Abstract. Background: Drug resistance to 5-fluorouracil (5-FU) is a major obstacle in colonic cancer treatment. Activation of nuclear factor-kappa B (NFκB), mitogen-activated protein kinase kinase kinase 8 (MAP3K8) and protein kinase B (AKT) is thought to protect cancer cells against therapy-induced cytotoxicity. Materials and Methods: Using cytotoxicity assays and immunoblotting, the impact of inhibitory strategies addressing NFκB, AKT and MAP3K8 in chemoresistance was evaluated in a colonic cancer model in vitro. This model consisted of the cell lines SW480 and SW620, and three subclones with increasing degrees of chemoresistance in order to mimic the development of secondary resistance. Results: NFκB protein p65 was selectively activated in all resistant cell lines. Consequently, several inhibitors of NFκB, MAP3K8 and AKT effectively circumvented this chemoresistance. As a cellular reaction, NFκB inhibition may trigger a feedback loop resulting in activation of extracellular signal-regulated kinase. The results suggest that chemoresistance to 5-FU in this colonic carcinoma model (cell lines SW480 and SW620) is strongly dependent on NFκB activation. The efficacy of MAP3K8 inhibition in our model potentially uncovers a new mechanism to circumvent 5-FU resistance.

The global burden of cancer is one of the most critical issues in healthcare, colorectal cancer being the third most frequently diagnosed type amongst men and women worldwide (1). Chemotherapy for this entity was initially based on 5-fluorouracil (5-FU) (2) and current regimes still include this agent as a cornerstone of systemic combination treatment (3, 4). Despite recent innovations and targeted approaches, resistance to chemotherapy is still a major obstacle to successful treatment. In this context, nuclear factor kappa B (NFκB) is suspected to play a pivotal role (5-7).

NFκB was first discovered as a transcription factor important for B-cell-specific gene expression (8). Although this transcription factor is present in all cells (9), it is inactive under physiological conditions (10). Its activation was quickly linked to inflammatory diseases (11-13) and in vitro experiments also demonstrated a tumor-promoting function, especially in inflammation-associated cancer, such as colorectal cancer and hepatocellular carcinoma (14-17). NFκB activity has since been linked to a variety of malignant diseases, including colorectal cancer (18), breast cancer (19, 20), prostate cancer (21, 22), multiple myeloma (23), and glioblastoma (24). It is now widely accepted that NFκB may serve as a therapeutic target (25, 26). This was further corroborated by studies that revealed promising antitumor effects due to NFκB inhibition in several disease entities (24, 27-32). NFκB activation has been linked to the development of chemoresistance to 5-FU in colonic, breast and oesophageal cancer cells (33-38).

Interestingly, MAP3K8 [also named tumor progression locus 2 (TPL2) or cancer Osaka thyroid (COT)] shares molecular pathways with NFκB. MAP3K8 is able to activate the extracellular signal-regulated kinase pathway (MEK/ERK) (39, 40). Originally identified as an oncogene in 1991 (41), its overexpression has since been associated with colorectal cancer (42), T-cell neoplasia (43), breast cancer (44, 45), Epstein-Barr virus-associated nasopharyngeal tumors, Hodgkin’s (46) and clear-cell renal cell carcinoma (47). MAP3K8 inhibition through honokiol was associated with reduced growth of gastric tumor in an orthotopic model (48). Furthermore, it is involved in adaptive and innate immune response (49). MAP3K8 is stoichiometrically bound to A20-binding inhibitor of NFκB2 (ABIN2) and the NFκB subunit p105 (50-52) and when overexpressed is able to activate NFκB (50). Besides p105, which functions as a precursor protein of
p50, the NFκB transcription factor family includes the proteins v-rel avian reticuloendotheliosis viral oncogene homolog A (REL A or p65), RELB, c-REL and p100 (precursor of p52). Physiologically, NFκB dimers are kept inactive in the cytoplasm, bound to proteins called inhibitors of NFκB (IκB). Two distinct signalling pathways have been described: the non-canonical and the canonical pathway, each of them with different players responding to different stimuli (53, 54).

