Blocking p38/ERK crosstalk affects colorectal cancer growth by inducing apoptosis in vitro and in preclinical mouse models

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We recently demonstrated that p38α is required to maintain colorectal cancer (CRC) metabolism, as its inhibition leads to FoxO3A activation, autophagy, cell death, and tumor growth reduction both in vitro and in vivo. Here we show that inhibition of p38α is followed by TRAIL-mediated activation of caspase-8 and FoxO3A-dependent HER3 upregulation with consequent overactivation of the MEK-ERK1/2 survival pathway. p38α and MEK combined inhibition specifically induces apoptosis by enabling TRAIL signaling propagation through t-Bid and caspase-3, and fosters cell death in CRC cells and preclinical mouse models. Current MEK1-directed pharmacological strategies could thus be exploited, in combination with p38α inhibition, to develop new approaches for CRC treatment.

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1. Introduction

p38α is one of the four members of the p38 MAPK family of serine/threonine-directed kinases, which are classified as “stress-activated” kinases [1]. p38 MAPKs are activated by multiple exogenous and endogenous stresses and stimuli, and regulate numerous processes, including inflammation, differentiation, cell proliferation and survival in a tissue-specific and signal-dependent manner [2,3]. Despite its anti-tumorigenic activity in some tissues [4–6], p38α is involved in sustaining tumor growth after acquisition of the malignant phenotype. In particular, p38α is required for HIF1α stabilization [7], for chondrosarcoma cell proliferation [8] and for tumor dormancy [9]. Transcriptional upregulation of the anti-apoptotic proteins BCL2 and MCL1 upon TRAIL treatment is dependent on p38α activation, and p38α inhibition increases TRAIL-dependent cell death [10]. Also, migration and invasion can be positively regulated by p38α [11–14]. We recently reported that p38α activity is also required to sustain CRC cell metabolism [15,16]. Indeed, its pharmacological inhibition or genetic ablation correlates with a strong reduction of HIF1α protein levels resulting in decreased expression of HIF1α target genes involved in glycolysis and drop of intracellular ATP levels. This acute energetic demand is sensed by AMPK that in turn triggers a FoxO3A-mediated autophagic response to promote cell survival. When inhibition of p38α is prolonged, the autophagic response is no longer able to provide the energy required for survival and cells undergo non-apoptotic cell death [17,18]. Moreover, a recent study identified p38α as a mediator of resistance to irinotecan and its activation correlates with impaired response to FOLFIRI therapy in CRC patients, while its inhibition sensitized resistant CRC cells to SN38 [19] and 5FU [20].

Another signaling pathway playing a key role in tumor formation and progression is the MEK/ERK pathway [21]. This pathway...
is activated by a variety of different stimuli, many of which promote cell proliferation and inhibit anti-proliferative and pro-apoptotic responses [22]. Mutations leading to constitutive activation of the upstream MEK1 activating kinases Ras and Raf are found in 36% and 9–11% of CRC patients, respectively, and significantly contribute to tumor formation and progression [21]. Even if MEK1 inhibition has been shown to reduce CRC cell proliferation in vitro and to decrease tumor growth in xenograft models [23], phase II clinical trials with the MEK1 inhibitor CI-1040 failed to prove efficacy against CRC [24].

Here we found that CRC cells react to the inhibition of p38α by upregulating HER3 expression and by activating the MEK/ERK signaling pathway. While inhibition of p38α or MEK1 alone failed to significantly induce apoptosis in CRC cells, their combined inhibition dramatically increased death rate by promoting apoptotic cell death. These data, obtained in vitro and confirmed in xenograft and preclinical mouse models, suggest that combinatorial inhibition of these two pathways might be exploited as a novel approach for CRC treatment.

