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Small molecule inhibition of the CHFR-PARP1 interaction as novel approach to overcome intrinsic taxane resistance in cancer

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ABSTRACT

The mitotic checkpoint protein CHFR has emerged as a major mediator of taxane resistance in cancer. Here we show that CHFR’s PAR-binding zinc finger domain (PBZ) mediates a protein interaction with poly-ADP ribosylated PARP1 leading to stabilization of CHFR. Disruption of the CHFR-PARP1 interaction through either PARP1 shRNA-mediated knockdown or overexpression of a PBZ domain peptide induces loss of CHFR protein expression. In an attempt to exploit this observation therapeutically, and to develop compounds with synthetic lethality in combination with taxanes, we performed a high-throughput computational screen of 5,256,508 chemical structures against the published crystal structure of the CHFR PBZ domain to identify candidate small molecule CHFR protein-protein interaction inhibitors. The 10 compounds with the best docking scores (< –9.7) were used for further in vitro testing. One lead compound in particular, termed ’A3’, completely disrupted the protein-protein interaction between CHFR and PARP1, resulting in the inhibition of mitotic checkpoint function, and led to therapeutic synergy with docetaxel in cell viability and colony formation assays. In mouse xenografts, i.p. administration of ’A3’ led to a significant reduction in nuclear CHFR protein expression with a maximal effect 4 hours after administration, confirming relevant pharmacodynamics following the peak of ’A3’ plasma concentration in vivo. Furthermore, combination of A3 and taxane led to significant reduction of implanted tumor size without increase in hematological, hepatic or renal toxicity. These findings provide a proof-of-principle that small molecule inhibition of CHFR PBZ domain interaction is a novel potential therapeutic approach to increase the efficacy of taxane-based chemotherapy in cancer.

INTRODUCTION

Microtubular-targeted chemotherapy agents such as taxanes are among the most widely prescribed first- and second-line chemotherapy choices for patients with the most common malignancies including lung-, breast-, and prostate cancer. Unfortunately, primary resistance to taxanes is common and poses an important clinical problem. The mitotic checkpoint gene “checkpoint with forhead and ringfinger domains” (CHFR) has recently emerged as a critical mediator of resistance against microtubular-targeted therapies in various different cancer types including gastric-[1], colon-[2, 3], endometrial-[4, 5] and lung cancer [6]. Epigenetic silencing of CHFR by promoter hypermethylation or reduced protein expression have been described as predictive biomarkers for taxane sensitivity. We have previously reported that advanced lung cancer patients with CHFR deficient lung cancers have remarkably high clinical benefits rates following taxane-based first line chemotherapy (81% vs 48%, \( p = 0.03 \)) and significantly improved overall-survival (HR = 0.24; 95% CI, 0.1–0.58%; \( P = 0.002 \)) suggesting that in this setting, taxanes can be
RESULTS

PBZ mutant CHFR fails to induce taxane resistance in CHFR deficient NSCLC cell lines

Transfection of wt-CHFR into CHFR deficient cells has previously been shown to restore the antephase checkpoint leading to a pre-mitotic cell cycle arrest after taxane challenge and ultimately to confer de-novo resistance to taxanes [8]. In Hela cells, it was suggested that full length, but not PBZ-mutant CHFR has similar cell cycle effects [12]. To determine the functional relevance of the PBZ domain on taxane resistance in NSCLC, we transfected CHFR deficient CALU-6 cells either with full-length CHFR (pDEST40-wt-CHFR) or PBZ mutant CHFR (pDEST40-CHFR-PBZ*). Cell viability assays showed that only transfection of wt-CHFR confers resistance to taxanes when compared to both transfection of empty vector or the PBZ mutant variant (Figure 1A). These findings highlight the importance of an intact PBZ domain for an intact checkpoint function and CHFR mediated taxane resistance, since the CHFR-PBZ* construct did not affect taxane sensitivity compared to empty vector. CHFR protein levels are shown in Supplementary Figure S1A.

