9227), phospho-Akt (Ser473, #9271), phospho-ERK1/2 (Thr202/Tyr204, #4377), MEK1 (#9124), phospho-MEK1 (#9121), phospho-B-Raf (Ser445, #269), phospho-C-Raf (Ser259, #9241), phospho-C-Raf (Ser338, #9427), phospho-INK (Thr183/Tyr185, #9255), GAPDH (#G9545) and phospho-DAPK (Ser308, #D4941) were from Sigma-Aldrich. ERK1/2 (SC-94), B-Raf (SC-5284), VDAC1 (SC-58649), and TOM20 (SC-11415) were from Santa Cruz Biotechnology. DAPK (#3798-1), phospho-PP2A (Y307, #1155-1), and α-tubulin (#1878-1) were from Epitomics. The PP2A C subunit (#05-421) was obtained from Millipore (KGaA).

#### Cell culture

The ACHN, A498, A2058, DU-145, HeLa, MDA-MB-453, MDA-MB-231, MCF-7 293T, A459, NIH-3T3, H1299, A253, HepG2, U87, GBM8401, LN229, 22RV1, SV-HUC-1, 786-O, Colo-205, HT-29, LnCaP, PC-3, RWPE-1, and WPMY-1 cells were obtained from the Bioresource Collection and Research Centre (BCRC, Taiwan). Mouse embryonic fibroblasts (MEF) immortalized with the SV40 large T antigen [clones K2 (C-Raf +/−) and K9 (C-Raf +/−)] were obtained from C. Pritchard (University of Leicester, Leicester, United Kingdom; ref. 29). Human mtDNA-less cells 143B and rho 0 143B cell lines were a gift from Y.H. Wei (Institute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei, Taiwan and Department of Medicine, Mackay Medical College, New Taipei City, Taiwan; ref. 30). The cells were maintained in culture media as recommended by the ATCC and maintained at 37°C in a 5% CO2, 95% humidity incubator.

#### Cell treatments

Preliminary experiments were performed to determine the optimum time and dose effect of different C-Raf inhibitor treatments. In this study, all inhibitors of C-Raf (10 μmol/L GW5074, 10 μmol/L PLX7420, and 10 μmol/L L779450), caspase (zVAD), PP2A, or the ROS scavenger NAC were added 30 minutes before sorafenib (5 μmol/L) treatment.

#### Generation of TKI-resistant RCC cell lines

Approval to use the cells for culture was obtained from the patients and the local ethics committee before the study. Tumor samples from patients were obtained as previously described (31). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 10 μg/mL streptomycin (Invitrogen). The cells underwent 30 passages. The cell lines were identified as RCC cell lines by the BCRC, Taiwan.

#### Annexin V/propidium iodide staining

For necroptosis assays, the cells were washed with phosphate-buffered saline (PBS), resuspended in a binding buffer from Enzo Life Sciences, and stained with Annexin V–FITC and propidium iodide (PI) for 15 minutes. Annexin V fluorescence was measured using the FACScan flow cytometer (BD Biosciences) and the membrane integrity of cells was simultaneously assessed by PI exclusion.
Combination therapy of sorafenib and GW574 showed synergistically anticancer effect in vitro and in vivo. A, cell death in ACHN (right) and A498 (left) was measured by sulforhodamine B assay at 24, 48, and 72 hours after treatment with the indicated Raf inhibitors (10 μmol/L GW5074, 5 μmol/L sorafenib, 10 μmol/L PLX4720, and 10 μmol/L L779450; mean ± SEM; **P < 0.01, each experiment triplicated; n = 4). The percentage of cell death after treatment was compared with DMSO control (DMSO treatment defined as 100% viable). B, tumor volume measurements of ACHN xenografts treated with vehicle, sorafenib at 5 mg/kg (by oral lavage), GW5074 at 25 mg/kg (intraperitoneal injection, i.p.), or drug combination once a day (mean ± SEM; **P < 0.01; n = 8). C, schematic representation shows how the spontaneous metastatic Luc-ACHN-LL cells were established from parental ACHN cells. (Continued on the following page.)
Wright-Giemsa staining

Cells grown on 6-cm dishes were washed in PBS twice and stained with Giemsa solution (5% Giemsa stain solution, 10% buffered formalin) for 15 minutes. After decanting the staining solution, the cells were thoroughly washed in distilled water three times and air-dried before being photographed.

Sulforhodamine B growth inhibition assays

The cells were plated in 96-well plates at a plating density of 3,000 cells per well. After 24 to 72 hours with different drug treatments, the cells were fixed with 10% trichloroacetic acid (Sigma-Aldrich) and stained with 0.4% sulforhodamine B (Sigma-Alrlich). The plates were read in a PerkinElmer Victor3 micro-plate reader at 570 nm, as described previously (32). Triplicate wells per condition were evaluated and the data presented are representative of three independent experiments.