Mainly, for activation IκB is phosphorylated by a kinase complex, called IκB kinase (IKK), and then ubiquitinated and degraded via the proteasome. By this mechanism, NFκB is released and able to translocate into the nucleus. Furthermore, IKK-mediated phosphorylation of p105 enables not only NFκB activation, but also MAP3K8-mediated activation of MEK (55, 56). Regarding resistance, MAP3K8 has so far only been associated with resistance to B-raf proto-oncogene (BRAF) inhibitors in melanoma (57, 58).

Previous studies have shown that besides MAP3K8, protein kinase B (AKT) is also able to activate NFκB. AKT can influence a key molecule of the NFκB pathway that MAP3K8 is also involved with: p65 (59-62). The objective of this study was to elucidate the roles of MAP3K8, NFκB and AKT signalling in our multi-stage resistant colonic carcinoma model in vitro.

Materials and Methods

Reagents. Anti-NFκB antibodies against p65, phospho-p65 (Ser536), anti-rabbit IgG were purchased from Cell Signaling (Danvers, MA, USA) as a pathway sampler kit; anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA); stabilized goat anti-rabbit antibody was purchased from Pierce (Waltham, MA, USA). 5-FU was from Serva (Heidelberg, Germany). We used five different NFκB inhibitors: MG-132 (proteasome inhibitor) and lupeol (IKK kinase and AKT inhibitor) were from Enzo (Lorrach, Germany); BAY1170-82 (IKK kinase inhibitor), violacein and disulfiram from Sigma-Aldrich (St. Louis, MO, USA). TPL2 inhibitor (Calbiochem®), InSolution AKT Inhibitor IV, wortmannin and rapamycin were obtained from CalBiochem (San Diego, CA, USA). Reagents were stored as instructed in their datasheets; stock solutions were prepared in dimethylsulfoxide (DMSO).

Cell culture. The primary adenocarcinoma cell line SW480 and its lymph node metastasis SW620 were obtained from the American Type Culture Collection (Rockville, MD, USA) (63). We used an already-established multi-stage colonic cancer model as resistant subclones had been produced previously by continuous exposure of tumor cells to increasing concentrations of 5-FU (64). Experiments were carried out with low-resistance phenotype (5 μM 5-FU), intermediate-resistance phenotype (25 μM 5-FU) and high-resistance phenotype (125 μM 5-FU) SW620 cells. Cells were maintained in RPMI-1640 with 2 mM Glutamax I, 10% heat inactivated foetal calf serum, 50 μg/ml gentamycin (all from Gibco BRL, Paisley, UK) at 37°C in a humidified atmosphere of 5% CO₂:95% air. For experimental purposes, cells were harvested in

Cytotoxicity assays. Cells were detached using accutase. A total of 5,000 cells/well were seeded in 96-well plates under standard culture conditions. Inhibitors were prepared from stock solutions and cells were exposed to the NFκB inhibitors lupeol (0.001 μM to 100 μM), MG-132 (0.0001 μM to 100 μM), BAY1170-82 (0.0001 μM to 0.1 μM), or TPL2 inhibitor (100 μM to 0.1 nm), an inhibitor of MAP3K8. For combination experiments, drugs were added after overnight incubation of the plate with 1 μM or 5 μM BAY117082 plus 5-FU at serial dilution. Cells were then incubated for 144 hours in a humidified atmosphere at 37°C or 72 hours for AKT inhibitor. The viability of cells was then evaluated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell titer non-radioactive cell-proliferation assay; Promega, Madison, WI, USA). After the addition of dye solution, the water-insoluble crystals were solubilised overnight and the absorbance was monitored at 570 nm with a reference wavelength of 690 nm using a 96-well plate reader (Anthos Reader, Krefeld, Germany). The inhibitory concentration for 50% of the cells (IC₅₀) for each agent was calculated by non-linear fitting to a sigmoidal dose-response curve using GraphPad Prism 4.0. (La Jolla, CA, USA).