The CHFR PBZ domain mediates interactions with parylated PARP1

To determine possible interactions between CHFR and other proteins that depend on an intact PBZ domain, we transfected the empty pDEST40 vector, pDEST40-wt-CHFR or pDEST40-CHFR-PBZ* vectors into HEK293 cells and performed immunoprecipitation with V5-tagged CHFR. Western blot analysis for poly ADP ribosylated (PAR) proteins revealed one prominent band at ~130kDa interacting only with wt-CHFR but not CHFR-PBZ*, suggesting an interaction with one major species of PARylated protein. Given that the molecular weight of parylated PARP1 is approximately 130 kDa, we performed a western blot for PARP1, which confirmed an interaction between CHFR and PARP1 (Figure 1B). Since protein expression of a PBZ mutant CHFR construct is unstable, we transfected either wt-CHFR or CHFR-PBZ constructs with an additional RING domain. These constructs show preserved and equal protein expression. Mutation of the CHFR-PBZ domain abolished the interaction between CHFR and PARP1, indicating that the interaction requires an intact PBZ domain (Figure 1C). The reciprocal interaction between PARP-1 and CHFR was also detected in HEK 293 cells expressing GST tagged wt-PARP-1 (Figure 1D). To determine if the interaction between CHFR and PARP1 is parylation dependent or independent, we generated a catalytically dead mutant of PARP1 (E988K), which completely lacks the ability to synthesize PAR [20]. Only wt-PARP1 showed an interaction with CHFR (Figure 1D), suggesting that the interaction between these two proteins is parylation-dependent. Taken together, these data indicate that CHFR preferentially interacts with the parylated form of PARP-1, and that this interaction is mediated by the CHFR PBZ domain.
The interaction with PARP1 stabilizes CHFR protein

We next determined the impact of CHFR on PARP1 stability and vice versa. shRNAs were used to generate stable knockdowns in HEK293 cells. CHFR knockdown led to an increase in PARP-1 protein expression (Figure 1E). This phenomenon has previously been attributed to the decrease in proteasomal degradation of PARP1. Conversely and interestingly, PARP1 knockdown led to a significant reduction in CHFR protein expression (Figure 1F). These findings suggest that the interaction with PARP1 stabilizes the CHFR protein, possibly by protecting it from degradation. If the interaction with PARP1 protects CHFR from degradation, then CHFR protein levels should be reduced by competing for this
interaction. To test this hypothesis, we overexpressed a Flag-tagged PBZ domain alone in HEK293 cells. Indeed, CHFR protein levels were reduced in cells expressing the PBZ domain as compared to those transfected with empty vector (Figure 1G), supporting the above hypothesis. Together, these results demonstrate that CHFR protein is indeed stabilized by its interaction with PARP1 which is mediated by CHFR’s PBZ domain.

CHFR degradation in PBZ mutant CHFR is mediated by autoubiquitination

Regulation of CHFR protein levels has previously been shown to be at least in part dependent on autoubiquitination mediated by its RING domain [21]. To determine if disruption of the CHFR-PARP1 interaction forces CHFR degradation by auto-ubiquitination and subsequent proteasomal degradation, we expressed wild-type and the CHFR-PBZ mutant in the presence and absence of the proteasome inhibitor MG-132 (Figure 1H) or after the additional depletion of the RING domain (Figure 1I) in Calu-6 cells. While the CHFR PBZ mutant protein is only expressed at very low levels, both inhibition of the proteasome or deletion of the RING domain restored PBZ mutated CHFR expression to levels that were comparable to those of the wt-CHFR. These data are consistent with the interpretation that disruption of the CHFR-PARP interaction results in auto-ubiquitination and degradation of CHFR. Pharmacologic inhibition of PARP1’s polymerase activity by the PARP-inhibitor ABT-888 did not result in altered CHFR protein levels (Figure 1H).