Measurement of ROS

The cells were under single or combined treatment for 24 hours. Intracellular ROS levels were measured by FACScan flow cytometry using CM-H2DCFDA (10 μmol/L). To detect mitochondrial superoxide, MitoSOX Red (Invitrogen) staining was following the manufacturer’s protocol. The histograms presented are representative of three separate experiments.

Results

Sorafenib and GW5074 combination therapy induced cancer cell death in vitro and suppressed tumor growth in vivo

We examined the effects of different Raf inhibitors with dosages from 2.5 to 10 μmol/L, including sorafenib, GW5074, PLX7420, and L779450, on the cell viability of ACHN and A498 RCC lines. Raf inhibitor monotherapy showed low cytotoxicity in ACHN and A498 RCC cell lines (Supplementary Fig. S1A). To identify potential combination therapies with high therapeutic efficacy, 5 μmol/L sorafenib, 10 μmol/L GW5074, 10 μmol/L PLX7420, and 10 μmol/L L779450 were combined in pairs. Sorafenib combined with either PLX7420 or L779450, and the other combination therapies, only modestly induced cell death (Fig. 1A). However, a combination of sorafenib and GW5074 significantly induced ACHN (95% ± 1.8%) and A498 (78% ± 2.1%) cell death, as compared with all other combination therapies (Fig. 1A and Supplementary Fig. S1B).

The antitumor effect of sorafenib and GW7054 combination therapy (SG-combination therapy) was further tested in vivo. We used immunodeficient mice xenografted with ACHN RCC tumors to test the in vivo antitumor activity and safety of SG-combination therapy (Fig. 1B and Supplementary Fig. S1C). In addition, we generated an orthotopic spontaneous metastatic RCC animal model in order to mimic clinical situations (Fig. 1C and D and Supplementary Fig. S1D and S1E).Luc-ACHN-LL cells with high metastatic capability and lethality were orthotopically injected into a renal capsule. Within 2 weeks after the injection, metastatic lesions were observed under the IVIS Xenogene system. Treatment with the same protocol as the xenografted experiments was started (Fig. 1E). SG-combination therapy significantly suppressed the primary and metastatic lesions, and prolonged the survival of mice (Fig. 1F–H), which remained healthy and active (Supplementary Fig. S1F). Tumors from the mice treated with SG-combination therapy showed severe necrosis compared with tumors from mice treated with monotherapy (Supplementary Fig. S1G).

Sorafenib and GW5074 combination therapy induced C-Raf-dependent mitochondrial dysfunction and ROS generation

Because sorafenib and GW5074 might have cellular targets, other than C-Raf, that mediate the anticancer effects of SG-combination therapy, we examined whether C-Raf was essential for the effectiveness of SG-combination therapy. Wild-type MEF (MEF/C-RafWT) and C-Raf knockout MEF (MEF/C-Raf–/–) were treated with mono- or SG-combination therapy. SG-combination therapy synergistically induced cell death of MEF/C-Raf–/– was observed under SG-combination therapy, which was similar to sorafenib monotherapy (Fig. 2A, left). Knockdown of C-Raf, but not B-Raf, in ACHN cells attenuated cell death induced by SG-combination therapy for 24 hours from 42.2% ± 2.5% to 14% ± 3% (Fig. 2B and Supplementary Fig. S2B).

Surprisingly, SG-combination therapy did not prevent S338 phosphorylation of C-Raf (p-C-RafS338). In addition, the phosphorylation of the major C-Raf downstream pathway components MEK, ERK, and Akt (33–35) was not inhibited by SG-combination therapy in MEF/C-RafWT, MEF/C-Raf–/–, ACHN, and A498 cells (Fig. 2C and Supplementary Fig. S2C). Because the combination therapy still increased p-C-RafS338, the impact of p-C-RafS338 on the therapeutic efficacy of combination therapy was checked (Supplementary Fig. S2D and S2E). Cell death induced by the combination therapy was most prominent in ACHN cells transfected with C-RafS338D (mimicking S338 phosphorylation; Supplementary Fig. S2F and S2G). In contrast, C-RafS338A (mimicking S338 nonphosphorylation) attenuated the cell death of ACHN cells under combination therapy (Supplementary Fig. S2F and S2G). These results indicated that C-Raf was the major target responsible for the cytotoxic effect of SG-combination therapy. However, other novel mechanisms mediated by C-Raf, instead of the canonical Raf signaling pathways, were responsible for the cytotoxic effects of SG-combination therapy.

