GSTP1-1 and thiazolide-induced apoptosis in colon carcinoma cells

Dissertation

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ABSTRACT

Colorectal cancer (CRC) is one of the most frequently diagnosed tumor entities and the second leading cause of cancer-related death in Europe. While the goal of many anti-cancer drugs is to initiate the death of tumor cells by targeting fast dividing cells, sophisticated resistance mechanisms in tumor cells limit the efficiency of chemotherapeutic agents and prevent cell death induction. In particular, glutathione S-transferases (GSTs), known as phase II detoxification enzymes catalyze the conjugation of glutathione (GSH) to endogenous metabolites and xenobiotics, which contributes to the inactivation of pharmacologically active compounds. Specifically, GST of class P1 (GSTP1-1) has been found to be frequently overexpressed in a wide variety of solid tumors, including CRC cells, and renders them resistant to chemotherapeutic drugs. Thus, recent studies proposed GSTP1-1 as an interesting drug target in anti-cancer therapy. The bromo-thiazolide RM4819 (N-(5-bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide) was the first described thiazolide that induces cell death in human colorectal adenocarcinoma cell lines in a GSTP1-1-dependent process, thereby bypassing this GSTP1-1-mediated drug resistance. However, the mechanism of thiazolide-induced apoptosis in colon cancer cells is still unclear. Therefore, it will be of interest to identify the underlying mechanism(s) and to clarify the structure-function relationship of thiazolides with regard to their ability to induce apoptosis. In this study, we investigate the molecular structure of RM4819 that is required for the interaction with GSTP1-1 and/or might be important for the conjugation to GSH. For that purpose, we synthesized novel thiazolide derivatives with different variations in their substituents, and we investigated how these substitutions affect their apoptosis-inducing activities. Interestingly, while all synthesized thiazolide derivatives induced cell death in CRC cells to some degree, the substitution of the bromide atom on the thiazole ring to a hydrogen considerably reduced the cell death-inducing activity, highlighting the importance of that position in apoptosis induction. When further studying the underlying apoptosis mechanism, we identified the mitogen-activated protein kinases (MAPKs) JNK and p38 as critical regulators in thiazolide-induced cell death. Moreover, we showed that JNK-dependent apoptosis induction is mediated by the induction and activation of pro-apoptotic Bcl-2 homologs that, in turn, bind and neutralize pro-survival Bcl-2 family members, promoting cell death. Interestingly, this effect was increased by elevating intracellular GSH level. In addition, we also observed that thiazolides sensitize cells to other apoptosis triggers. For instance, we found that thiazolides enhance cell death induction by chemotherapeutic drugs as well as TRAIL in a synergistic manner. Accordingly, we propose thiazolides as a novel therapeutic approach for the treatment of CRC by bypassing, respectively targeting the GSTP1-1-mediated drug-resistance mechanism, thereby sensitizing CRC cells to other cell death inducers.
ZUSAMMENFASSUNG

mitochondrialen Apoptoseweg aktivieren. Interessanterweise konnte dieser Effekt durch Erhöhung der intrazellularen GSH-Konzentration gesteigert werden. Zusätzlich konnte eine Thiazolid-vermittelte Sensibilisierung kolorektaler Tumorzellen gegenüber weiterer Apoptose-Auslöser, wie Chemotherapeutika oder TRAIL, beobachtet werden, was in einer synergistischen Induktion von Zelltod resultierte. Daher stellen Thiazolide ein neues vielversprechendes therapeutisches Medikament für die Behandlung gegen Darmkrebs dar, in dem der GSTP1-1-vermittelte Resistenzmechanismus umgangen, bzw. ausgenutzt wird um gezielt Zelltod in Darmkrebszellen auszulösen oder diese zu sensibilisieren gegenüber anderen Zelltodauslösern.
GENERAL INTRODUCTION

1. Colorectal Cancer

Colorectal cancer (CRC) is one of the most dangerous forms of cancer and frequently arises in the colon or rectum (Brenner et al., 2014; Ferlay et al., 2010; Ferlay et al., 2013). Acquired genetic and epigenetic alterations along the tumorigenesis lead to an abnormal growth of cells with the ability to invade or spread to other parts of the body (Grady and Carethers, 2008; Grady and Pritchard, 2014). While CRC is an extensive and complex topic, the most common and important features of CRC will be discussed in the following sections with the subsequent focus on drug resistant mechanisms in tumor cells.