The CHFR-PARP1 interaction is cell cycle dependent and enhanced by docataxel induced mitotic stress

We next sought to determine the functional significance of the interaction between CHFR and PARP1 in antephase checkpoint control and taxane sensitivity. HEK293 cells were transfected with a GST-tagged PARP1, synchronized with aphidocholin, and the interaction between GST-tagged PARP1 and CHFR was analyzed at various stages of the cell cycle (Figure 2A, 2B, Supplementary Figure S3). The interaction between PARP1 and CHFR was limited to the G2/M phase of the cell cycle and was further enhanced by additional exposure to mitotic stress, suggesting a specific role of this interaction in the control of mitotic entry. To determine the effect on taxane sensitivity, we performed XTT assays in A549 cells stably transfected with either shRNAs specifically targeting CHFR or PARP1 or control shRNAs (scrambled or luciferase targeting respectively, Supplementary Figure S1B and S1C). As expected, CHFR- deficient A549 cells showed an increased sensitivity to docetaxel relative to A549 cells expressing a scrambled control (Figure 2C). Interestingly, PARP1 deficient cells also showed increased sensitivity to docetaxel (Figure 2D), likely due to the reduction in CHFR expression that accompanies PARP1 knockdown (Figure 1G). These findings show that not only CHFR is required for a functional mitotic checkpoint, but that additionally PARP1 is also an important mediator of taxane resistance.

PARP-inhibition fails to induce synergistic cytotoxicity in CHFR expressing lung cancer cell lines

Hypothesizing that PARP-inhibition could induce synergistic cytotoxicity in CHFR expressing lung cancer cell lines, we performed cytotoxicity assays in seven lung cancer cell lines (A549, EKVX, H596, H522, Hop-62, H460 and H2023) using docetaxel and the PARP-inhibitor ABT-888 either alone or in combination. Synergy was not observed in any of the cell lines. Also, combination of docetaxel with a dose of ABT-888 (20 μM), which achieves PARP inhibition in vitro, did not lead to enhanced cytostasis (data not shown). In vitro, ABT-888 did not reduce CHFR protein expression (Figure 1H) nor did it disrupt the interaction between CHFR and PARP-1 (not shown), suggesting that PARP-1 inhibition is insufficient to force CHFR into auto-degradation. We therefore hypothesized that the development of a pharmacologic strategy that mimics the effects of a PBZ domain mutation and completely inhibits the binding of parylated proteins in the PBZ domain may be superior to PARP-1 inhibition in the sensitization against taxanes.

High-throughput computational screening identifies a small molecule inhibitor of the CHFR/PARP1 interaction

In an attempt to generate possible lead compounds for the pharmacologic inhibition of the CHFR-PARP1 interaction, we performed a high throughput computational screen of 5,256,508 chemical structures in the MCULE database of purchasable compounds using the AutoDockVina algorithm against the published crystal structure of the CHFR-PBZ domain. The 10 most promising ‘hits’ based on Autodock Vina docking scores (< –9.7) were selected for further characterization (Supplementary Figure S2). One compound (A9) failed to synthesize. We tested the 9 available compounds for their impact on nuclear CHFR levels (Figure 3A). Several compounds, including ‘A2’, ‘A3’, ‘A4’ and ‘A6’ decreased CHFR levels assayed by western blotting of whole cell extracts. We determined the impact of the above referenced compounds on the PARP1/CHFR interaction. HEK293 cells were transfected with GST tagged PARP1 and the interaction with CHFR determined in pull down assays for those 4 compounds. Only ‘A3’ completely prevented the PARP1-CHFR interaction at 10 μM concentration (Figure 3B). Further
studies therefore focused on ‘A3’. Chemical structures are shown in Figure 3C and 3D. In contrast to treatment with ABT-888, ‘A3’ completely disrupted the interaction between GST-tagged PARP-1 and CHFR (Figure 3B). Importantly, In vitro parylation studies showed that ‘A3’ has no impact on PARP1 auto-parylation, indicating that the disruption of the PARP1/CHFR interaction by ‘A3’ is not dependent on de-parylation of PARP1, but rather the interaction between CHFR and parylated PARP1 (Supplementary Figure S4). Since it is possible that the co-immunoprecipitation between PARP-1 and CHFR after ‘A3’ treatment was negative due to the fact that CHFR was degraded, we repeated the experiment after transfection with the Ring-domain deleted ∆R-CHFR construct, which maintained both stable CHFR and PARP1 levels even after treatment with ‘A3’. However, ‘A3’ treatment resulted in a dose dependent inhibition of the interaction between CHFR and PARP1, suggesting a specific inhibitory effect of ‘A3’ on this protein interaction (Figure 3E). These observations were confirmed by data that show a dose dependent regulation of CHFR protein levels by ‘A3’ (Figure 3F). Interestingly, however, at low concentrations of ‘A3’ an initial increase in CHFR protein levels was observed, followed by complete disappearance of CHFR at the 10 uM concentration. Since CHFR’s mitotic checkpoint function is predominantly executed in the nucleus, we next studied the effects of ‘A3’ treatment on nuclear localization of CHFR. ‘A3’ in combination with MG-132 lead to a depletion of nuclear CHFR and accumulation of cytoplasmic CHFR, suggesting the possibility that ‘A3’ may indeed be capable of disrupting the antephase checkpoint (Figure 3G).