Because C-Raf plays an important role in mitochondria, mitochondrial function and ROS production were examined under mono- or SG-combination therapy. In MEF/C-RafWT but not in MEF/C-Raf–/–, SG-combination therapy resulted in a 6-fold increase in ROS production, and decreased mitochondrial membrane potential (Fig. 2D and E and Supplementary Fig. S3A–S3D). The immunofluorescence assay clearly demonstrated that mitochondrial fragmentation with donut- and blob-shape formation was induced by SG-combination therapy.
Figure 2.
SG-combination therapy induced cell death via C-Raf dependent mitochondrial dysfunction and ROS production. A, cell death of MEFC-Raf-WT (right) and MEFC-Raf<sup>+/−</sup> (left) at 24, 48, and 72 hours after treatment with sorafenib and GW5074 mono- or SG-combination therapy normalized to DMSO-treated cells (mean ± SEM; **, P < 0.01; n = 4). Clonogenic assay (Wright-Giemsa staining) of MEFC-Raf-WT and MEFC-Raf<sup>+/−</sup> cells was measured after a 7-day treatment with sorafenib and GW5074 mono- or SG-combination therapy (representative photographs; n = 3 for each condition). B, ACHN cells transfected with nontargeting siRNA, B-Raf siRNA, or C-Raf siRNA for 24 hours were treated with or without SG-combination therapy for 24 hours. Cell death was assayed by sulforhodamine B (mean ± SEM; **, P < 0.01; n = 3). (Continued on the following page.)
Sorafenib and GW5074 combination therapy induced two-hit damage of cancer cells through ROS generation and pDAPK<sub>S308</sub> dephosphorylation

Recent studies have suggested that DAPK serves as a mitochondrial sensor, which activates and triggers cell death after dephosphorylation of its S308 by PP2A (Fig. 3A). We observed that S308 phosphorylation of DAPK (pDAPK<sub>S308</sub>) was significantly reduced in ACHN, A498, and MEF<sup>C-Raf-WT</sup> cells after treatment with SG-combination therapy (Fig. 3A and Supplementary Fig. S4A). This reduction in phosphorylation appeared to be dependent upon C-Raf, as SG-combination therapy neither induced profound cell death nor dephosphorylated pDAPK<sub>S308</sub> in MEF<sup>C-Raf−/−</sup> (Fig. 3A). We also observed that PP2A was required for the dephosphorylation of pDAPK<sub>S308</sub> induced by SG-combination therapy, as PP2A inhibitors efficiently suppressed pDAPK<sub>S308</sub> dephosphorylation (Fig. 3B). Meanwhile, PP2A inhibitors also attenuated the cell death induced by SG-combination therapy (Fig. 3C and Supplementary Fig. S4B). The ROS production caused by SG-combination therapy was not rescued by PP2A inhibitors (Fig. 3D), suggesting that the dephosphorylation of pDAPK<sub>S308</sub> by PP2A was a downstream event to the ROS production.

The ROS scavenger NAC and PEG-SOD erased ROS and attenuated the cell death induced by SG-combination therapy for 24 hours from 46.5% ± 5.2% to 13.8% ± 2.6%, and prevented the dephosphorylation of pDAPK<sub>S308</sub> (Fig. 3E and F and Supplementary Fig. S4C and S4D). However, PP2A inhibitors only attenuated the cell death induced by SG-combination therapy from 45.0% ± 3.6% to 20.3% ± 4.1% in ACHN cancer cells, which was less than an attenuation of cell death that caused by the NAC and PEG-SOD ROS scavengers (Fig. 3E).

These results indicated that SG-combination therapy caused two-hit damage in cancer cells. The first hit of the SG-combination therapy induced mitochondrial dysfunction and ROS generation. The second hit was the action of ROS, which facilitated the PP2A-mediated dephosphorylation of pDAPK<sub>S308</sub> and activated its death-causing activities. These sequential events caused by SG-combination therapy contributed to the profound cell death.

**pDAPK<sub>S308</sub> was essential for the anticancer effect of sorafenib and GW5074 combination therapy**

We next wanted to determine whether DAPK or pDAPK<sub>S308</sub> levels were essential for the cytotoxicity caused by SG-combination therapy. We assayed the therapeutic efficacy of SG-combination therapy and compared it with the total DAPK and pDAPK<sub>S308</sub> levels of various cell lines. Normal fibroblasts and epithelial cells had high total DAPK protein, but very low levels of pDAPK<sub>S308</sub>, and SG-combination therapy caused low toxicity to these cells (Fig. 3G, blue bars). Consistent with these results, cell death induced by SG-combination therapy in different cancer cells was positively correlated with pDAPK<sub>S308</sub> levels, but not with total DAPK protein levels (Fig. 3G and H).