1.1 Epidemiology of colorectal cancer

CRC is the one of the most frequently diagnosed cancer in Europe, representing 13.0% of all newly diagnosed cancers (excluding non-melanoma skin cancers). In the year 2012, around 447.000 incidences were estimated for CRC and around 50% of diagnosed patients will probably die, resulting in 12.2% of the total number of cancer death. Accordingly, CRC is the second leading cause of cancer-related death in Europe, right after lung cancer (Ferlay et al., 2013). Although men have a slightly higher incidence rate to develop CRC than women, both genders are affected. Elevated rates of CRC incidences have been reported in Central European countries: Slovakia (92 per 100.000), Hungary (87) followed by Czech Republic (81) in men, and in Norway (54), Denmark (53) and the Netherlands (50) in women (Ferlay et al., 2013). An overview of incidence and mortality rates of both sexes is shown in Figure 1.1 A and B.

![Figure 1.1 Epidemiology of colorectal cancer in Europe.](image)

(A) Incidence rates and (B) mortality rates in both sexes. ASR (W)-age-standardized world incidences/mortality rate per 100.00 populations. Source: GLOBOCAN 2012 (Ferlay et al., 2015) International Agency for Research on Cancer. Lyon (France) 2013. Available form: http://globocan.iarc.fr and http://www.crcprevention.eu
Interestingly, while CRC is one of the most frequently diagnosed cancers, small-bowel cancers are the least common (Calman, 1974; Neugut et al., 1997). The underlying reason for this fact is not truly clear, especially considering that the small intestine is located between two organs with a high risk of cancer development, i.e., the stomach and colon (Ferlay et al., 2013). Recent research links this observation with the bacterial flora of the gut (Neugut et al., 1998). In this regard, the small bowel is relatively sterile compared to the colon. Therefore, it is possible that the presence or absence of bacteria plays an important role. Along this line, germ-free animals exposed to known intestinal carcinogens showed a reduced frequency of CRC development compared to normal animals, supporting a role of the intestinal flora in intestinal cancer development (Sumi and Miyakawa, 1979). Another hypothesis for the low incidence of small-bowel cancer is based on the more effective local immune system in the small intestine in comparison to the colon, which may limit the development of tumors (Calman, 1974; Samson et al., 1973). The small intestine is known to be an important lymphoid organ that secretes large amounts of immunoglobulins. It has been shown that bowel diseases, which arise from an abnormal immune response, correlate with an increase in incidence rate of small-bowel cancer (Calman, 1974), but also of CRC (Desai et al., 2015), suggesting that the intestinal immune system plays a critical role in controlling the tumor development.

About 75% of patients with CRC have sporadic diseases that appear somewhere over the age of 60 (Seifeldin and Hantsch, 1999). These patients have no evidences of inherited disorders, while the remaining 25% of patients have a family history of CRC that suggest heredity contributions (A. Syed Sameer and Siddiqi, 2010). In this regard, epidemiological studies showed that some families are more predisposed in getting CRC than others (Fuchs et al., 1994). CRC cases in the first generation result in a higher risk of developing CRC in the following generation. Only 2-5% of cases are accounted for heredity genetic mutations, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) (Jaspersen et al., 2010). Both hereditary diseases show characteristic mutations in the genome that lead to an uncontrolled proliferation and the formation of many (benign) adenoma polyps in the intestine, even at young age. This increases dramatically the risk to develop (malignant) adenocarcinoma at a later stage (Jaspersen et al., 2010).
1.2 Tumorigenesis: Transformation of colonic epithelial cells into adenocarcinoma cells