2. Materials and methods

2.1. Cell culture and reagents

HCT-116, HT-29, DLD-1 and LS174T cells were maintained in DMEM supplemented with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS. Caco-2 cells were maintained in DMEM supplemented with 20% FBS. The NCM460 cell line was received by a materials transfer agreement with INCELL Corporation. The cells were routinely propagated under standard conditions with 10% FBS.

2.2. In vivo studies

Experiments involving APC+/min were previously described [17]. For xenograft experiments, 10 × 106 HT-29 cells were injected subcutaneously into the flank of athymic nude mice (Jackson Laboratory). Mice were treated daily for 9 days with 0.05 μmol/kg of SB202190 by i.p. injection (n = 8), or orally with 3 mg/kg of PD0325901 (n = 10), or with a combination of both (n = 10), or with the vehicle alone (n = 8). The treatment started when tumors had reached a mean volume of 100 mm3. After 9 days the treatment was discontinued, a fraction of the animals were sacrificed (n = 4 for each group) and the tumors explanted (n = 6 for mice treated with SB202190 or with the vehicle alone; n = 8 for tumors treated with PD0325901 or with a combination of both inhibitors). The rest of the animals (n = 4 for mice treated with SB202190 or with the vehicle alone; n = 6 for mice treated with PD0325901 or with a combination of both inhibitors) were monitored and the tumors (n = 6 for the vehicle control group; n = 8 for the SB202190-treated group; n = 8 for the PD0325901-treated group; n = 10 for the SB202190-PD0325901-treated group) measured until mice health conditions were no longer compatible with ethical guidelines. For chemical-induced colitis carcinogenesis 20 C57BL/6 mice were injected intraperitoneally with 12 mg/kg of azoxy-methane (AOM). Then, 2% dextran sulfate sodium (DSS) was given in the drinking water over five days, followed by two weeks of regular water. This cycle was repeated three times. Ten days after the last round of DSS 10 animals were treated concomitantly with SB202190 and PD0325901 and 10 with the vehicle alone, as described above. All tissues were fixed overnight in 10% formalin and paraffin-embedded. Procedures involving animals were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

2.3. Quantitative real-time PCR and RNA interference

Total RNA was extracted using Tri reagent (Sigma). Samples were treated with DNase-1 (Ambion) and retro-transcribed using the High Capacity DNA Archive Kit (Applied Biosystem). PCRs were carried out using SYBR Green PCR Master Mix and an ABI 7500HT machine (Applied Biosystems). Relative quantification was done using the ddCT method. For RNAi, cells were transfected with 50 nM Stealth siRNA against Fox3A or TRAIL (Invitrogen, validated oligos) using RNAiMAX (Invitrogen). On-TARGET-plus control siRNAs (Thermo Scientific) were used as control sequences. Primer sequences are available on request.

2.4. Histology, immunohistochemistry and immunofluorescence

Paraffin-embedded samples were sectioned and stained with hematoxylin and eosin or Periodic Acid Schiff (PAS). For immunohistochemistry, sections were incubated with antibodies against Ki-67 (Dako), phospho-ERK1/2, phospho-p38, active caspase-3 and active caspase-8 (all from Cell Signaling) overnight at 4 °C. Avidin/biotin-based detection systems (Vector) were used to reveal the signal. Immunofluorescence was performed using anti-active caspase-8 and anti-phospho-ERK1/2 (Cell Signaling) as previously described [17]. Nuclei were counterstained using TO-PRO3.
For human samples, 15 non-consecutive colorectal tumors specimens were fixed for 24 h in 10% neutral-buffered formalin, embedded in paraffin and sectioned; immunohistochemistry was carried out using the EnVision-TM system (Dako).

2.5. Proliferation and apoptosis assays

For colony formation assays, cells were fixed and stained using Coomassie Brilliant Blue. Proliferation assays were conducted using the WST-1 reagent (Roche). For sub-G1 quantification experiments, cells were harvested, fixed and stained with propidium iodide (Sigma–Aldrich). The Annexin V-FITC Apoptosis Detection Kit (Sigma–Aldrich) was used following manufacturer’s instructions. Samples were analyzed using FACS-Canto (Becton Dickinson). TUNEL assays (Roche) were performed on tissue sections following manufacturer’s instructions.