We further investigated the therapeutic efficacy of SG-combination therapy on cancer cells derived from clinical patient (RCC-Sor-001; Supplementary Table S1) and animal models (786-O-T4, ACHN-T2R; Supplementary Fig. S5A–S5C) with evasive resistance to sorafenib monotherapy. SG-combination therapy for 24 hours induced significant cell death in these cancer cells (RCC-Sor-001: 34.6% ± 2.1%, 786-O-T4: 31.2% ± 3.2%, ACHN-T2R: 42.3% ± 5.2%), SG-combination therapy also induced cell death in HT29 (34.9% ± 3.7%; ref. 37) and A2058 (44.7% ± 2.8% ref. 38), which have inherited resistance to Raf inhibitors (Fig. 3G, red bar). All of the resistant cancer cells with sensitivity to SG-combination showed high levels of pDAPK<sub>S308</sub>. Importantly, because normal cells have low levels of pDAPK<sub>S308</sub>, SG-combination therapy has the advantage of selective toxicity toward cancer cells.

DU-145 prostate cancer cells expressed nearly undetectable total DAPK and pDAPK<sub>S308</sub> levels, and did not respond to SG-combination therapy (Fig. 3G). We took advantage of DU-145 cells to examine whether pDAPK<sub>S308</sub> (but not DAPK protein) mediated the cell death induced by SG-combination therapy. DU-145 cells overexpressing either DAPK<sup>WT</sup> (wild-type), DAPK<sup>SD308D</sup> (mimicking S308 phosphorylation), DAPK<sup>SD308A</sup> (mimicking S308 nonphosphorylation), and DAPK<sup>K42A</sup> (kinase-dead), were treated with SG-combination therapy. Only DAPK<sup>SD308D</sup> enhanced cell death induced by SG-combination therapy in DU-145 cancer cells (Fig. 3I and Supplementary Fig. S6A). Furthermore, knockdown of DAPK by siRNA attenuated the cell death induced by SG-combination therapy in ACHN, 786-O, and RCC-Sut-002 cells that have DAPK expression and pDAPK<sub>S308</sub> modification (Fig. 3I and Supplementary Fig. S6B). These results indicated that pDAPK<sub>S308</sub> was essential for the cytotoxicity of SG-combination therapy, because only cancer cells with pDAPK<sub>S308</sub> modification responded to the therapy.
Figure 3.
pDAPK$^{308}$ expression positively correlated with the therapeutic efficacy of SG-combination therapy. A, ACHN and MEF cells treated with sorafenib, GW5074, or their combination for 24 hours. Phosphorylation (pDAPK$^{308}$) and expression of DAPK were assayed by immunoblotting. B, ACHN cells treated with SG-combination therapy with or without NAC pretreatment. Total cell lysates were immunoblotted with antibodies against DAPK and pDAPK$^{308}$. C, ACHN cells treated with SG-combination therapy concomitant with or without the PP2A inhibitors cantharidin (C.A.) or okadaic acid (O.A.) for 24 hours; the percentage of cell death was normalized to DMSO-treated cells (mean $\pm$ SEM, $^*P < 0.05$, $^{**}P < 0.01$, $n = 4$). (Continued on the following page.)
Combined sorafenib and GW5074 induced p-C-Raf$^{S338}$ and pDAPK$^{S308}$ cytosolic translocation, mitochondrial dysfunction, and ROS generation

Because SG-combination therapy was mitochondria-dependent, and C-Raf and pDAPK$^{S308}$ were essential for the therapeutic efficacy, we hypothesized that C-Raf and DAPK might be colocalized in the mitochondria. C-Raf and DAPK were localized to both the cytoplasm and the mitochondria in MEFC-C-Raf$^{WT}$. However, DAPK only localized to the cytoplasm in MEFC-C-Raf$^{WT}$ (Fig. 4A). SG-combination therapy caused a redistribution of DAPK and C-Raf from the mitochondria to the cytoplasm in MEFC-C-Raf$^{WT}$, but not in MEFC-C-Raf$^{WT}$ (Fig. 4A). In addition, dephosphorylation of pDAPK$^{S308}$ was observed after its SG-combination therapy-induced cytoplasmic translocation from the mitochondria. The C-Raf protein and its S338 phosphorylation were not affected by SG-combination therapy. The aforementioned results were also noted in cancer cells with pDAPK$^{S308}$ modification, but not in those without (Fig. 4B and Supplementary Fig. S7).