CRC is the consequence of a stepwise accumulation of acquired genetic and epigenetic alterations that transform colonic epithelial cells into invasive adenocarcinoma. It often begins with the development of benign adenomatous polyps at the internal surface of the intestine, followed by invasive carcinomas and metastatic cancer. Sequential processes of gene mutations in tumor suppressor genes and oncogenes in crypt stem cells is widely believed to drive the initiation of benign adenomas and the progression into malignant adenocarcinomas. These mutations affect important cell signaling pathways that regulate the overall proliferation and survival of cancer cells (Grady and Carethers, 2008; Grady and Pritchard, 2014). Thereby, a mutated crypt stem cell has a clonal growth advantage, which leads to the outgrowth of altered cells and the development of a tumor (Humphries and Wright, 2008). Additionally, the accumulation of mutations is facilitated by the loss of genomic stability, which appears to be a critical molecular step in cancer development (Gollin, 2005; Lengauer et al., 1998). The most common type of genomic instability is chromosome instability (CIN), which occurs in 80-85% of CRC (Grady and Carethers, 2008). CIN can be recognized by the present of aneuploidy, defined in numerical chromosome changes or multiple structure aberrations. CIN is an early event in tumor formation and increases along the adenoma-carcinoma progression (Grady and Carethers, 2008; Hermsen et al., 2002; Lengauer et al., 1997; Stoler et al., 1999). However, the mechanism that gives rise to this form of genomic instability and the role of aneuploidy in tumor progression remains poorly understood. In addition to CIN, the three suppressor genes such as APC, SMAD4 and TP53 and the oncogene KRAS are critical targets of mutations along the adenoma-carcinoma sequence ultimately leading to the carcinoma development (Armaghany et al., 2012; Fodde, 2002; Grady and Carethers, 2008; Grady and Pritchard, 2014). They are the most frequently genetic changes detectable in CRC development, which can be mutated at different stages (Figure 1.2). In particular, along the adenoma-carcinoma sequences, the earliest identifiable lesion in colon cancer formation is the aberrant crypt focus (ACF), whose origin is still unclear (Bird, 1987; Roncucci et al., 1991; Takayama et al., 1998). Progression of dysplastic ACF can carry mutations in the adenomatous polyposis coli (APC) gene (Grady and Carethers, 2008). Mutated APC gene occurs in up to 70% of sporadic CRC (Grady and Carethers, 2008; Grady and Pritchard, 2014) and is also the cause of the FAP cancer predisposition syndrome (Groden et al., 1991; Kinzler et al., 1991; Nagase et al., 1992). While early mutations in the APC gene are already known to initiate the formation of benign adenomas (Powell et al., 1992), it might be also involved in genomic instability, thus facilitating the progression to adenocarcinoma (Fodde et al., 2001; Grady and Carethers, 2008). In general, the
APC protein negatively regulates the Wingless/Wnt-signaling pathway by interacting with the transcription factor β-catenin in a so-called destruction complex, promoting the ubiquitin-mediated proteasomal degradation of β-catenin. Disruption of the APC protein results in an over-activation of the Wnt-signaling pathway through the stabilization of nuclear β-catenin, which increases the transcription of downstream target genes such as MYC and cyclin D1 (Behrens et al., 1998; Hart et al., 1998; He et al., 1998; Kishida et al., 1999; Tetsu and McCormick, 1999). It is widely accepted that MYC and cyclin D1 are involved in the regulation of different cellular processes, such as proliferation, cell cycle progression and apoptosis, and that their increased expression levels affect likely to the normal intestinal epithelial renewal by increasing the proliferation rate (Arber et al., 1996; Brabletz et al., 2000; Fodde, 2002). Accordingly, the combination of an over-activation of the Wingless/Wnt-signaling pathway and the possible APC-induced chromosome instability facilitate the early stage of tumor formation and allows the initial clonal expansion.