Cell cycle arrest at G2/M in response to microtubular damage is a hallmark of a functional antephase checkpoint. To test the functional relevance of our newly discovered CHFR small molecule inhibitor ‘A3’, we exposed unsynchronized A549 cells for 12 hours to docetaxel (50 nM)
with or without ‘A3’ (10 uM) and analyzed the cell cycle stages by flow-cytometry (Figure 3H and Supplementary Figure S5). ‘A3’ exposure prevented docetaxel induced cell cycle arrest, suggesting that ‘A3’ functionally disrupted the antephase checkpoint. This is an important finding since it validates the proposed mechanism of action for ‘A3’.

Exposure to ‘A3’ has synergistic growth inhibitory activity that correlates with CHFR expression

Similarly to the previous experiments in which CHFR knockdown increased taxane sensitivity in A549 cells, ‘A3’ and docetaxel showed synergistic growth inhibitory functions in cell viability assays as demonstrated by a Combination index (CI) < 1 (Figure 4A and 4B). To determine if the synergistic effects of A3 are indeed dependent on an interaction between CHFR and PARP1, we performed colony formation assays in A549 cells transfected with shRNA which was either scrambled or directed against CHFR or PARP1. Synergy between A3 and docetaxel was maintained only in the A549 scramble cell line, but not after CHFR or PARP1 knockdown, suggesting that ‘A3’s effects are mainly through its on-target effects on the CHFR-PARP1 interaction (Figure 4D). Finally, we performed colony formation assays on a panel
of cell lines of different cancer types such as lung-, breast-, and colon cancer. With the exemption of the EKVX NSCLC cell lines, synergy between ‘A3’ and docetaxel was observed for all other CHFR expressing cell lines regardless of tumor type supporting the possibility that CHFR targeted therapy may have a wide range of possible applications in those cancer types in which taxanes are frequently used (Figure 4C and 4E).

**In vivo characterization of A3 levels and activity**

In the previous section we have demonstrated that ‘A3’ is capable of disrupting the interaction between CHFR and PARP1 *in vitro*, leading to a depletion of CHFR expression and to sensitization towards the cytotoxic effects of taxanes. We next examined the impact of ‘A3’ administration on lung tumor xenografts. A549 NSCLC cells were injected into the flank of nude mice and allowed to establish for approximately 14 days. Once tumors had reached a volume about 100 mm³, mice received a single ip injection of ‘A3’ at 10 mg/kg. Mice were then euthanized at 1, 2, 4 and 24 hours after and CHFR in the xenografts was analyzed by immunohistochemistry (Figure 5A). Significant suppression of nuclear CHFR expression was observed after 4 hours, but was restored to baseline levels at the 24 hr time point.
We next sought to characterize the in vivo properties of ‘A3’. We first determined the pharmacokinetics of ‘A3’ after a single oral (25 mg/kg) and intravenous (10 mg/kg) dose of in CD1 mice. Plasma was obtained at pre-specified time points (15 min to 24 hours) and ‘A3’ concentrations were determined by LC/MS/MS. These data indicated that ‘A3’ was rapidly and widely distributed following intravenous dosing, and the relative bioavailability of ‘A3’ from dose normalized AUC values was calculated as 19.5% (Figure 5B). Oral bioavailability of ‘A3’ was insignificant. The time-point of maximal CHFR suppression at 4 hrs follow the peak in A3 plasma levels with some delay.