To further investigate the role of pDAPK$^{S308}$ in SG-combination therapy-induced mitochondrial dysfunction, wild-type or mutant forms of DAPK were overexpressed in DU-145 cells that had undetectable endogenous pDAPK$^{S308}$ modification. Only DAPK$^{S308D}$ was induced to translocate from the mitochondria to the cytoplasm, concomitant with mitochondrial dysfunction and ROS generation in DU-145 cells under SG-combination therapy [Fig. 4C and D (red rectangle), E and Supplementary Fig. S8]. Knockdown of DAPK also attenuated mitochondrial dysfunction and ROS generation induced by SG-combination therapy (Fig. 4F). These results indicated that C-Raf directed DAPK to become colocalized to the mitochondria, and that pDAPK$^{S308}$ was essential for the selective toxicity of SG-combination therapy-mediated mitochondrial dysfunction and ROS generation in cancer cells.

Sorafenib and GW5074 combination therapy disassembled the C-Raf, DAPK, and PP2A complex in the cytoplasm

Because pDAPK$^{S308}$ played an important role in mediating SG-combination therapy-induced mitochondrial dysfunction, the role of pDAPK$^{S308}$ in C-Raf-mediated mitochondrial function was investigated. Phorbol 12-myristate 13-acetate (PMA) was shown to activate C-Raf for the regulation of mitochondrial remodeling (3). PMA treatment induced striking mitochondrial remodeling with fragmented mitochondria and perinuclear clustering (3). PMA treatment induced signiﬁcantly higher mitochondrial remodeling in DU-145 prostate cancer cells overexpressing wild-type or DAPK mutants were treated with PMA. PMA induced significantly higher mitochondrial remodeling in DU-145 prostate cancer cells with DAPK$^{S308D}$ expression, as compared with the other DAPK versions (Fig. 5A and B and Supplementary Fig. SSA and SSB). These results suggested that pDAPK$^{S308}$ cooperated with C-Raf to regulate physiologic cellular function by enhancing mitochondrial remodeling.

Previous studies showed that PP2A interacts with C-Raf and DAPK (21, 39). Immunoprecipitation (IP) assays showed that C-Raf interacted with DAPK, both in the cytoplasm and in the mitochondria (Fig. 5C and Supplementary Fig. S5C). C-Raf and DAPK also interacted with PP2A to form a complex in the cytoplasm. SG-combination therapy reduced the interaction of PP2A with DAPK and C-Raf, concomitant with the reduction of pDAPK$^{S308}$ (Fig. 5D). The IP assay also revealed that DAPK$^{S308A}$ had a weaker interaction with PP2A than did DAPK$^{WT}$, DAPK$^{S308A}$, and DAPK$^{K42A}$. SG-combination therapy further enhanced PP2A dissociation from DAPK$^{S308D}$ (Fig. 5E and Supplementary Fig. S5D).

pDAPK$^{S308}$ served as a good prognostic biomarker for RCC in the clinics

Because the pDAPK$^{S308}$ levels were correlated with the therapeutic efficacy of SG-combination therapy, we investigated the levels of pDAPK$^{S308}$ in human cancer tissues. Twenty pairs of RCC tumor samples and their normal matched counterparts were examined. By Western blot analysis, 16 out of 20 tumor tissues showed high pDAPK$^{S308}$ levels compared with their normal matched counterparts (Fig. 6A and B). Immunohistochemical (IHC) analysis of 181 human RCC specimens with tissue arrays showed that pDAPK$^{S308}$ level was remarkably higher in cancer tissues than in normal renal parenchyma (Fig. 6C). There was no difference in pDAPK$^{S308}$ level related to tumor grade or clinical stage (Fig. 6D).

The Kaplan–Meier analysis of patients with survival data revealed that the high-level of pDAPK$^{S308}$ positively correlated with poor disease-free survival and poor overall survival (Fig. 6E and F and Supplementary Table S2). These results demonstrated that pDAPK$^{S308}$ could serve as a good prognostic biomarker for RCC. It is worthy to investigate whether pDAPK$^{S308}$ can serve as a predictive biomarker in the treatment of RCC with SG-combination therapy in clinics.

The three-dimensional structure of sorafenib and GW5074 binding to C-Raf predicted how they induced C-Raf/pDAPK$^{S308}$ cytosolic translocation

Computerized simulation was performed to evaluate the structure of C-Raf bound with different Raf inhibitors. The predicted maximal binding energy of inhibitors in complexes with a C-Raf kinase domain was most prominent in the combination of GW5074 and sorafenib (~182 kcal/mol), compared with other Raf inhibitors. Prior docking of GW5074 led to a deep binding of this compound into a hydrophobic pocket formed by Ile355,

(Continued)
Val363, Ala373, Leu406, Trp423, and Phe475. More of the extended area was occupied by GW5074 and sorafenib in the binding pocket (Fig. 7A and Table 1).