Western blotting. Cell lysates were produced using lysis buffer (PhosSTOP Phosphatase Inhibitor Cocktail and complete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and protein concentration was quantified measuring the absorbance at 260 nm and controlling the correct protein loading on the gel by immunoblotting of GAPDH. For experiments where cells were pre-treated with an inhibitor, the incubation period was 24 hours. This shorter incubation period when compared with the cytotoxicity experiments was chosen to demonstrate a putative stress response of the NFκB pathway, which is known to be regulated within hours. A total of 250,000 cells were seeded in 4 ml medium; after overnight incubation, the medium was exchanged and drugs were added (5 μM for TPL2 and BAY1170-82). DMSO was used as control. Prepared lysates were stored at −80°C until blotting. Protein samples (10 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) and transferred to a polyvinylidene fluoride membrane (ThermoFisher Scientific, Waltham, MA, USA) by semidyrib transfer (BioRad, Hercules, CA, USA). The membrane then was blocked overnight at 4°C in SuperBlock Blocking Buffer (Pierce, ThermoFisher Scientific) and 0.05% Tween (Merck, Billerica, MA, USA), followed by incubation with primary/secondary antibody for 1 hour at room temperature and washing (in 1×Tris-buffered saline and 0.05% Tween for 1 hour). Visualization of the signal was performed using Super Signal West Dura Extended Duration Substrate (ThermoFisher Scientific) reagents (ThermoFisher Scientific) and Amersharm Hyperfilm (GE Healthcare, Little Chelfont, UK).

Statistical analysis. The mean values and standard deviations were calculated for each point from the pooled normalized data in GraphPad Prism 4.0. Dose–response curves to evaluate the IC₅₀ were obtained by non-linear fitting to a sigmoidal model.

Results

Proteasome inhibitors induce cell death and overcome resistance. Based on the hypothesis that activation of NFκB is associated with the development of chemoresistance, we examined the cytotoxic effect of inhibitory strategies on the
targets NFκB and MAP3K8 by using MTT assays. Considering the complexity of NFκB signaling, we assessed several upstream and downstream sites within the NFκB activation pathway, e.g., inhibition of phosphorylation of IκB, inhibition of the proteasome and inhibition of the translocation of NFκB dimers to the nucleus (65). In order to elucidate the relevance of NFκB degradation for cell survival, we used two proteasome inhibitors known to act on NFκB: disulfiram and MG-132. Exposure to disulfiram resulted in a cytotoxic response in all cell lines at concentrations in the low micromolar range (data not shown). At one order of magnitude lower and independently of the degree of chemoresistance, MG-132 induced cell death in all cell lines at similar concentrations, with a mean IC₅₀ of 0.21 μM (Figure 1).

Inhibitors of IKK and their efficacy in abolishing resistance. These results prompted us to address the phosphorylation of IκB via IKK as a pivotal step in canonical NFκB activation, that is followed by degradation of the inhibitory molecule IκB by the proteasome. To evaluate potential differences between these two relevant steps, we investigated the effect of three IKK inhibitors, namely violacein, lupeol and BAY1170-82. Treatment with violacein exerted inhibitory effects on native as well as on resistant phenotypes with concentrations in excess of 100 μM (data not shown). Marginally more active, lupeol was able to eradicate all cellular subclones independently of their grade of resistance, but only at concentrations around 60 μM (Table I). In contrast, exposure to BAY1170-82, a specific inhibitor of both IKKα and IKKβ (66), resulted in a significant cytotoxic response, with an average IC₅₀ value of 0.296 μM, remarkably lower than those recorded with violacein, and lupeol (Figure 2). Moreover, BAY1170-82 showed a trend towards having higher cytotoxic activity against the resistant subclones.

