Thiazolides promote G1 cell cycle arrest in colorectal cancer cells by targeting the mitochondrial respiratory chain

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Abstract

Systemic toxicity and tumor cell resistance still limit the efficacy of chemotherapy in colorectal cancer. Therefore, alternative treatments are desperately needed. The thiazolide Nitazoxanide (NTZ) is an FDA-approved drug for the treatment of parasite-mediated infectious diarrhea with a favorable safety profile. Interestingly, NTZ and the thiazolide RM4819—its bromo-derivative lacking antibiotic activity—are also promising candidates for cancer treatment. Yet the exact anticancer mechanism(s) of these compounds still remains unclear. In this study, we systematically investigated RM4819 and NTZ in 2D and 3D colorectal cancer culture systems. Both compounds strongly inhibited proliferation of colon carcinoma cell lines by promoting G1 phase cell cycle arrest. Thiazolide-induced cell cycle arrest was independent of the p53/p21 axis, but was mediated by inhibition of protein translation via the mTOR/c-Myc/p27 pathway, likely caused by inhibition of mitochondrial respiration. While both thiazolides demonstrated mitochondrial uncoupling activity, only RM4819 inhibited the mitochondrial respiratory chain complex III. Interestingly, thiazolides also potently inhibited the growth of murine colonic tumoroids in a comparable manner with cisplatin, while in contrast to cisplatin thiazolides did not affect the growth of primary intestinal organoids. Thus, thiazolides appear to have a tumor-selective antiproliferative activity, which offers new perspectives in the treatment of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide (IARC, Cancer Surveillance Section, WHO, 2018). Despite the fact that the incidence rate of CRC is decreasing thanks to early diagnosis and prompt removal of precancerous lesions, the prognosis for advanced stages and metastatic CRC is still grim. Chemotherapy is regarded as standard treatment for patients with advanced CRC. The typical first-line treatment is currently a combination of the antimetabolite 5-fluorouracil (5-FU) and its adjuvant leucovorin, together with DNA-damaging agents (e.g., oxaliplatin and/or irinotecan) [1, 2]. However, development of resistance of tumor cells to chemotherapy, next to systemic toxicity, is limiting the therapeutic efficacy of these approaches [3]. Furthermore, despite the fact that immunotherapy is emerging as groundbreaking treatment for several types of tumors, CRC is currently one of the types of cancer for which immunotherapy has been less effective [4]. Thus, new alternative and targeted therapies are urgently needed.

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Drug repositioning, also known as drug repurposing or redirecting, is a promising strategy by screening already existing drugs for alternative clinical applications [5]. Nitazoxanide (NTZ) (Supplementary Fig. S1) is an FDA-approved antiprotozoal drug for the treatment of infectious diarrhea caused by the intestinal parasites *Cryptosporidium parvum* and *Giardia intestinalis* [6]. On the market since 2004, NTZ has been used to safely treat millions of patients, both infants and adults. Due to its favorable safety profile (only few side effects are known), several studies addressed NTZ drug repurposing. Of interest, NTZ is also active against a broad range of anaerobic bacteria, like *Helicobacter pylori* and *Clostridium difficile* [7–10], replicating and nonreplicating *Mycobacterium tuberculosis* [11–13], helminths [14–18] as well as viruses [19–24]. Surprisingly, several studies also reported NTZ as a potential anticancer agent [25, 26]. Müller et al. firstly reported the antitumor effect of NTZ in CRC cell lines by inducing apoptosis in a glutathione-S-transferase P1 (GSTP1)-dependent manner [27]. Subsequently, various high-throughput screenings confirmed the anticancer activity of NTZ in 2D and 3D culture systems of tumor cells. While activities of NTZ on a broad range of cellular pathways, e.g., metabolism, proliferation, and autophagy, have been reported, no clear mechanism of antitumor activity has been described so far [28–31]. Thus, further investigations are required to shed light on the antitumor activity of NTZ to fully exploit its anticancer potential.

Chemical modifications of the lead compound NTZ have given rise to a number of derivatives. Several modifications aimed to replace the reactive nitro group of NTZ with halides. Thus, bromo- and chloro-thiazolides are currently investigated for their antiviral and anticancer activity [27, 32–36]. On the background of increasing antibiotic resistance [37, 38] and cumulating knowledge about the relevance of a healthy microbiome in the regulation of intestinal homeostasis [39, 40], thiazolides lacking antibiotic effects appear to be the preferred lead compounds for anticancer treatment. Interestingly, the bromo-thiazolide RM4819 (Supplementary Fig. S1) showed a high antitumor activity by promoting apoptosis in several colon carcinoma cell lines [27, 32], in the absence of antiparasitic or antibacterial activity [41]. We previously reported that the apoptosis-inducing activity of thiazolides in CRC cells involves GSTP1 enzymatic activity and the induction of the pro-apoptotic Bcl-2 homologs Bim and Puma [32]. However, the upstream events responsible for RM4819-induced cell death are so far still unclear. In this study, we thus aimed to unravel the chemotherapeutic potential of RM4819 in the treatment of CRC, with a special focus on RM4819-triggered signaling processes prior to execution of apoptosis.