Based on these pharmacodynamics and pharmacokinetic properties of ‘A3’, we analyzed the in-vivo effects of ‘A3’, docetaxel and the combination on tumor growth in mouse xenografts, who were previously injected with the H460 cell line. Pre-treatment with either DMSO or ‘A3’ was given 4 hours prior to docetaxel injection, when CHFR expression levels are expected to be the lowest based on the above pharmacodynamic experiments. ‘A3’ treatment alone did not have a discernable effect on tumor growth when compared to DMSO alone. However, in combination with docetaxel, ‘A3’ significantly decreased tumor size compared to docetaxel alone, demonstrating that pharmacologic targeting of the CHFR/PARP interaction may indeed be a promising strategy to improve taxane sensitivity in lung cancer.

Importantly, combination treatment with ‘A3’ was not associated with a significant increase in hematologic (Figure 6A), hepatic (Figure 6B) and renal toxicity (Figure 6C) 10 days after treatment. Representative H&E stained sections of liver and kidneys did not show any toxicity in any of the four treatment arms (Figure 6D).
DISCUSSION

We have shown in this study, that the interaction between CHFR and parylated PARP1 stabilizes CHFR protein levels. We furthermore show that this interaction is mediated by CHFR’s PBZ domain and that its disruption either by mutation of the PBZ domain or PARP1 knockdown leads to auto-ubiquitination and subsequent proteasomal degradation of CHFR. Together with previously published results that reduced CHFR expression in lung cancer is associated with improved survival following platinum taxane based therapy [6] and that taxane sensitivity is increased in gastric [1]-, colon [2]- and cervical cancers [22] in which CHFR is silenced epigenetically, we proposed a model in which pharmacologic inhibition of the CHFR-PARP1 interaction with subsequent loss of CHFR and disruption of antephase checkpoint function helps to overcome intrinsic taxane resistance across a wide spectrum of different tumor types (Figure 7).

This is the first report to demonstrate the feasibility of inhibiting the mitotic checkpoint by targeting the interaction between CHFR and PARP1 with a small molecule. Our data provide several lines of evidence to demonstrate specific target inhibition: First, we show that A3 inhibits the biochemical interaction between PARP1 and CHFR. Second, we find that ‘A3’ treatment leads to the functional disruption of the docataxel-induced mitotic checkpoint, a point in the cell cycle in which the CHFR-PARP1 interaction is greatest. Third, we establish that ‘A3’ synergizes with docetaxel only in CHFR expressing cell lines. Finally, we demonstrate that ‘A3’ administration results in a pharmacodynamic reduction in CHFR expression in vivo in human tumor xenograft models.

Our data show clearly that unlike ‘A3’, the PARP1 inhibitor ABT-888 is not capable of disrupting the interaction between CHFR and PARP1. This is surprising since the enzymatically inactive PARP1-E988K mutant does not interact with CHFR. This observation may be explained by the fact that pharmacologic PARP1 inhibition prevents previously un-parylated PARP-1 from autoparylation and may prevent a future interaction with CHFR. Previously parylated PARP-1 however may remain bound to CHFR and PARP inhibition unlike direct small molecule targeting of the PBZ domain will do nothing to force it out of this interaction.

Figure 6: Nude mice were treated with a one-time dose of docetaxel either in combination with ‘A3’ (10 mg/kg), or with equal volumes of DMSO. No significant increase in A. hematologic B. hepatic or C. renal toxicity was observed with combination treatment. H&E staining of mouse kidney and liver samples treated with indicated compounds are unremarkable.
The finding that CHFR protein levels initially increase at low concentrations of ‘A3’ is of potential relevance. The exact explanation for this observation is not entirely clear, but it is interesting to note that this finding was only observed in experiments where CHFR was amendable to proteasomal degradation. Moreover, we did also observe that ‘A3’ exposure enhanced cell proliferation in several lung cancer cell lines including A549 cells. Since CHFR protein is stabilized in G2, we hypothesize that two competing mechanisms are involved in response to ‘A3’: At lower concentrations, a cell cycle mediated increased interaction with PARP-1 may protect CHFR from proteasomal degradation, while at higher concentrations, ‘A3’ completely disrupts the interaction between CHFR and PARP1 leading to loss of CHFR protein expression.