NFκB activation. Given that our chemoresistant model was sensitive to NFκB inhibition, we blotted the representative subunit p65 together with its phosphorylated variant to determine the degree of NFκB activation in relation to the resistance phenotype. In agreement with the results described above, NFκB was highly activated, with strong signals for phospho-p65 in all resistant subclones (Figure 3). In contrast, neither of the native cell lines, SW480 and SW620, exhibited any sign of NFκB activation at all.

MAP3K8 inhibition leads to cytotoxic response. Having demonstrated the relevance of NFκB in this cell model, the possible role of MAP3K8 in drug resistance was investigated, although its association with NFκB remains not fully

### Table I. 50% Inhibitory concentration (IC₅₀) values for parental SW620 cells, and low-resistance [LR, 5 μM 5-fluorouracil (5-FU)], intermediate- (IR, 25 μM 5-FU) and high-resistance (HR, 125 μM 5-FU) phenotype SW620 cells treated with lupeol, BAY1170-82, MG-132 and mitogen-activated protein kinase kinase kinase 8 (MAP3K8) inhibitor. Data are the mean±standard deviation. IC₅₀ concentrations were derived from the dose-response curves after 144-h incubation using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

<table>
<thead>
<tr>
<th>Substance</th>
<th>SW620</th>
<th>LR</th>
<th>IR</th>
<th>HR</th>
</tr>
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<tbody>
<tr>
<td>Lupeol</td>
<td>97.6±35.5</td>
<td>84.6±37.0</td>
<td>52.8±18.9</td>
<td>80.4±19.7</td>
</tr>
<tr>
<td>BAY1170-82</td>
<td>0.461±0.148</td>
<td>0.288±0.136</td>
<td>0.226±0.147</td>
<td>0.211±0.275</td>
</tr>
<tr>
<td>MG-132</td>
<td>0.177±0.132</td>
<td>0.180±0.045</td>
<td>0.155±0.017</td>
<td>0.309±0.215</td>
</tr>
<tr>
<td>TPL2 Inhibitor</td>
<td>3.83±0.03</td>
<td>7.70±0.92</td>
<td>4.83±1.82</td>
<td>2.12±0.22</td>
</tr>
</tbody>
</table>
understood. Inhibition of MAP3K8 had a remarkable and similar cytotoxic effect on all resistant cell lines. Given an IC₅₀ of 3.8 μM against the parental cell line SW620, there was no statistically significant difference for the resistant subclones (low-resistance phenotype: 7.7 μM, high-resistance phenotype: 2.1 μM; Table I). The sensitivity was therefore in the range of IC₅₀ values typically recorded upon exposure to 5-FU in the parental colorectal cancer cells in vitro. In addition to a direct cytotoxic effect, the dose-response curve exhibited a cytostatic effect for a small fraction of cells, indicating a remaining fraction of viable, but resting (or senescent) cells (data not shown). MAP3K8 was active in all resistant subclones, with complete abrogation of 5-FU resistance.

**Targeting AKT.** To evaluate the role of the AKT pathway in our cell model, we examined cytotoxicity in a vertical sequence using an inhibitor of phosphoinositide 3-kinase (PI3K) (wortmannin), an AKT inhibitor and rapamycin (inhibitor of mammalian target of rapamycin (mTOR)). Interestingly, wortmannin, acting upstream of AKT, did not exert any inhibitory effect up to concentrations of 100 μM in our model (IC₅₀ always >100 μM). Inhibition of AKT for 72 h, however, resulted in a strong cytotoxic response. With IC₅₀ values consistently below 1 μM, we were able to completely overcome resistance (Table II). Targeting mTOR (downstream of AKT) via rapamycin for 72 h was less effective in the cell lines, but able to circumvent resistance at IC₅₀ concentrations, which were approximately 10-fold higher when compared with AKT inhibition (Table II).