The conformation for DAPKWT and DAPKS308D in complex with C-Raf was derived from the poses with the best E_Rdock of 18.24 and 2.41 kcal/mol, respectively (Fig. 7B). In this binding manner, there were a total of nine hydrogen bonds and six salt bridges formed between DAPKWT and C-Raf, but only seven hydrogen bonds and four salt bridges were formed between DAPKS308D and C-Raf. Interaction of DAPKWT with C-Raf showed an antiparallel sheet (strand 3 and 4) of C-Raf protruding into the binding pocket of DAPK, leaving the N-terminal and C-terminal ends on the protein surface. The –OH group in S308 of DAPK interacted with the OE2 group in Glu125 of C-Raf at the interface.

SG-combination therapy induced mitochondrial dysfunction through pDAPK308 cytosolic translocation and dephosphorylation. A, MEF cells treated with or without SG-combination therapy for 24 hours were fractionated into cytosolic and mitochondrial fractions and then immunoblotted with indicated antibodies. Anti-VDAC1 and anti-α-tubulin antibodies were run to distinguish between mitochondrial and cytosolic fractions, respectively. B, immunoblotting with the indicated antibodies for cytoplasmic and mitochondrial fractions of ACHN and DU-145 cells under vehicle, mono-, or SG-combination treatments for 24 hours, respectively. C, DU-145 cells were stably transfected with wild-type and DAPK mutants, followed by treatment with SG-combination therapy for 24 hours. The cells were stained with CM-H2DCFDA (left) or JC-1 (right) for 30 minutes, respectively. The ROS generation (left) and mitochondrial potential decrease (right) was analyzed and quantitated by flow cytometry (*, P < 0.05; **, P < 0.01). D, DU-145 cells transfected with VS-tagged-vector, VS-tagged-DAPK, or the indicated mutants were treated with DMSO (control) or SG-combination therapy. The cells were fractionated into cytosolic and mitochondrial fractions, and protein translocation was analyzed by immunoblotting with the indicated antibodies. E, DU-145 cells stably transfected with vector, wild-type, or DAPK mutants and treated with DMSO or SG-combination therapy for 24 hours. Cells were stained with mitochondria antibody and nuclear marker DAPI and imaged with fluorescence microscopy. Scale bar, 20 μm. F, ACHN cells transfected with nontargeting or DAPK siRNAs for 24 hours were treated with SG-combination therapy for 24 hours. The cells were stained with CM-H2DCFDA or JC-1 for 30 minutes, and ROS generation (left) or mitochondrial potential decrease (right) was analyzed by flow cytometry (*, P < 0.05; **, P < 0.01).
of the protein complex. In contrast, the binding manner for C-Raf in the DAPK308D–C-Raf complex model was different. Asp substitution in residue 308 of DAPK changed the interaction between the two protein interfaces, which led C-Raf to adopt an alternative stable conformation in which the position of the antiparallel sheet in the N-terminus of C-Raf was switched to interact with the binding pocket of DAPK.

These results demonstrated that the combination of GW5074 and sorafenib only induced the conformational change in C-Raf in the presence of pDAPK308D. This change compromised the mitochondrial targeting effect of the N-terminal domain of C-Raf, which led C-Raf to adopt an alternative stable conformation in which the position of the antiparallel sheet in the N-terminus of C-Raf was switched to interact with the binding pocket of DAPK.

This study revealed that pC-RafS338 interacted with pDAPK308 and directed it to become colocalized in the mitochondria. GW5074 and sorafenib combination therapy (SG-combination therapy) resulted in the translocation of pC-RafS338 and pDAPK308 from the mitochondria to the cytoplasm, concomitant with a decrease in mitochondrial membrane potential and increase in ROS generation. These results suggested that pC-RafS338 cooperated with pDAPK308 in the regulation of mitochondrial function. DU-145 prostate cancer cells with ectopic DAPK308D expression were much more sensitive to PMA induced C-Raf–mediated mitochondrial remodeling, as compared with cells ectopically overexpressing DAPKWT, DAPKK42A or DAPKS308A. This conclusion is supported by the observation that only DAPK308D, but not DAPKWT, DAPKK42A, and DAPKS308A, could be induced to translocate from the mitochondria to the cytoplasm by SG-combination therapy, subsequent to the induction of mitochondrial dysfunction and ROS generation.