2.6. Immunoprecipitation, immunoblot analysis and ELISA

Cells were collected and homogenized in lysis buffer (50 mM Tris-HCl pH 7.4; 5 mM EDTA; 250 mM NaCl and 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Cell lysates were pre-cleaned by incubation with GammaBind G Sepharose for 30 min at 4°C. Samples were immunoprecipitated with anti-Bid antibodies (from Cell Signaling) or IgG control, and incubated at 4°C for 4–6 h. Immunoprecipitated proteins were extensively washed with lysis buffer, resuspended in Laemmli buffer, separated on polyacrylamide gel and transferred to nitrocellulose membranes, after which precipitated proteins were subjected to immunoblot analysis.

A total of 20 μg of protein extracts from each sample were denatured in Laemmli sample buffer and used for SDS-PAGE. Immunoblot analyses were performed using anti-actin (Sigma), anti-phospho-Ser/Thr-Pro (Upstate Millipore),
anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-HER3, anti-phospho-MEK-1/2 (Ser217/221), anti-active caspase-3, anti-active caspase-8, anti-Bid (all from Cell Signaling), anti-PARP1 fragment p85 (Promega), anti-β-tubulin (Santa Cruz Biotechnology), anti-lc3 (kindly provided by Francesca De Marchi). HRPO-conjugated secondary antibodies were used (GE Healthcare) and the signal was revealed using the ECL-plus chemiluminescence reagent (GE Healthcare). ELISA was performed using the Trail ELISA KIT (Bender Medsystems) following manufacturer’s instructions.

3. Results

3.1. p38α inhibition leads to MEK/ERK activation in CRC

In our previous studies, using pharmacologic and genetic approaches we identified p38α as the kinase responsible for...
SB202190-dependent induction of cell cycle arrest, autophagy and cell death in CRC cells [15–17]. Indeed, SB202190 strongly reduces the phosphorylation levels of the p38α endogenous target MAPK-activated Protein Kinase-2 (MK2) in CRC cells (Fig. S1).

Here we show that in CRC cells pharmacologic inhibition of p38α potentiates the MEK/ERK pathway, irrespectively of the mutational status of the ERK upstream activators RAS and RAF. Indeed, increased phospho-activation of both MEK1 and ERK1/2 was observed in BRAF mutant HT-29 cells, KRAS mutant HCT-116 cells and Caco-2 cells, which are wild-type for these two genes (Fig. 1A). This effect was also confirmed by using a structurally and functionally different p38α inhibitor, BIRB796, (Fig. 1B) and observed in vivo in HT-29-xenografted nude mice (Fig. 1C) and in colon sections derived from AOM-treated APC+/min mice injected with SB202190 (Fig. 1D).

Importantly, inhibition of MEK1 in HT-29, HCT-116 and Caco-2 cells triggers the phospho-activation of p38α (Fig. 1E), indicating the existence of a p38α/ERK crosstalk in CRC cells.

p38α inhibition leads to the time-dependent upregulation of the receptor tyrosine kinase HER3, both at the protein and mRNA level (Fig. 2A and B). Moreover, functional HER3 complexes are required for MEK/ERK phospho-activation upon p38α inhibition, as shown in Fig. 2C. As inhibition of p38α induces a strong activation and nuclear accumulation of the FoxO3A transcription factor [17], we investigated whether FoxO3A was involved in the transcriptional upregulation of HER3, as already shown in breast cancer cells [25]. To this aim, we inhibited p38α activity in HT-29 cells transfected with a FoxO3A-specific or a control siRNA and found that the up to three-fold increase in HER3 transcription was blunted by RNAi for FoxO3A (Fig. 2D). The deacetylase SIRT1 is a druggable transcriptional cofactor that positively regulates FoxO3A transcriptional activity and promotes a FoxO3A-dependent pro-survival response [26]. Thus, we investigated whether SIRT1 activity was required for the upregulation of HER3 transcription. As shown in Fig. 2E, HER3 transcription is blunted upon SIRT1 inhibition and, as a consequence, the phospho-activated form of ERK1/2 decreases (Fig. 2F). These data show that CRC cells react to p38α inhibition by promoting the transcriptional upregulation of HER3 and the consequent activation of the MEK/ERK pathway in a FoxO3A- and SIRT1-dependent manner.