Moreover, we systematically compared the effect of RM4819 with that of the parent compound NTZ in 2D as well as 3D culture system of CRC cell lines and primary tumors. A systematic comparison was necessary to highlight differences and similarities between the two compounds, which could be exploited for a rational clinical application. Our work confirmed that both thiazolides have a relevant antitumor activity. While RM4819 and NTZ differed in their apoptosis-inducing activities, both thiazolides strongly impaired G1/S transition and associated proliferation due to a direct effect on mitochondrial oxidative phosphorylation. Both thiazolides act as mitochondrial uncouplers, however, RM4819 additionally blocks complex III of the mitochondrial respiratory chain (MRC), thus being more potent than NTZ. Of interest, while thiazolides blocked tumor cell growth, they did not harm primary intestinal epithelial cells. Given its specific antitumor activity and the lack of antibiotic activity, the bromo-thiazolide RM4819 could be an interesting drug for cancer treatment.

Results

RM4819 and NTZ induce G1 cell cycle arrest, prior or in the absence of apoptosis induction

In order to investigate the activity of RM4819 in CRC cells, and to compare it with the partially described activities of NTZ, we assessed the pro-apoptotic effects of both thiazolides (tested at 20 μ M) in the colon carcinoma cell lines Caco-2, LS174T, and HCT116, revealing cell line-specific results. In LS174T cells, we observed 50% and 20% of apoptotic cells after 72 h of RM4819 or NTZ treatment, respectively. In HCT116 cells 20%, respectively 40% apoptosis induction was observed. In contrast, in Caco-2 cells no significant thiazolide-induced apoptosis was detected at any time point investigated (Fig. 1a). In LS174T cells RM4819-induced apoptosis was also associated with increased levels of cleaved caspase 3 and PARP, and in NTZ-treated HCT116 cells with cleaved PARP (Fig. 1b). In contrast, no significant changes of these apoptotic markers could be observed in thiazolide-treated Caco-2 cells (Fig. 1b). These results suggested that LS174T cells strongly responded to RM4819 treatment by induction of apoptosis, while they appeared to be resistant to NTZ, and the opposite effect was observed for HCT116 cells. Surprisingly, Caco-2 cells did not undergo cell death induction upon treatment with either of the thiazolides. Interestingly, RM4819 strongly reduced the potential of LS174T and Caco-2 cells to form colonies after 3 days of treatment, independently to their sensitivity towards

thiazolide-induced apoptosis (Fig. 1c). In contrast, NTZ was able to inhibit the clonogenic potential of Caco-2 cells but not of LS174T cells (Fig. 1c). In addition, a crystal violet-based proliferation assay revealed growth inhibition already after 48 h of treatment with RM4819 in all three cell lines investigated. In a similar fashion, NTZ strongly inhibited Caco-2 and HCT116 proliferation whereas no significant

effect on cell growth could be detected in LS174T cells (Fig. 1d).

Altogether these results indicate a prominent anti-proliferative effect of RM4819 and NTZ, which cannot be solely explained by cell death induction. We therefore investigated whether thiazolides could directly impair cellular proliferation by triggering cell cycle arrest. In all

◀ **Fig. 1 Thiazolides induce G1 cell cycle arrest independent of apoptosis induction.** **a** Flow cytometric analysis of cell death by AnnexinV staining in LS174T, Caco-2, and HCT116 cells upon treatment with DMSO control (ctrl), 20 μ M RM4819, 20 μ M NTZ, 33.3 μ M (LS174T and Caco-2), or 66.6 μ M (HCT116) Cisplatin for 24, 48, or 72 h. Representative results from two independent experiments are shown as mean $\pm$ SEM. **b** Immunoblots of PARP, cleaved (cl.) PARP, cleaved caspase 3 (cl C3), and tubulin from LS174T, Caco-2, HCT116 cells after 24 h treatment of drugs as described in **a**. Representative results of three independent experiments are shown. **c** Colony formation assay of LS174T and Caco-2 cells treated for 3 days as in **a**. Representative results of three independent experiments are shown. **d** Crystal violet-based proliferation assay of LS174T, Caco-2, HCT116 cells treated as in **a** for 6, 24, 48, or 72 h. Representative results of three independent experiments are shown as mean $\pm$ SEM. **e** Contour plot of flow cytometric cell cycle analysis in LS174T, Caco-2, and HCT116 cells after 24 h of treatment with DMSO control, 20 μ M RM4819, 20 μ M NTZ, 150 ng/ml Nocodazole, 33.3 μ M (LS174T and Caco-2), or 66.6 μ M (HCT116) Cisplatin. **f** Relative quantification of the experiment shown in **e**. Bar graph represents the mean $\pm$ SD of three independent experiments. Sno prol, S phase with no EDU incorporation. ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA. **g** Representative immunoblots of cyclin B1, cyclin E1, and tubulin in Caco-2, LS174T, HCT116 cells 24 h after treatment as

three cell lines RM4819 treatment resulted in a strong accumulation of cells in G1 phase, while the percentage of cells in proliferating S phase (EdU positive) was reduced almost completely when compared with control-treated cells (Fig. 1e, f). A similar effect could be observed for NTZ-treated Caco-2 and HCT116 cells, whereas only minor differences in cell cycle distribution was detected in NTZ-stimulated LS174T cells.