In the development of targeted therapies several aspects deserve consideration: First of all, the functional importance of the molecular target itself needs to be firmly established in order to avoid failure at the clinical stage. The wealth of functional and preclinical data about the role of CHFR in the control of the mitotic checkpoint, as well as the clinical data showing improved response and survival following taxane based therapy in patients...
with reduced or epigenetically silenced CHFR expression firmly establish CHFR as a promising molecular target in cancer. Our data here show that the pharmacologic targeting of CHFR with a small molecule inhibitor indeed yields synergistic growth inhibitory effects in combination with taxanes.

A second concern is the role of the molecular target in the prevention of treatment related toxicities. In the clinical setting the two major dose limiting toxicities of taxanes are neuropathy and hematotoxicity. If CHFR was integrally involved in preventing these, targeted therapy against CHFR in combination with taxanes could be doomed due to unacceptable toxicities, even if it produced a synergistic anticancer effect. Our data clearly demonstrate that ‘A3’ in combination with docetaxel does not significantly increase hematologic, hepatic or renal toxicities when compared to docetaxel alone. We speculate that hematopoietic precursors may be less susceptible to pharmacologic CHFR inhibition than epithelial or tumor tissues based on analysis of CHFR expression in the Tissue-specific Gene Expression and Regulation (TiGER) database, which reveals that CHFR expression in bone marrow is significantly lower than in epithelial tissues [23]. Taxane induced neurotoxicity is mostly mediated by interference with of the axonal transport, which in turn results in demyelination and axonal degeneration. Since this is a cell cycle independent process it is unlikely that CHFR has a protective function against taxane induced neuropathy.

The third area of concern in successful drug development pertains to the chemical and biochemical properties of the lead compound itself. Our data suggest specificity of ‘A3’ towards CHFR. Its pharmacodynamic properties are promising, but structural variations will be necessary to further improve binding characteristics, bioavailability and elimination half life, while maintaining specificity. Lead optimization should be undertaken before larger scale co-clinical trials in xenografts are performed to assess in vivo synergy and toxicity in combination with docetaxel.

In summary, our study represents a novel paradigm to improve taxane sensitivity in cancer therapy by targeting a central mediator of taxane resistance. This study proves the feasibility and specificity of this approach. Refinements in the chemical structure of ‘A3’ will be necessary prior to more extensive in vivo studies.

MATERIALS AND METHODS

Constructs and transfection

Full length CHFR cDNAs was obtained in pENTR vector from Life Technologies (Grand Island, NY). Full length PARP-1 was cloned by RT-PCR from cDNA obtained from immortalized bronchial epithelial cells (HBEC-3KT) and cloned into pENTR vectors (Life Technology, Grand Island, NY). The following mutants were generated using the Quickchange mutagenesis kit (Agilent Technologies, Santa Clara, CA): 1) CHFR-PBZ* (C623A C629A), 2) CHFR-ΔR (del 305-351), 3) CHFR-ΔR-PBZ* , 4) PARP1-E988K. 5) A FLAG-tagged CR-PBZ-peptide (del 1-406) of CHFR was PCR generated. Using Gateway recombine kits (Life Technologies, Grand Island, NY), the individual cDNAs were inserted into pDEST26, pDEST27, pDEST40 and pDEST51 vectors, respectively. shRNA against CHFR or scrambled sequence control in pGFP-V-RS vectors were obtained from Origene (Rockville, MD), while shRNA against PARP-1 and luciferase controls were a gift from Dr. Kraus (UT Southwestern). Vectors were transfected using Lipofectamine-2000 (Life Technologies, Grand Island, NY) and stably transfected clones were selected after treatment with G418 or blasticidin or puromycin.

Cell lines

Cell lines were either purchased directly from ATCC (Manassas, VA) (A549 cells). Other cell lines were a gift from Dr. Vertino. These lines were genotypically validated by STR analysis (Biosynthesis Inc.; Lewisville, TX). All cell lines were cultured in the appropriate media, supplemented with 10% FCS.

Cell viability assay

2,000 cells/well were seeded in 96 well flat bottom plates. 24 hours later, cells were exposed to either docetaxel alone (1 nM–1000 nM), ‘A3’ alone (1 µM–100 µM) or a combination of those. Mock treated wells received DMSO only. After 48–72 hours, cell viability was analyzed using XTT reagent (Trevigen, Gaithersburg, MD) as colorimetric method. Absorbance was read at 488 nM on a plate reader. Results were plotted using Prism5 software (GraphPad, LaJolla, CA).