3.2. Combined inhibition of p38α and MEK1 increases CRC cell death

To investigate the role of the MEK/ERK survival pathway in the cellular response to p38α inhibition, we treated CRC cells with pharmacological inhibitors for both p38α (SB202190) and MEK1 (PD98059). As shown by WST1 proliferation assay, colony formation assay and FACS analysis, combined inhibition significantly reduces the viability of both the HT-29 (Fig. 3A–C) and the HCT-116 (Fig. S2A–C) cell lines in a dose-dependent manner compared with the inhibition of only one of the two pathways. These results were further confirmed by the use of a structurally unrelated MEK1 inhibitor, UO126 (Fig. 3B, S2B). To test whether this effect was confined to cell culture conditions, we injected HT-29 CRC cells into the flank of athymic nude mice. Animals were treated with SB202190, or with the orally administrable MEK1 inhibitor PD0325901, or with a combination of both. To evaluate the effective residual tumor mass, animals were treated for the first 9 days, then the treatment was discontinued and tumor growth monitored until the end of the experiment. As shown in Fig. 3D, combined inhibition of p38α and MEK1 significantly reduced tumor growth...
compared with the single treatments. Importantly, after the end of the treatment, tumors treated with only one of the two inhibitors started to grow faster compared with tumors treated with both SB202190 and PD0325901. These data suggest that both in cell lines and in xenograft models inhibition of a single pathway promotes mainly a cytostatic effect, while combined inhibition triggers a massive cytotoxic effect.

3.3. Combined inhibition of p38α and MEK1 induces apoptotic cell death

We previously reported that p38α inhibition induces autophagy and cell death [15,17]. To investigate whether combined inhibition of p38α and MEK1 increases CRC cell death by modulating autophagy, we evaluated the amount of lipidated MAP1 LC3 (LC3II). No significant changes in LC3II were observed compared to the inhibition of p38α alone (Fig. 4A). Surprisingly, when we analyzed the typical apoptotic markers, we observed that combined inhibition of both signaling pathways leads to the activation of caspase-3 and to a strong increase of the cleaved PARP1 fragment p85. Of note, combined inhibition of p38α and MEK1 induces apoptosis in both HT-29 and HCT-116 cells (Fig. 4A and E), while in human primary non-tumorigenic colon cells (NCM460) no significant increase in apoptosis basal levels can be observed (Fig. S3A). In agreement with the data presented in Fig. 2, inhibition of SIRT1 or HER3 activity also enhances apoptosis in SB202190-treated cells.