Interestingly, in Caco-2 cells also an increase in S phase in the absence of EdU incorporation (Sno prol) was noticed. To further investigate this atypical behavior, nocodazole was used to synchronize Caco-2 cells in the G2M phase (Supplementary Fig. S2a). After removal of nocodazole block, synchronized Caco-2 cells were treated with medium control or RM4819, and bidimensional cell cycle analysis was performed after 9, 12, 24, and 48 h post treatment. Again, cells accumulated in G1 phase until 24 h, however, after 48 h a partial progression into S phase was detectable (Supplementary Fig. S2b), suggesting that some cells were able to escape the G1 arrest and slowly progressed to, respectively arrested in the S phase. Again, we noticed an almost complete inhibition of EdU incorporation. In line with the strong accumulation in G1, in all cell lines a decrease in cyclin E1 and B1 expression was noticed after RM4819 treatment. Similarly, cyclin E1 and B1 levels were reduced in NTZ-treated Caco-2 and HCT116 cells, whereas no significant changes appeared in NTZ-treated LS174T cells (Fig. 1g), consistent with the apparent inability of NTZ at 20 μ M to promote any effects in this cell line (Fig. 1b–d).

Thus, both thiazolides strongly blocked proliferation by arresting cells in the G1 phase, independently of apoptosis

induction. Moreover, the strong differences in activity of RM4819 compared with NTZ in LS174T at 20 μ M suggested that the bromo-thiazolide could have different and/or more potent effects than the parent compound NTZ. To test for a potency-offset, LS174T cells were treated with 40 μ M NTZ. Interestingly, at this concentration NTZ treatment also resulted in a strong G1 arrest, time-dependent induction of apoptosis and decreased clonogenicity, comparable with the RM4819-induced anticancer effects at 20 μ M (Supplementary Fig. S3). Thus, RM4819 and NTZ appear to act in a comparable manner, yet with different potencies.

Thiazolides induced G1 arrest is not mediated by the p53/p21 axis

To investigate the underlying mechanism(s) of RM4819- and NTZ-induced G1 arrest we focused on key regulators of G1/S transition. Cyclin D/CDK4 and Cyclin E/CDK2 complexes critically control G1/S transition by inhibiting the E2F-repressor protein Rb and activating genes required for S phase entry [42]. Cyclin/CDK complexes are tightly controlled by their negative regulators, CDK inhibitors (CDKIs). p21^{Cip1/WAF1} strongly inhibits G1/S transition, e.g., during DNA damage-induced G1 arrest downstream of p53 family-mediated pathways [43]. Immunoblots were used to assess the activation of p53 family/p21 pathway in CRC cell lines upon treatment with thiazolides. Caco-2 cells have a point mutation (Glu>Stop) in the *TP53* gene resulting in no detectable p53 protein [44], suggesting that p53 is not involved in thiazolide-induced cell cycle arrest. Interestingly, Caco-2 cells showed a clear reduction, rather than upregulation/stabilization, of p73 protein and no increase in p21 levels within 72 h of thiazolide treatment (Fig. 2a, left panel). In the LS174T and HCT116 cells, which are p53 wild type, neither a p53 stabilization nor p73 and/or p21 upregulation could be detected upon treatment with NTZ or RM4819, in contrast to cisplatin treatment (Fig. 2a, b). In addition, assessment of DNA strand breaks by fluorimetric analysis of DNA unwinding revealed no evidence for RM4819-induced DNA damage in LS174T cells (Supplementary Fig. S4a). To further exclude a role of p53 in thiazolide-induced G1 arrest, wild type HCT116 cells were compared with Crispr/Cas9-generated p53 knockout cells. While in parental HCT116 cells a robust induction of p53 upon treatment with cisplatin was apparent, no p53 protein could be detected in knockout cells (Fig. 2c). A comparable thiazolide-induced accumulation of cells in G1 phase and reduction in S phase was observed in both cell lines, indicating that p53 is not involved in thiazolide-induced G1 arrest (Figs. 2d and S4b). Surprisingly, we also observed that HCT116 p53-deficient cells were more sensitive to thiazolides induced cell death than parental cells, while more resistant to cisplatin (Fig. 2e).