Colony formation assay

Cell lines were seeded at a density of 1,000 cells / well into 6 well dishes. After 24 hrs. cells were treated with either docetaxel (between 50–100 nM) alone or in combination with A3 (10 uM) for 1 hour. After this media was removed, cells were washed in PBS x1 and fresh media was added back. In A3 treated cells, A3 was added back for a total exposure of 72 hours. Equal concentrations of DMSO were added as mock where appropriate. When colonies were visible with the naked eye, cells were fixed in 4% formaldehyde, stained with 0.5% crystal violet and image particles were counted using the ImageJ software.

Analysis of combination-drug effects

The effects of combined exposure to docetaxel and A3 was compared to single drug exposure in both XTT
assays and colony formation assays using the Chou-Talalev method [16]. For effects that were statistically significantly different, a combination index (CI) was calculated using CalcuSyn Software (Biosoft, Cambridge, UK), where a CI < 1 indicates synergistic, a CI = 1 additive and a CI > 1 antagonistic drug effects of the combination. Experiments with single drug concentrations were analyzed using the Bliss additivity method [17], where an additive effect is calculated according to the formula C = A + B – A * B. Effects greater than C are considered synergistic, while effects smaller than C are antagonistic.

Protein precipitations

Cells expressing H6-tagged and GST-tagged CHFR were lysed in NTA lysis buffer (50 mM Sodium Phosphate, 300 mM NaCl, 10 mM Imidizole, 0.05% Tween-20 pH8) containing PMSF, protease and phosphatase inhibitors. After sonication and clarification by centrifugation, CHFR was affinity purified using nickel-nitrilotriacetic acid (NiNTA) magnetic agarose (Qiagen). Cells expressing V5-tagged proteins were lysed in cell lysis buffer (Cell Signaling, cat #: 9803) containing PMSF and complete protease inhibitors. GST-tagged proteins were isolated using a glutathione sepharose resin (GE healthcare) while V5-tagged constructs were immunoprecipitated using V5 antibodies (Invitrogen) sepharose resin (GE healthcare) while V5-tagged constructs were affinity purified using nickel-nitrilotriacetic acid (NiNTA) magnetic agarose (Qiagen). Cells expressing V5-tagged proteins were lysed in cell lysis buffer (Cell Signaling, cat #: 9803) containing PMSF and complete protease inhibitors. GST-tagged proteins were isolated using a glutathione sepharose resin (GE healthcare) while V5-tagged constructs were immunoprecipitated using V5 antibodies (Invitrogen) and eluted with V5 peptide (Alpha Diagnostic).

Immunoblotting

Cells were lysed in 1x cell lysis buffer (Cell Signaling), containing Complete protease inhibitor and Phostop (Roche) and 1 mM PMSF. Cells were sonicated briefly and lysates clarified by centrifugation. Following SDS-PAGE and semi-dry transfer the following antibodies were used: CHFR (Sigma), PARP-1 (Calbiochem), PAR (Tulip), GST (Cell Signaling), V5(Life technologies). In addition, Beta Actin and Beta Tubulin (Sigma) GAPDH (Cell Signaling) were used as loading controls depending on the application and molecular weights of target proteins in the experiment.

Flow cytometry

Cells were synchronized with 5 μM aphidicholine for 24 hrs, released for the indicated time (0, 6, 12, 24 hrs) and fixed in 70% ice cold Ethanol. Cells were stained with 7-Aminoactinomycin D (7AAD) 250 ng/mL in the presence of 100 ug/mL RNase A. Cells were counted on a BD-FACSCANTO II instrument and analyzed on DIVA and FLOW-Jo software.

High throughput computational screen for small molecule inhibitors of CHFR's PBZ domain

5,256,508 chemical structures of purchasable compounds were screened against the published crystal structure of CHFR’s PBZ domain (PDB ID: 2XOC)[18] using Autodock Vina algorithms on http://www.mcule.com [19]. The top 10 compounds with the best docking scores (~9.7) were selected and ordered for further study.