Fig. 5. Inhibition of the MEK/ERK pathway rescues a caspase-8-dependent proapoptotic program activated upon p38α inhibition. (A) p38α inhibition by SB202190 leads to caspase-8 activation and increases Bim levels without triggering BID cleavage nor caspase-3 activation in HT-29 cells. (B) Combined inhibition of p38α (SB202190, 10 μM) and MEK1 (PD98059, 20 μM) triggers BID cleavage. (C) Inhibition of caspase-8 (Z-IETD, 10 μM) 12 h before the end of the treatment with MEK1 (PD98059, 20 μM) and p38α (SB202190, 10 μM) inhibitors significantly reduces BID cleavage and apoptosis induction. (D) Immunoprecipitated BID was blotted with an anti-phospho-Ser/Thr-Pro (pS/TP) antibody. Densitometric analysis was performed and normalized on immunoprecipitated BID protein levels. Plotted data revealed a significant increase of BID phosphorylation upon p38α inhibition (SB202190, 10 μM) that is blunted upon concomitant inhibition of the MEK/ERK pathway (SB + PD: SB202190, 10 μM; PD98059, 20 μM). (E) HT-29-derived tumors explanted from xenografted nude mice treated for 9 days with the vehicle alone (n = 6), SB202190 (0.05 μmol/kg/day; n = 6), PD0325901 (3 mg/kg/day; n = 8) or SB + PD (0.05 μmol/kg/day SB202190 and 3 mg/kg/day PD0325901, n = 8) were analyzed by immunohistochemistry. Caspase-8 activation can be observed also in tumor samples from HT-29-xenografted nude mice treated with the p38α inhibitor, whereas activation of caspase-3 can be observed only upon p38α and MEK1 inhibition.
We quantified by FACS analysis the extent of apoptotic cell death in cells treated with SB202190 and PD98059. As shown in Fig. 4C, the 30.9% of HT-29 cells treated with both inhibitors underwent apoptosis (AV+/PI+/C0) compared with 6.7% or 7.7% of the cells treated with the p38a or the MEK1 inhibitor alone, respectively, while only 1.8% of the vehicle-treated cells were positive for Annexin V staining only. Pro-apoptotic signaling pathways can converge to stimulate mitochondrial membrane permeabilization (MMP) and cytochrome c release depending on the stimuli and on the cell line analyzed. A key role in MMP is played by the pro-apoptotic protein Bax [27]. The importance of Bax in colorectal tumorigenesis is highlighted by the presence of inactivating mutations in more than 50% of CRCs characterized by a microsatellite instability (MIN) phenotype [28]. For these reasons, we tested whether apoptosis induced by the combined inhibition of p38a and MEK1 was dependent on the mitochondrial branch of the pro-apoptotic cascade. To this aim, we used two isogenic CRC cell lines, one of which was Bax KO [29]. As shown in Fig. S3B, HCT-116 Bax−/− cells were able to sense and react to the inhibition of p38a, as their wild-type counterpart, by activating autophagy. On the other hand, the cytotoxic effect of the combined inhibition of p38a and MEK1 was largely lost in Bax−/− cells (Fig. 4D), due to a failure in activating apoptosis (Fig. 4E). Consistently, combined inhibition of p38a and MEK1 triggered apoptosis also in DLD-1, LS174T and Caco-2 CRC cell lines (Fig. 4F). These data reveal that the increase in CRC cell death observed upon inhibition of these two signaling pathways is due to apoptotic cell death - which requires the presence of Bax - and therefore engages the mitochondrial branch of the pro-apoptotic cascade. Recently, Solit and colleagues nicely demonstrated that BRAF - but not KRAS - mutations predict sensitivity to MEK inhibitors. MEK inhibitors exert a cytostatic and cytotoxic effect in BRAF mutant cell lines, but only a subset of these cells undergo apoptosis [30]. Our data show that MEK1 or p38a inhibition exerts a cytostatic and cytotoxic effect but only the combined inhibition of both signaling pathways is able to trigger massive apoptosis. This happens independently of KRAS/BRAF status and chromosome instability (CIN) or MIN phenotypes, as shown by the results obtained in HT-29, HCT-116, Caco-2, DLD-1 and LS174T cells.