**Discussion**

Nonselective off-target mitochondrial toxicity is a major contributor to the failure of chemotherapeutic agents in clinical practice (40, 41). Recent studies of mitochondrial involvement in cancer have uncovered a plethora of differences in the structure, genome, and function of these organelles, by comparing metastatic mitochondria with those from nontransformed cells. However, there are limited therapeutic strategies selectively targeting cancer cell mitochondria (42, 43).
The N-terminal domain of C-Raf is required for its mitochondrial localization (3). Our computer simulation results demonstrated that only in the presence of pDAPK308, but not wild-type or other DAPK mutants, sorafenib and GW5074 bound to C-Raf and induced a conformational change of the N-terminal domain, which compromised its mitochondrial targeting capability. This indicated that sorafenib and GW5074 served as C-Raf allosteric modulators, instead of Raf inhibitors, and provides a platform for the development of new allosteric modulators in the future.

Therefore, the detailed mechanisms involved in the regulation of mitochondrial function by pDAPK308 and C-Raf are biologically important and worthy of further investigation.

The generation of ROS by SG-combination therapy promoted the PP2A-mediated dephosphorylation of S308 in DAPK. It has been shown that the synthetic retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), induces ROS production in human leukemia cells (44). The 4-HPR-mediated ROS evokes Akt conformation change by forming an intramolecular disulfide bond. Akt is subsequently dephosphorylated at Thr308 and Ser473 by PP2A (45). Whether this mechanism occurs in this study warrants further evaluation.

Other important issues regarding DAPK translocation and its phosphorylation-based regulation need to be explored. Because HSP90 and 14-3-3 are two chaperone proteins that interact with and regulate C-Raf phosphorylation status and function (46, 47), it would be interesting to determine whether these two proteins are also responsible for the regulation of DAPK translocation and phosphorylation. In addition to S308 phosphorylation of DAPK, other posttranslational phosphorylation sites also affect its kinase activity (22). This provides a potential explanation for why normal cells and some cancer cells expressing very low levels of pDAPK308 did not display cell death. The DAPK kinase inactivation in those cells might be attributed to other inhibitory phosphorylation sites. However, why the S308 residue of DAPK is phosphorylated in certain types of cancer and whether S308 phosphorylation of DAPK is due to autophosphorylation or other kinases warrant further studies.

The expression levels of pDAPK308 in cancer cells or tumor tissues are crucial for SG-combination therapy efficacy in terms of clinical cancer treatment. The detection of pDAPK308 from cancer tissues would be a prerequisite before the application of SG-combination therapy. There are higher expression levels of pDAPK308 in RCC tumor tissues than in normal renal parenchyma, regardless of tumor grade or clinical stage. These findings demonstrate that pDAPK308 is an ideal predictive biomarker for SG-combination therapy in clinical practice, because only a single factor needs to be assayed in tumor tissues obtained from either the primary or metastatic lesions. Moreover, because pDAPK308 also mediated the anticancer effects of SG-combination therapy by targeting mitochondrial function instead of the C-Raf signaling pathway, it is not necessary to check upstream Ras or Raf mutation status, or other
Computer simulation and molecular mechanism of C-Raf and DAPK with SG combination therapy. A, comparison of structures simulated for C-Raf kinase domain in complex with sorafenib and GW5074. The structure of the protein is presented as the surface model. Sorafenib and GW5074 are shown as stick models and colored by magenta and green, respectively. B, the wild-type and S308D-mutant DAPK in the docked protein complex are presented as a surface model colored by interpolated charge. C-Raf is shown as a ribbon model and colored by cyan. The N-terminal antiparallel β-sheet (residues 55–75) is highlighted by magenta color. The conformation for wild-type and mutant DAPK in complex with C-Raf were derived from the poses with the best E_Rdock of −18.24 and −2.41 kcal/mol, respectively. C, schematic view of combined sorafenib and GW5074 inducing two-hit cell damage in sequential events. Left, in the presence of pDAPK3308, sorafenib and GW5074 binding induces C-Raf conformational change, compromising its N-terminal domain mitochondrial targeting effect, and leading to C-Raf/pDAPK3308 cytosolic translocation. Right, cytosolic translocation of C-Raf/pDAPK3308 induces mitochondrial dysfunction and ROS generation (first hit), and triggers PP2A-mediated dephosphorylation and activation of pDAPK3308 (second hit).
Table 1. Binding energy of Raf inhibitors in complex with C-Raf

<table>
<thead>
<tr>
<th>Name Structure</th>
<th>Binding energy, kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMS2</td>
<td>-76</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>-82</td>
</tr>
<tr>
<td>+ L-779450</td>
<td>-134</td>
</tr>
<tr>
<td>+ GW5074</td>
<td>-125</td>
</tr>
<tr>
<td>+ PLX44720</td>
<td>-113</td>
</tr>
<tr>
<td>Gw5074</td>
<td>-128</td>
</tr>
<tr>
<td>+ Sorafenib PLX44720</td>
<td>-182</td>
</tr>
<tr>
<td>+ Sorafenib L-779450</td>
<td>-140</td>
</tr>
<tr>
<td>+ Sorafenib</td>
<td>-125</td>
</tr>
</tbody>
</table>

compensatory pathways responsible for the inherited or evasive resistance to Raf inhibitor monotherapy.