In vivo pharmacokinetics

Pharmacokinetics of A3 (5 mg/ml in DMSO) after iv (10 mg/kg) and oral (25 mg/kg) administration were performed in 7 week old male CD-1 mice (Charles River, Raleigh, NC) by the Southern Research Institute (Birmingham, AL). Mice were held in standard housing with maximal 3 mice/cage and fed a standard rodent diet (Harlan, Madison, WI). 4 groups of 3 mice were treated both for iv. and oral dosing schedules. Blood was drawn at various time points (group A: 0.083, 1 hrs.; group B: 0.25, 4 hrs.; group C: 0.5, 8 hrs.; group D: 2, 12 hrs.) and analyzed by HPLC/MS/MS. Mean plasma drug concentration versus time data for both IV bolus and oral dosing were analyzed using the sparse sampling function of non-compartmental analysis with Phoenix® WinNonlin® Version 6.4 (Pharsight Corp.; Cary, NC). Parameters including maximum concentration (Cmax), time to maximum concentration (tmax), half-life (t1/2), and area under the concentration-time curve extrapolated to infinity (AUC0-∞), among others, were assessed for both routes, and bioavailability was calculated from dose-normalized values.

In vivo pharmacodynamics

All in vivo experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and performed according to federal guidelines. Mice were housed in pathogen-free animal facilities of the Winship Cancer Institute and fed a standard rodent diet with water at libitum. 9 week old female nude mice (Harlan laboratories) were xenografted with either A549 or H460 cell lines. When tumors reached a size of about 100 mm², mice were injected with ‘A3’ intraperitoneally at a concentration of 10 mg/kg or an equal volume of DMSO. Mice were euthanized at predefined time points after injection (0 h, 1 h, 4 h, 24 h). Tumors were explanted, imbedded in OCT media and immediately snap frozen in liquid nitrogen. Sections of fresh frozen tissue were prepared at 5 μm thickness and stained for CHFR as described in the immunohistochemistry section.

In vivo efficacy

9 week old female nude mice (Harlan laboratories) were xenografted with H460 cells at a concentration of 2.5 × 10⁵ cells. 5 days after inoculation, mice were randomly divided into four groups of 10 mice to receive the following treatment: A) DMSO at 0 h and 2 h B) ‘A3’ (10 mg/kg) at 0 h and 2 h, C) DMSO at 0 h and 2 h and Docetaxel 15 mg/kg
at 2 h, D) ‘A3’ (10 mg/kg) at 0 h and 4 h and Docetaxel 15 mg/kg at 4 h. Treatment was given intra-peritoneally every Monday and Thursday for 2 consecutive weeks. Tumor size was measured tri-dimensionally every 2 days using calipers. When tumor burden scores (defined by http://www.iacuc.emory.edu/documents/tumor_burden_scoring.pdf) exceeded 14, euthanasia was performed with CO2 narcosis. Tumors were immediately explanted, photographed, weighted and frozen in liquid nitrogen.

**In vitro auto-arylation**

PARP1 HSA (trevigen) was incubated in the presence or absence of A3, ABT888, or equal volume of DMSO at indicated concentrations in PARylation buffer (20 mM Tris 7.9, 100 mM NaCl, 10 mM MgCl2, 10 mM ZnCl2, 10% Glycerol, 1 mM DTT, 1 mg/ml ssDNA (Sigma), 300 uM B-NAD (Amresco)) for 30’ at 30 degrees C. Negative control reactions are performed in the absence of B-NAD. 2x SDS PAGE sample buffer stops the reaction and sample is loaded on SDS-PAGE for subsequent western blotting for PAR and PARP1.

**Immunohistochemistry (IHC)**

OCT media imbedded snap frozen tissue was sectioned on a microtome at 5 uM thickness. IHC was performed using the following antibodies and dilutions: CHFR (1:100) (CellSignaling). After washing and scavenging for free peroxidases, biotin conjugated anti-rabbit antibody was used in 1:500 dilution. Subsequent staining by DAB was performed using a Strep-HRP conjugate and DAB staining kit (vector labs). Slides were imaged on a Zeiss Axioplan2 microscope.

**Statistics**

Differences between continuous variables were analyzed by Student’s t-test.

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**CONFLICTS OF INTEREST**

The authors disclose no potential conflicts of interest.

**REFERENCES**