Fig. 2 Thiazolides induced G1 cell cycle arrest is independent of the p53/p21 axis. Immunoblots for p73, p53, and p21 in **a** Caco-2 and LS174T, and **b** HCT116 cells upon treatment with ctrl (DMSO), 20 μ M RM4819, 20 μ M NTZ, 33.3 μ M (LS174T and Caco-2), or 66.6 μ M (HCT116) Cisplatin for 24, 48, 72 h (**a**) and 24 h (**b**). Representative results from three independent experiments are shown. **c** Immunoblot of p53 and tubulin in HCT116 parental or p53 KO cells

(p53^{-/-}) 24 h after treatment with 20 μ g/ml Cisplatin. **d** Quantification of flow cytometric cell cycle analysis and **e** quantification of cell death measured by AnnexinV positive cells of parental or p53-deficient HCT116 cells 24 h after treatment with ctrl (DMSO), 20 μ M RM4819, 20 μ M NTZ, or 66.6 μ M Cisplatin. Bar graph represents the mean $\pm$ SD of three independent experiments. **** p < 0.0001, two-way ANOVA.

Thiazolides induce c-Myc/p27-mediated G1 arrest

Next to p21 also p27 is a member of the Cip/Kip family of CDKIs that inhibit G1/S transition upon nutrient deprivation or lack of mitogenic stimuli [45]. We therefore determined whether the cytostatic effect of thiazolides was mediated by p27. Indeed, RM4819 increased p27 levels time dependently in Caco-2 and LS174T cells, and NTZ in Caco-2 cells, in line with its efficacy to induce G1 arrest (Figs. 3a and 1e, f). Cellular p27 protein levels are primarily controlled by its degradation in a SCF^{SKP2} ubiquitin ligase complex-dependent manner. Furthermore, cyclin E/CDK2-mediated p27 phosphorylation is essential for recognition by SCF^{SKP2}. Since c-Myc regulates the expression of *CycE*, *Skp2*, and *E2F*, it indirectly, but strongly, regulates p27 protein stability [46]. Therefore, we determined the role of the c-Myc oncoprotein on thiazolide-triggered p27 stabilization. Immunoblots of c-Myc in NTZ-treated Caco-2 cells and RM4819-treated Caco-2 and LS174T cells revealed a time-dependent reduction of c-Myc protein (Fig. 3b), which correlates with the observed p27 stabilization (Fig. 3a). To assess whether this decrease of c-Myc protein was due to altered protein stability, the kinetics of c-Myc decay were analyzed. Cycloheximide treatment revealed that neither RM4819 nor NTZ accelerated c-Myc turnover (Fig. 3c). Furthermore, inhibition of

c-Myc degradation by the proteasome inhibitor MG132 could only partially prevent the loss of cellular c-Myc protein in thiazolide-treated Caco-2 and LS174T cells (Fig. 3d). Thus, both thiazolides did not alter c-Myc protein stability. Moreover, no significant reduction of *MYC* mRNA level could be detected in Caco-2 and LS174T cells upon RM4819 or NTZ treatment (Fig. 3e), while a strong decrease in mRNA for the c-Myc target genes *CycE1* and *CycD1* could be observed (Supplementary Fig. S5), suggesting that thiazolides also do not impair *MYC* transcription.

We therefore investigated whether the observed decrease of c-Myc protein could be due to a direct effect of thiazolides on c-Myc protein synthesis. Several studies already reported the possibility to target c-Myc translation in CRC via inhibition of the mTORC1 pathway [47]. We therefore monitored the effect of RM4819 and NTZ on direct downstream targets of mTORC1, the eukaryotic initiator factor 4E binding protein 1 (4EBP1) and the ribosomal subunit S6 kinase (S6k or p70S6k). Indeed, immunoblots for activated (phosphorylated) p70S6K (p-p70S6K) and inhibited (phosphorylated) 4EBP1 (p-4EBP1) showed decreased phosphorylation of both proteins in RM4819-treated Caco-2 and LS174T cells, and in NTZ-treated Caco-2 cells already after 4 h of treatment, revealing strong mTORC1 inhibition. Moreover, NTZ at concentrations not

Fig. 3 Thiazolide induce p27 stabilization via reduction of c-Myc protein translation. Immunoblots for p27 (a) and c-Myc (b) in Caco-2 and LS174T cell lines upon treatment with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ for 24, 48, and 72 h. Tubulin was used as loading control. Representative results from two independent experiments are shown. **c** Immunoblots of c-Myc in Caco-2 and LS174T cells treated with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ in the presence of 20 μ M Cycloheximide (CHX) for indicated time points. **d** Representative immunoblots of c-Myc in Caco-2 and

LS174T cells treated with 5 μ M MG132 or DMSO control ctrl, and 20 μ M RM4819 or 20 μ M NTZ for 8 h. **e** mRNA level analyzed by qPCR of c-Myc after 6 h of treatment with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Bar graph represents the mean $\pm$ SD of two independent experiments. **f** Immunoblots of p-p70S6K and p-4EBP1 in Caco-2 and LS174T cells upon treatment with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ for 2, 4, and 6 h. Tubulin was used as loading control. Representative results of three independent experiments are shown. Numbers in western blots indicate densitometric quantification.

causing cell cycle arrest clearly did also not affect the mTORC1 pathway (Fig. 3f). These results suggested that thiazolide-induced G1 arrest is mediated by mTOR inhibition, and c-Myc depletion-induced p27 stabilization.