**Effect of BAY1170-82 and MAP3K8 inhibition on downstream proteins.** Since NFκB and MAP3K8 inhibition led to significant cytotoxic effects, we wanted to further elucidate their effects on proteins downstream in the signalling cascade. With this aim, the protein expression of ERK, phospho-ERK, p65 and phospho-p65 were analysed after MAP3K8 inhibition and treatment with BAY1170-82, both active principles in abrogating chemoresistance. As an
upstream activator of ERK, the effect of MAP3K8 inhibition on NFκB activity (p65 as readout) was also evaluated to investigate a possible interaction with NFκB signalling. For this purpose, only SW620 and the high-resistance subclone were analysed, since data derived from western blots indicated very similar levels of phospho-p-65 in all resistant cell lines (see above). As demonstrated by immunoblotting, the signal for ERK was similar in all investigated cell lines. Unexpectedly, phospho-ERK was enhanced upon treatment with the MAP3K8 inhibitor in SW620 and the high-resistance subclone (Figure 4). Although BAY1170-82 induced phospho-ERK in SW620, the signal in the high-resistance subclone was unchanged (data not shown). Total p65 levels were also similar in all investigated cell lines with minor increases in the MAP3K8, and BAY1170-82-treated high-resistance subclone. Activated phospho-p65 was detected in samples treated with MAP3K8 inhibitor, as well as in samples treated with 5-FU, without significant difference when comparing the high-resistance subclone with the parental cell line (data not shown).

Combination effects. As a stress response, activation of NFκB signalling might contribute to acquisition of resistance in cancer cells. To evaluate a possible synergistic cytotoxic effect, the NFκB inhibitor BAY1170-82 was added to cells in combination with 5-FU. This combination only led to a marginal decrease in IC_{50} of approximately 25%. This suggests that combination approaches might not substantially add to the effect already achieved when chemoresistance was completely abrogated, even in totally 5-FU-insensitive colonic cancer clones with a variety of NF-κB targeting agents.

Discussion

NFκB as well as MAP3K8 play a pivotal role in cancer progression and have been associated with resistance to several antitumor drugs. Interestingly, there are data linking NFκB and MAP3K8 with the well-known regulator of survival and apoptosis AKT. In this study, we wanted to investigate the potential influence of NFκB, MAP3K8 and AKT signalling on chemoresistance to 5-FU in our multi-stage resistant colonic carcinoma model. Phosphorylation of p65 enables NFκB protein to bind DNA efficiently and is required for optimal activation of the NFκB pathway (67, 68). Indeed, we found evidence for constitutive activation of NFκB, as we found phospho-p65 to be highly expressed in the resistant phenotypes, whereas phospho-p65 was not detected in our native cell lines, SW480 and SW62, at all. This finding indicates that resistant cell lines rely on NFκB signalling to actively maintain resistance. Interestingly, Lewander and co-workers described the serine 536-phosphorylation status of p65 as a negative predictor of survival in patients with colorectal cancer (69). According to this assumption, our results of NFκB inhibition in resistant cell lines further illuminate the importance of this pathway. All inhibitors were able to inhibit growth and to cause cell death in our in vitro model. Taken together, in accordance with previous studies (34, 35, 70), we have showed that inhibition of NFκB results in a cytotoxic response of chemoresistant colorectal cell lines, suggesting that resistance in our model is dependent on NFκB.