The animal model of orthotopic spontaneous RCC metastases shows high metastatic capability of the kidneys, liver, and brain within 4 weeks, and lethality within 6 to 8 weeks. The animal model not only provides an ideal preclinical model to demonstrate the synergistic anticancer effect of SG-combination therapy in vivo, but can also be used to elucidate the underlying mechanisms responsible for the high metastases and lethality of phenotypes. Resistant cancer cell lines derived from clinical patients or animal models under sorafenib or sunitinib monotherapy may also serve as important research tools for illustrating the underlying mechanisms involved in evasive resistance to anti-VEGFR tyrosine kinase inhibitors.

Raf inhibitor monotherapy induced the S338 phosphorylation of C-Raf and promoted tumor progression (13). Therefore, allosteric inhibitors were developed to prevent S338 phosphorylation of C-Raf for cancer treatment in a preclinical study (48). SG-combination therapy might also induce C-Raf conformational changes by allosteric regulation mechanisms, while still inducing the S338 phosphorylation of C-Raf. Our simulation program predicted two important issues: first, GW5074 bound to C-Raf and induced the conformational change of C-Raf, which enhanced the binding affinity of sorafenib to C-Raf; and second, only in the presence of pDAPK<sup>308</sup> did sorafenib and GW5074 bound to C-Raf alter its N-terminal conformation, which likely compromised the C-Raf mitochondrial targeting effect. The biologic function of the mitochondrial C-Raf/DAPK complex and its allosteric regulation by Raf inhibitors and other potential chemicals need further investigation.

Our animal model showed that only 20% of regular sorafenib dosage combined with GW5074 efficiently suppressed primary and metastatic tumors in vivo. This could provide an advantage to reduce the dose-related side effects of sorafenib in clinical practice (49). This finding also raises an important question as to whether the sequence of drug application of GW5074 and sorafenib has any impact on the therapeutic efficacy of SG-combination therapy before its clinical translation. The in vitro data show that the addition of GW5074 followed by sorafenib has the advantage of stronger growth inhibition of cancer cells within 24 hours. However, there is no difference in growth inhibition in terms of the sequence of drug application of GW5074 and sorafenib when treating cancer cells over 24 hours (data not shown). The impact on the sequence of drug application of sorafenib and GW5074 in clinical patients needs further investigation in thoughtfully designed clinical trials.

This study demonstrated the effectiveness of a novel combination therapy with sorafenib and GW5074 for cancer therapy. These mechanisms by which these two inhibitors interacted with their target protein, C-Raf, provided an ideal model for a new drug designation. This study also illuminated a unique interplay among C-Raf, DAPK, and PP2A in regulating mitochondrial function in cell death cascades. The safety issue of SG-combination therapy is highly promising due to its selective toxicity toward cancer cells over normal cells. This study also identified pDAPK<sup>308</sup> as a biomarker for predicting the therapeutic efficacy of SG-combination therapy to prevent unnecessary treatments. A spontaneous metastatic animal model used to mimic human cancer disease demonstrated the anticancer efficacy of combination therapy in vivo. This study shows a way to overcome obstacles encountered in the current cancer therapeutics, and fulfills the criteria of an ideal preclinical therapeutic model worthy of further clinical translation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.-T. Tsai, M.-J. Chuang, G.-H. Sun, C.-C. Lin, S.-Y. Chang
Development of methodology: Y.-T. Tsai, M.-R. Chen, C.-C. Lin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-T. Tsai, S.-T. Wu, Y.-C. Chen, C.-P. Yu, I.-Y. Ho, V.C. Lin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-T. Tsai, S.-H. Tang, G.-H. Sun, S.-M. Huang, H.-J. Lee, C.-C. Lin
Writing, review, and/or revision of the manuscript: Y.-T. Tsai, S.-H. Tang, G.-H. Sun, P.-W. Hsiao, S.-M. Huang, D.-S. Yu, T.-L. Cha
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-T. Tsai, M.-J. Chuang, S.-M. Huang, M.-R. Chen
Study supervision: H.-K. Lin, S.-Y. Chang
Other (performed the experiments): C.-C. Lin

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