RM4819 and NTZ induce energy stress by targeting oxidative phosphorylation

Since inhibition of mitochondrial respiration by NTZ has been previously linked to the inhibition of the mTOR pathway [30], we evaluated whether RM4819-mediated mTOR inhibition was related to altered mitochondrial respiration and associated energy deficiency. Cellular ATP deficits normally lead to the activation of AMP-activated

protein kinase (AMPK) [48]. Increased AMPK phosphorylation, i.e., activation, was observed in both cell lines upon thiazolide treatment (Fig. 4a). In parallel, also intracellular ATP levels dropped (Fig. 4b). A strong compensatory increase in lactate secretion (proxy for compensatory glycolysis) was observed for both cell lines upon thiazolide treatment (Fig. 4c). Moreover, strongly increased extracellular acidification rates (ECARs), a measure for compensatory glycolysis, were observed for NTZ and RM4819 in both cell lines (Fig. 4d). This suggests that thiazolides may cause impaired mitochondrial energy production and associated mTOR-dependent inhibition of protein translation.

Thus, mitochondrial functionality was further investigated. Since NTZ has been described as mitochondrial

Fig. 4 RM4819 and NTZ induce energy depletion by directly targeting mitochondrial oxidative phosphorylation. **a** Immunoblot for pAMPK in Caco-2 and LS174T cells upon 24, 48, 72 h treatment with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Tubulin was used as loading control. Representative results from two independent experiments are shown. **b** ATP levels and **c** relative lactate secretion of LS174T and Caco-2 cells 8 h after treatment with ctrl (DMSO), 20 μ M RM4819, 20 μ M NTZ, or 1 μ M rotenone. Bar graph represents the mean $\pm$ SD of two to three independent experiments. Each data point represents one independent experiment. **d** Extracellular acidification

rates of LS174T and Caco-2 cells directly after injection of ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Bar graph represents the mean of independent experiments $\pm$ SD. Each data point represents one independent experiment. **e** Coupling efficiency assay in LS174T and Caco-2 cells treated with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Bar graph represents the mean of independent experiments $\pm$ SD. * p < 0.05, ** p 0.01, one-way ANOVA. Each data point represents one independent experiment. Numbers in western blots indicate densitometric quantification.

uncoupler [30], we investigated this potential toxicity mechanism of thiazolides. Cells were permeabilized to directly probe their mitochondrial function by using an established protocol [49]. Specific uncoupling effects were evaluated in the presence of oligomycin, an ATPase inhibitor. Under such conditions NTZ or RM4819 increased respiration by three- to fivefold (Fig. 4e). This indicates that both NTZ and RM4819 enable the translocation of protons across the inner mitochondrial membrane, bypassing the ATPase, thus being genuine uncouplers.

RM4819, but not NTZ, inhibits mitochondrial complex III

Since RM4819 has a different cytotoxicity spectrum than NTZ, we investigated additional effects on MRC complexes I–IV (cI–IV). Permeabilized cells were fed with specific substrates for each complex, and their respiratory activity was monitored. While NTZ did not alter the activity of cI–IV (Fig. 5a), RM4819 specifically inhibited cIII activity by ~50% in LS174T and Caco-2 cells. Thus, NTZ only acts as an uncoupler, while RM4819 additionally inhibits cIII.

In intact cells, RM4819 decreased oxygen consumption rate (OCR) in both cell types immediately after treatment by 50% (Fig. 5b), in line with its inhibition of respiration via

cIII. NTZ (20 μ M) did not immediately affect respiration in Caco-2 cells and it increased OCR in LS174T cells (uncoupling effect). These apparently inconsistent effects were explained by a complex concentration dependence. For both, NTZ and FCCP (a canonical uncoupler), a bell-shaped concentration-response was observed (Supplementary Fig. S6). At low/uncoupling concentrations, both compounds increased the respiration, but at higher concentrations the OCR was reduced. The respiration inhibition was stronger when drugs were preincubated for 2 h. This delayed failure of mitochondrial function after prolonged exposure to an uncoupler is a well-known phenomenon [30]. As Caco-2 cells are more sensitive to NTZ than LS174T cells, their balance of uncoupling and inhibition of respiration must be shifted. Exposure to NTZ (20 μ M) results in both, uncoupling and impairment of the MRC. These balance each other out, so that no net change is observable (Supplementary Fig. S6).