Furthermore, we wanted to investigate possible differences between several levels in the NFκB activation pathway we inhibited (proteasome and kinase inhibitors). Using prostate carcinoma cells, Gasparian and co-workers found proteasome inhibitors to be more effective than inhibitors of phosphorylation in blocking NFκB activity (71). In our model, we found similar results upon inhibition of NFκB via the proteasome (MG-132) and via kinase inhibitor (BAY1170-82). One shortcoming of this approach is that proteasome inhibitors act very non-specifically. The fruit and vegetable triterpene lupeol, which inhibits NFκB as well as the PI3K pathway, was able to circumvent resistance, but was less efficient than the kinase inhibitor BAY1170-82. BAY1170-82 specifically blocks both NFκB-activating pathways as it targets IKKα as well as IKKβ. Between these, the canonical NFκB pathway is mostly dependent on IKKβ activity, whereas the alternate pathway requires only IKKα.
(53, 72). Most interestingly, it is known that IKKβ activation is also required for activation of MAP3K8 in macrophages (55). As described above, MAP3K8 (TPL2) and NFκB share molecular elements that link activation of one to the other. Kane and co-workers detected transport of p65 to the nucleus in HeLa and Jurkat T-cells expressing wild-type MAP3K8, whereas kinase-inactive MAP3K8-expressing cell lines were not able to do so (62). Hence we investigated MAP3K8 signalling and a possible cross-talk with NFκB. Using viability assays, we showed that MAP3K8 inhibition is able to produce a cytotoxic response in chemoresistant cell lines. No influence of MAP3K8 inhibition on activation of NFκB (phosphorylation of p65) was observed, since MAP3K8 inhibition did not suppress the phospho-p65 signal but, on the contrary, increased it. This suggests that MAP3K8 inhibition does not inhibit NFκB activity directly. Yet MAP3K8 is known to act mainly via phosphorylation of ERK. Interestingly, our data showed an increase of phospho-ERK after inhibition of MAP3K8 in SW620 cells and in the high-resistance cell line. This result was rather unexpected, since MAP3K8 inhibitor is believed to bind the ATP binding box competitively, thereby preventing phosphorylation of target proteins such as ERK. Regarding these data, one could speculate that other compensatory pathways might be activated upon MAP3K8 inhibition.

It is known that AKT can activate MAP3K8 induction of NFκB-dependent transcription in T-cells (62), which might differ considering the distinct cellular background in our model. There are, however, data linking inhibition of PI3K to overcoming 5-FU resistance via reducing phospho-AKT and phospho-NFκB in gastric cancer cells, as well as the description of AKT-dependent NFκB activation reducing stress-induced apoptosis in colon cancer (59, 73). In agreement with these investigations, we suggest that AKT plays a critical role in sustaining chemoresistance in our cell model via activation of MAP3K8 and activation of NFκB target genes. Since inhibition of neither PI3K nor mTOR effectively inhibited the resistant cell strains, we consider the signalling axis AKT–MAP3K8–NFκB as being valuable in our model. Targeting this axis via molecular inhibitors may be a promising tool for overcoming chemoresistance in patients with colonic cancer, since inhibition of the single pathways does abrogate the acquired resistance phenotype. In order to address possible side-effects, the NFκB inhibitor disulfiram has been used for decades as an anti-alcoholism drug, with mostly mild adverse events (70, 74). Pierce and co-workers treated mice intraperitoneally with up to 20 mg/kg of BAY1170-82, a substance chemically and functionally very similar to BAY1170-82, and did not observe overt toxicity (75). For the less-investigated MAP3K8, it is known that MAP3K8−/− mice are viable but display altered immune responses (76).

In conclusion, our results suggest that chemoresistance to 5-FU in this model of colonic carcinoma (cell lines SW480 and SW620) is strongly dependent on NFκB activation. The efficacy of MAP3K8 inhibition in our multi-stage resistance model potentially uncovers a new approach to circumventing resistance. Considering that there are few data on the specific kinase functions of MAP3K8 and its target genes or client proteins, our data link MAP3K8 inhibition to resistance to 5-FU in colorectal cancer for the first time. Besides NFκB, AKT signaling also plays a pivotal role, indicating that pharmacological stress tolerance to 5-FU activates at least three druggable pathways in colonic cancer in vitro.

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References


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