3.4. A caspase-8-dependent apoptotic program sustains cell death induced by p38a and ERK inhibition

The results obtained led us to speculate that inhibition of the MEK/ERK pathway is instrumental to rescue a pro-apoptotic program activated upon p38a inhibition. Since the p53 status does not influence apoptosis induced by the combined inhibition of p38a and MEK1 (p53 is mutated in HT-29, DLD-1 and Caco2 cells, whereas HCT-116 and LS174T cells are p53 wt), we focused on the extrinsic apoptotic pathway. The analysis of key players of the extrinsic pathway upon p38a inhibition in HT-29 cells revealed a significant activation of caspase-8 (Fig. 5A; S4) and, in agreement with the previously described activation of FoxO3A [17], a strong increase of all three isoforms of BIM, but no activation of Bid and caspase-3 (Fig. 5A). Inhibition of both p38a and MEK1 restores Bid cleavage (Fig. 5B) and induces caspase-3 activation (Fig. 4A). Of note, caspase-8 inhibition prevented Bid processing and consequent apoptosis induction in HT-29 cells treated with both p38a and MEK inhibitors (Fig. 5C).

To begin elucidating the mechanism that mediates MEK1-dependent Bid processing by caspase-8, we carried out an evaluation of endogenous MAPK activity by immunoprecipitating Bid from untreated HT-29 cells and from HT-29 cells treated with SB202190 or with a combination of SB202190 and PD98059, followed by an immunoblot with an antibody specifically recognizing the MAPK consensus phosphorylation sites (Proline-directed Serine/Threonine, S/T-P). Phosphorylation of Bid was induced by p38a inhibition and was eliminated by the concomitant inhibition
of MEK1 (Fig. 5D). Although the functional relevance of this phosphorylation has yet to be established, it has been previously demonstrated that phosphorylation of Bid by casein kinases I and II regulates its cleavage by caspase-8 [31].

The activation of caspase-8 upon p38α inhibition was also confirmed in HT-29 xenografted tumors, as was the ability of the combined inhibition of p38α and MEK1 to trigger apoptosis (Fig. 5E), shown by caspase-3 activation. Since caspase-8 is one of the first caspases being activated upon triggering of the extrinsic apoptotic pathway [32], we assessed the expression of death-receptor ligands and their receptors. Of note, upon p38α inhibition TRAIL expression increased both at the mRNA and the protein level in HT-29 cells (Fig. 6A and B), whereas the expression of FasL and TNF was largely unaffected (data not shown). The analysis of TRAIL receptors revealed a time-dependent upregulation of killer/DR5 (Fig. 6C), while the mRNA levels of DR4 were unchanged (data not shown). To evaluate the importance of TRAIL upregulation in apoptosis induced by inhibition of p38α and MEK1, we transfected HT-29 cells with a TRAIL-specific siRNA. As shown in Fig. S4, RNAi efficiently abrogated TRAIL transcription and this correlated with a significant reduction in apoptosis induced by the simultaneous inhibition of both signaling pathways (Fig. 6D). These data indicate that p38α inhibition initiates a caspase-8-dependent cascade by upregulating TRAIL and DR5, and that the concomitant inhibition of the MEK/ERK pathway enables propagation of the signaling trough t-Bid and caspase 3.

3.5. Combined inhibition of p38α and MEK1 reduces tumor growth in preclinical mouse models

To evaluate the efficacy of the pharmacological blockade of both p38α and MEK1 in vivo, we used the AOM/DSS colitis-associated
carcinoma model, which is considered a highly reproducible and reliable CRC model, as the pathogenesis closely recapitulates human CRC [33]. To this aim, animals were injected with the carcinogen azoxymethane and then fed with DSS to induce colitis (Fig. 7A). After 10 days from the last round of DSS, animals were treated with PD0325901 and SB202190 or with the vehicle alone. After two weeks of treatment, animals were sacrificed and the explanted tissues were analyzed. As shown in Fig. 7B, inhibition of p38 and MEK1 significantly reduced the volume and number of colorectal tumors compared with vehicle-treated mice. Histological examination (Fig. 7C) revealed the presence of polypoid adenocarcinomas with low amount of differentiated mucus-secreting cells (stained purple with PAS staining) in vehicle-treated mice, whereas animals treated with both inhibitors displayed a flat, adenocarcinomas with low amount of differentiated mucus-secretion.