To further investigate the potency difference of the drugs, also the concentration response of RM4819 was investigated. No increased respiration was observed at any concentration as RM4819's cIII-inhibition always counteracts its uncoupling properties. RM4819's minimal effective concentration (IC_{25}) was at 7.5 μ M and >20 μ M for NTZ (Supplementary Figs. S7a and S6a). After 2 h preincubation,

Fig. 5 RM4819 but not NTZ strongly inhibits mitochondrial respiratory chain complex III. **a** Mitochondrial respiratory chain (MRC) complex inhibition assay in LS174T and Caco-2 cells treated with ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Effects >25% were considered to be biologically relevant. The gray shaded area indicates the no-response range. **b** Oxygen consumption rates of LS174T and Caco-2 cells directly after injection of ctrl (DMSO), 20 μ M RM4819, or 20 μ M NTZ. Bar graph represents the mean of independent experiments $\pm$ SD. **a, b** Each data point represents one

independent experiment. **c** Crystal violet-based proliferation assay of LS174T and Caco-2 cells treated with ctrl (DMSO), 1 μ M FCCP, 1 μ M Antimycin A (AA), or combination of FCCP and AA for 6, 24, and 48 h. Representative results from two independent experiments are shown as mean $\pm$ SEM. **d** Contour plot of flow cytometric cell cycle analysis in LS174T and Caco-2 cells 24 h after treatment as in **c**. **e** relative quantification of **d**. Representative results from two independent experiments are shown. Bar graph represents the mean of triplicates $\pm$ SEM.

the IC₂₅ of RM4819 was 5.2 and 18 μ M for NTZ (Supplementary Figs. S7b and S6b). We conclude that RM4819 was approximately three times more potent in impairing mitochondrial energy generation (Caco-2 cells).

Mitochondria-targeting agents may be useful for cancer therapy [50], but the underlying molecular mechanisms of their antitumor activities are still unclear. Providing a rationale linking G1 cell cycle arrest to impaired

mitochondrial respiration, we investigated whether FCCP, the cIII inhibitor antimycin A (AA), or a combination of both would mimic the effects of NTZ or RM4819. The combination of FCCP and AA clearly impaired proliferation (Fig. 5c) and caused the G1 arrest (Fig. 5d, e). Uncoupling alone (FCCP) was not sufficient to reduce the proliferation of LS174T cells, as also observed for NTZ (Fig. 5e). These data suggest that RM4819 double-effects on the MRC,

Fig. 6 RM4819 and NTZ strongly inhibit the proliferation of intestinal tumoroids without altering intestinal organoids growth. Representative microscopy images of **a** mouse intestinal tumoroids and **c** mouse intestinal organoids at day 3 of treatment with ctrl

(DMSO), 20 μM RM4819, 20 μM NTZ, or 66.6 μM Cisplatin. Scale bar = 200 μm. **b, d** Quantification of MTT-based viability assay of experiment shown in **a** and **c**. Bar graph represents the mean ± SD of three independent experiments.

followed by a drop of ATP and the associated shutdown of translation is the major cause of cell cycle arrest in CRC cell lines.

Thiazolides inhibit the proliferation of intestinal tumoroids, but not normal intestinal organoids

The potential use of thiazolides in CRC therapy will largely depend on their selectivity for tumor cells, while preventing side effects on normal tissue cells, such as the intestinal epithelium. Whereas the treatment of intestinal infections already confirmed a good safety profile for NTZ, it is currently not known how selective thiazolides are for intestinal tumor cells versus normal intestinal epithelium. RM4819 and NTZ were thus tested on 3D organoids of murine colonic tumors or normal intestine. While tumoroids grew in typical balloon-like structures, normal intestinal organoids showed typical crypt/villus structures (Fig. 6a, b). Interestingly, while the growth of normal intestinal organoids was completely unaffected by treatment with RM4819 and NTZ, both thiazolides inhibited the growth of tumoroids. Whereas growth rates were reduced, there was no obvious increase of thiazolide-induced cell death in tumor organoids. In marked contrast, cisplatin treatment resulted in the massive induction of cell death and associated growth reduction in both, tumoroids and normal intestinal organoids (Fig. 6a–d). Thus, RM4819 and NTZ, unlike the classical chemotherapeutic drugs cisplatin, show a high selectivity for transformed tumor cells, while sparing normal tissue cells.

Discussion

The efficacy of current cancer therapies is still limited by severe side effects on normal tissue, and the development of resistance to the drugs used. Cancer stem cells (CSC) are tumor-initiating cells capable of self-renewing and multi-lineage differentiation. Thus, selective survival of CSC is often responsible for treatment failure and cancer relapse [51]. Recent studies have highlighted that CSC, unlike mature cancer cells, strongly rely on energy production by mitochondrial oxidative phosphorylation, proposing mitochondrial-targeting drugs (mitocans) for anti-CSC therapy. Development of new mitocans is, however, challenged by the fact that also normal cells rely on mitochondrial ATP production, although to a different degree, possibly offering a window of opportunity [52]. In this regard, repositioning of old drugs with a good safety profile could represent an attractive new approach. The antidiabetic drug Metformin is a good example for this strategy, being currently proposed for anti-CSC therapy due to its modulatory inhibitory activity on mitochondrial cI [53].

In this study, we describe the thiazolide RM4819, a bromo-derivative of the FDA-approved antiparasitic drug NTZ, as an inhibitor of the mitochondrial cIII. Our mechanistic investigations aimed to compare the chemotherapeutic potential of RM4819 with its parent compound NTZ for the treatment of CRC. We find that both thiazolides arrest CRC cells in the G1 phase of the cell cycle, independent of their ability to cause cell death.

Although NTZ has been recently shown to induce autophagy-related cell cycle arrest in glioblastoma cell lines [28], we here demonstrate that in CRC cells thiazolide-induced G1 arrest is primarily regulated by the mTOR/c-Myc/p27 axis. Our study also revealed differences and similarities between these two compounds. Although the structures of the two thiazolides differ only minimally (Supplementary Fig. S1), RM4819 and NTZ have a diverse spectrum of activities. Both substances have a strong anti-tumor potential and both uncouple mitochondrial respiration (our study and ref. [30]). However, RM4819 has an additional and unique cIII inhibitory activity, which further potentiates its anticancer effect compared with NTZ. As RM4819 does not retain NTZ's antibacterial activity, it may also help to preserve a stable intestinal microbiome during cancer treatment. Thus, we propose that RM4819 should be further investigated for the treatment of cancer in general, and CRC in particular.

As mentioned above, potential side effects on vital tissues, such as the intestine, are limiting aspects of cancer therapy. Particularly encouraging in this regard is the good safety profile of NTZ. NTZ has been in clinical use since many years for the treatment of intestinal infections, with apparent minimal side effects on the intestinal mucosa. In line with this observation, we have seen previously that thiazolides had an antiproliferative activity in CRC cell lines, but much less in untransformed fibroblasts [27]. This indicates that the cytotoxic effect of thiazolides and possibly also the here studied antiproliferative effect of thiazolides is somehow restricted to tumor cells and very limited in primary untransformed cells. In line with this observation, we here showed that thiazolides potently inhibited the proliferation of tumor organoids, yet did not affect the growth of organoids from primary intestinal epithelium. While this selectivity is exciting and encouraging for the potential use of thiazolides in the clinics, it is at present difficult to explain mechanistically. One potential underlying reason may be related to our previous observation that inhibition of cell cycle prevented thiazolide-induced apoptosis in CRC cell lines [27]. As the proliferative potential of tumor cells is much higher than that of primary tissue cells, where only the stem and progenitor cells proliferate, the impact of thiazolides on primary tissue cells may be less pronounced. This could, however, not explain why the growth of normal intestinal organoids was also not affected by thiazolide treatment, even though these 3D cultures contain a high number of proliferating cells.

Thus, possibly this selectivity is related to other differences between primary and tumor cells. An attractive aspect is potential differences in the transcriptome and metabolome. In Caco-2 cells we have previously identified the GSTP1 as a major thiazolide-binding protein [27]. Furthermore, we have seen that thiazolide-induced cell death is

dependent on GSTP1 activity [32, 33]. Thus, likely thiazolides are a substrate of GSTP1, converting the prodrug into an active compound, which promotes cell cycle arrest and/or apoptosis. In line with this proposed mechanism is our observation that thiazolide-induced cell death is significantly enhanced upon co-treatment of cells with N-acetylcysteine, which is a precursor molecule in the glutathione synthesis pathway. This indicates that by increasing the intracellular glutathione levels more thiazolides can be conjugated to glutathione, thus resulting in more cell death in CRC cell lines. Interestingly, N-acetylcysteine co-treatment of cells has the opposite effect on cisplatin-induced apoptosis, as here glutathione leads to a detoxification of the cell and increased survival [32]. GSTs in general are often overexpressed in tumor cells compared with normal tissue cells, likely to cope with increased oxidative and chemical stress. In particular, GSTP1 is overexpressed in CRC compared with normal intestinal epithelium, thus potentially explaining why tumoroids and normal intestinal organoids respond differentially to thiazolide treatment, as shown in this study.

In summary, our study describes a novel mechanism of growth control by thiazolides in CRC cells via the inhibition of the mitochondrial respiration and associated cell cycle arrest. Furthermore, we show that the thiazolide RM4819 is not only a mitochondrial uncoupler, but also a cIII inhibitor. Last but not least, thiazolides appear to selectively target transformed tumor cells and spare primary tissue cells. Given its good safety profile and the lack of antibiotic activity RM4819 might represent an interesting lead compound for the development of novel thiazolide-based therapies for the treatment of CRC.

Materials and methods

Cell culture and reagents

The human CRC cell lines Caco-2 (ATCC HTB-37) and LS174T (ATCC CL-188) were obtained from American Type Culture Collection (ATCC) and cultured in Iscove's Modified Dulbecco's Medium. Parental HCT116 and p53-deficient HCT116 were kindly provided by Markus Morrison (Stuttgart) and cultured in RPMI-1640 medium. All cell culture media were supplemented with 10% fetal calf serum, 4 mM L-glutamine and 50 µg/ml gentamicin (all from Sigma-Aldrich, Steinheim, Germany). Cells were grown at 37 °C and 5% CO₂ in a humidified atmosphere and passaged at 80–90% confluency every 2–3 days.