This table summarizes the clinical, histological and immunohistochemical data obtained on a study group including 15 patients affected by colorectal adenocarcinoma, ranging in age from 60 to 85 years. Dukes staging is also reported. Five samples over fifteen were found phospho-p38 positive.

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Table 1

3.6. Increased p38 phosphorylation can be observed in human cancer samples

The MEK/ERK pathway has been extensively investigated in CRC and it is now widely accepted that it plays a significant role in CRC formation and progression [21]. To our knowledge, however, limited studies have addressed the activation of p38 in human CRC samples. Thus, we evaluated the activation of this kinase in sections derived from archival human CRC specimens derived from four low grade and eleven high grade CRC biopsies. Of these fifteen samples, five showed phospho-activated p38 (Table 1). As shown in Fig. 8, phospho-activated p38 immuno-reactivity can be observed mainly in epithelial cells, where it accumulates into the nucleus. Although based on a restricted number of cases, these data suggest that p38 is activated in human tumors and thus further support that a rationale exists for the use of p38 inhibitors in CRC treatment.

4. Discussion

CRC is the third most frequent malignancy, but the second cause of death for tumor in western population [34]. The genomic landscape of CRC has been recently unveiled [35] revealing that other signaling pathways are deregulated in CRC cells, besides the well-known Wnt, p53, K-ras, and TGFβ [36,37]. Selective compounds are increasingly used for CRC treatment. Indeed, several patients are now treated with monoclonal antibodies against VEGF or against EGFR in combination with poorly-selective chemotherapy [38]. The success of chemotherapy relies in the ability to kill or, better, to selectively induce apoptosis in cancer cells [3]. As these cells are exposed to a selective pressure [39], it is not surprising that they evolve strategies to inhibit pro-apoptotic signaling pathways and favor pro-survival and proliferative cascades [3]. Therefore, a promising approach consists in finding critical molecular targets that are positively selected and are essential for tumor growth. This concept is the base of current research and several compounds that target signaling pathways such as MEK/ERK and P38K/AKT are now under clinical trials. In CRC, the MEK/ERK signaling pathway has been frequently found overactive due to activating mutations in the upstream kinases RAS and RAF. This pathway is required for tumor initiation and progression by promoting cell proliferation and survival [21] and is being widely studied as a promising pharmacological target [40]. Although initial results demonstrated that inhibition of RAF or MEK1 exerts a cytostatic and cytotoxic effect in vitro and in xenograft models [23,41], the MEK1 inhibitor CI-1040 showed insufficient antitumor activity in phase II clinical trials [24]. Moreover, inhibition of RAF in tumor cells harboring mutant BRAF was able to inhibit ERK signaling, but surprisingly enhanced ERK signaling in cells with wild type BRAF or mutant KRAS [42,43].

Fig. 8. Nuclear localization of phospho-activated p38. Activated p38 shows its typical nuclear localization in sections derived from human colorectal cancer biopsies.
cancer cells and its inhibition leads to an acute state of energy demand that is buffered through the activation of a FoxO3A-dependent autophagic response [16,17].

Here we showed that p38α inhibition induces increased expression of HER3, one of the receptor tyrosine kinases (RTK) of the EGF pathway, and that this effect is dependent upon the activity of FoxO3A and its cofactor Sirt1. In turn, HER3 upregulation leads to over-activation of the MEK/ERK pathway, even in the presence of mutations in RAF or RAS. Indeed, cells carrying mutant forms of these oncogenes are still able to overactivate ERK signaling in response to extracellular ligands (i.e. EGF) [44]. Of note, it was recently demonstrated that FoxO3A is able to increase expression of RTKs, including HER3, in breast cancer cells in response to AKT inhibition [25]. In light of these data, it has to be considered that the activation of FoxO3A observed upon p38α inhibition is supported by the activation of AMPK but also correlates with inactivation of AKT [17].

The interplay between the p38α and the MEK/ERK pathway was already reported in primary fibroblasts and breast epithelial cells, and involved the activity of the phosphatase PP2A [45,46]. Here we described new players involved in p38α/ERK crosstalk. Indeed, even if we cannot rule out a possible contribution of PP2A in this model, our experiments indicate that p38α inhibition triggers FoxO3A-dependent HER3 transcription and that functional HER3 complexes are required for MEK/ERK phospho-activation. We also demonstrated that counteracting the activation of the MEK/ERK pathway upon p38α inhibition leads to the rescue of a pro-apoptotic program driven by the extrinsic pathway. Indeed, transcriptional upregulation of TRAIL and activation of caspase-8 can be observed in CRC cells upon p38α inhibition, and concomitant MEK inhibition triggers Bax-dependent apoptosis by enabling propagation of the signaling through t-Bid and caspase 3. Our data suggest that the MEK/ERK pathway could affect Bid processing by caspase-8, thus resembling the activity of cassekinases I/II, which regulate caspase 8-dependent Bid cleavage by phosphorylation [31]. Indeed, Bid phosphorylation at MAPK exerts both a cytostatic and a cytotoxic effect, it fails to induce massive apoptosis. This also emerges from the xenograft experiments. While a significant reduction in tumor growth can be observed by inhibiting p38α or MEK1 alone, combined inhibition further reduces tumor volume and triggers apoptosis. On the other hand, after discontinuing treatment we observed that tumors treated with only one of the two inhibitors started to grow significantly faster than tumors where both p38α and MEK1 had been inhibited. Intriguingly, p38α activation can be observed in HT-29, HCT-116 and Caco2 colorectal cancer cells upon MEK1 inhibition, but not in human primary non-tumorigenic colon cells (Grossi V, Peserico A and Simone C, unpublished results), suggesting that a reciprocal compensatory role of these two pathways may exist in cancer cells.

The potential efficacy of combined inhibition of p38α and MEK1 as a novel pharmacological strategy is also supported by the results obtained in preclinical mouse models. The AOM/DSS protocol is widely used to induce colitis-associated carcinoma and is considered the model of choice also for sporadic CRC [33]. Combined inhibition of p38α and MEK1 efficiently reduces the volume of AOM/DSS-induced tumors in vivo. The rationale of such approach presupposes higher levels of the activated kinase in tumor samples. Indeed, we found high levels of phospho-activated p38 in a subset (5/11) of high grade tumor specimens. Considering also that it was recently reported that p38α activation correlates with impaired response to FOLFIri therapy in CRC patients [19], and that several p38α and MEK1 inhibitors passed phase I clinical trials and are in phase II for inflammatory diseases and cancer [47,24], such compounds might prove effective in combination with classical chemotheraphy or other molecularly-targeted drugs in CRC patients.

Our studies provide evidence that the therapeutic efficacy of p38α/MEK1 inhibition is independent from mutations in p53, KRAS, BRAF genes, and from the CIN or MIN phenotype; conversely, Bax<sup>−/−</sup> cells showed almost 50% apoptosis reduction with respect to wild type cells. Bax inactivating mutations have been described in more than 50% of CRCs characterized by a MIN phenotype, however these only account for 10–15% of all CRCs [28]. Thus, Bax status could potentially represent a bio-marker for p38α/MEK1-targeted therapy, as does KRAS for treatments directed against EGFR [48].

Conflict of interest statement

As corresponding author, I warrant that all authors have read and concur with the submission of this manuscript. I also warrant that the material submitted for publication has not been previously reported and is not under consideration for publication elsewhere. Furthermore, we declare no competing financial interests. Mary P. Moyer (INCELL Corporation, San Antonio, Texas 78249, USA) has been listed as Author for providing the commercial human colon cell line NCM460.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.05.006.

References