NTZ was purchased from Sigma-Aldrich. RM4819 was synthesized in house as described earlier [48]. Thiazolides were kept as 20 mM stock solution in DMSO. Cisplatin was purchased from Sigma-Aldrich and stored at 4 °C as 1 mg/ml

sterile stock solution in H₂O, light protected. Nocodazole was obtained from Calbiochem (Darmstadt, Germany), Cycloheximide from INC Biomedicals (Eschwege, Germany), MG132 from Selleckchem (Houston, TX), FCCP, AA, and Rotenone from Sigma-Aldrich.

Generation of murine intestinal organoids and colonic tumoroids

Mouse intestinal organoids were generated and cultured as described in [54]. Colonic tumors were induced in C57BL/6 mice by the AOM/DSS model [55], and tumoroids were generated and cultured as described previously [56]. Representative pictures of organoids or tumoroids were taken using Zeiss AxioCam microscope.

MTT-based viability assay in 3D systems

Organoid and tumoroids viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as reported earlier [57].

Clonogenic assay

CRC cell lines were treated with indicated compounds for 72 h and subsequently replated in 24-well plates as serial dilutions. Cells were cultured for additional 6 days (Caco-2) or 12 days (LS174T). Colonies were fixed and stained with 5% crystal violet (ROTH, Karlsruhe, Germany) solution in 20% methanol for 15 min. Representative pictures of stained colonies were processed using the ColonyArea plugin of the ImageJ software version 2.0.

Crystal violet-based proliferation assay

Caco-2 (1000 cells/well), LS174T, and HCT116 (2000 cells/well) cells were seeded in 96-well plates and treated with thiazolides after overnight attachment. At indicated time points, medium was removed and cells were stained with 5% crystal violet solution for 15 min. After removal of the staining solution and subsequent washes, plates were air-dried for at least 24 h. Solubilization of the dye was performed in 100% methanol for 15 min. Absorbance was detected as OD 592 nm in a plate reader (Tecan M200 Pro).

Flow cytometric analysis of cell cycle and apoptosis

Cell cycle analysis was performed using the 647 EdU Click Proliferation Kit (BD Bioscience, San Jose, CA), in parallel with the detection of apoptotic cell death by AnnexinV-FITC staining. Samples were analyzed by flow cytometry for apoptosis (AnnexinV⁺) and bidimensional cell cycle distribution (EdU-Alexa647 and DAPI) on a BD

LSRFortessa flow cytometer (BD Bioscience). For cell cycle analysis, 10,000 single cells were recorded. Flow cytometric data were analyzed using FlowJo software (Tree Star, OR).

Immunoblot analysis

Cell pellets were washed in ice-cold PBS and lysed using RIPA buffer freshly supplemented with protease inhibitor cocktail. Equal amounts of protein (15–30 µg) were loaded on a 12% SDS-PAGE and then transferred on a polyvinylidene difluoride membrane (Roche, Germany). After overnight incubation at 4 °C with specific primary antibody and then the secondary antibody, proteins were visualized using ECL developing reagent on an Image Quant LAS 4000 (GE Healthcare, Germany). PARP (#9532), cleaved caspase 3 (#9661), CycE1 (#20 808), CycB1 (#12231), p53 (#2527), p73 (#14620), p27 (#3686), pAMPK (#2535), Myc (#13987), p-4EBP1 (#2855), and p-p70S6K (#9234) antibodies were purchased from Cell Signaling Technology. p21 (sc-6246) antibody was purchased from Santa Cruz Biotechnology. Tubulin (#T5168) was used as loading control and was obtained from Sigma-Aldrich.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using TriFastTM (VWR, Darmstadt, Germany). High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to synthesize cDNA. Real-time quantitative PCR was performed using Fast SYBR[®] Green Master Mix (Thermo Fisher Scientific). The housekeeping gene β-actin was used as an internal control. For the nucleotide sequences of the primers used, see Supplementary Information.

ATP measurement

Cellular ATP levels were assessed using the CellTiter-Glo[®] Luminescent Cell Viability Assay Kit (Promega, Mannheim, Germany) according to manufacturer's instructions.

Lactate determination assay

Supernatant of cells treated for 8 h with indicated compounds were analyzed for lactate secretion using the LAC-142 assay (Diaglobal, Berlin, Germany) according to manufacturer's instructions.

Assessment of mitochondrial functionality

For the quantification of mitochondrial respiration and glycolytic function, the OCR and the ECAR of intact

LS174T and Caco-2 cells were analyzed on a Seahorse XFe24 analyzer (Agilent, Ratingen, Germany), as described in [58]. To assess the MRC complex activities, cells were permeabilized as described in [49]. To assess mitochondrial inner membrane integrity, assays were performed in the presence of oligomycin (1 μ M). For detailed descriptions, see Supplementary Information.

Statistical analysis

One-way ANOVA or two-way ANOVA with Dunnett's correction for multiple comparisons was used to assess statistical significance values (using Graph-Pad Prism Version 6.0).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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