Adenoma-Carcinoma Sequence of CRC with CIN

![Adenoma-Carcinoma Sequence of CRC with CIN](image)

**Figure 1.2 Colorectal tumor progressions with CIN.**

CIN is an early event in tumor formation and increases along the adenoma-carcinoma sequence. Additionally, mutation in the APC gene leads to the progression of benign adenomas. Subsequent point mutations in the KRAS oncogene during the early adenoma stage promote further cell growth and survival. The loss of the long arm of chromosome 18 (18q loss of heterozygosity; LOH) and mutations in the TP53 gene in advanced adenoma facilitate the malignant progression to adenocarcinoma. (Figure based on (Grady and Carethers, 2008; Grady and Pritchard, 2014; Vogelstein et al., 1988))

Additionally, point mutations in the oncogene KRAS promote further cell growth and survival through the downstream activation of the MAPK pathway during the early adenoma formation (Grady and Pritchard, 2014; Horst et al., 2012). KRAS is a member of the RAS family and encodes the monomeric GTP-binding protein KRAS. Its mutations occur in approximately 40%
of colorectal cancers and may appear after APC mutation during the early tumorigenesis (Hasegawa et al., 1995; Vaughn et al., 2011; Vogelstein et al., 1988). These mutations lead to a permanent activation of KRAS by impairing the ability of GTPase activation proteins to hydrolyze KRAS-bound GTP (Bos, 1989; Li and Zhang, 2004). Successive deregulation of APC and KRAS will further trigger CIN (Fodde, 2002). The subsequent loss of the long arm of chromosome 18 (18q loss of heterozygosity; LOH) occurs in up to 70% of all cases and is the most frequent cytogenetic alteration in CRC (Grady and Pritchard, 2014; Vogelstein et al., 1988). The loss of 18qLOH leads to the deletion of the tumor suppressor gene SMAD4, which normally mediates the transforming growth factor, beta (TGF-beta) signaling pathway, controlling epithelial cell growth (Grady and Carethers, 2008; Peter Zauber, 2008; Tanaka et al., 2008). Deletion of SMAD4 results in disruption of TGF-beta signaling and therefore in an uncontrolled growth of the epithelium that supports the malignant progression (Miyaki et al., 1999; Miyaki and Kuroki, 2003; Takaku et al., 1998). Also mutations in the TP53 tumor suppressor gene strongly promote the malignant transformation of adenomas (Rivlin et al., 2011; Rodrigues et al., 1990). It encodes the p53 protein, a stress-inducible transcription factor, which regulates cell cycle arrest, senescence, DNA repair and apoptosis. Mutations in the TP53 gene occurs in up to 50% of CRC and leads to a regulated expression of target genes, followed by uncontrolled cell growth and an increased occurrence of mutations in the genome (Grady and Pritchard, 2014; Li et al., 2015).

Unfortunately, only few acquired genetic alternations needs to occur in order to initiate tumor progression up to the malignant stage of CRC (Fodde, 2002; Vogelstein et al., 1988).

1.3 Symptoms of CRC and currently therapeutic options against CRC
Classical symptoms of CRC are blood in stool, constipation, weight loss, loss of appetite, nausea and vomiting, while rectal bleeding and anemia are high-risk symptoms (Hamilton and Sharp, 2004). During the early stages of CRC, no or minimal symptoms are detectable and therefore screening via colonoscopy is recommended from the age of 50 years. Early diagnosis of CRC has a high 5-years survival rate of up to 93%, while late diagnosis of CRC in stage III and IV dramatically decreased the lifespan (Stintzing, 2014). This certainly correlates with the development of drug-resistance mechanisms during tumorigenesis, which limit the efficiency of anti-cancer drugs, thereby hampering the successful treatment of CRC patients (Holohan et al., 2013; Housman et al., 2014). An overview about the 5-year survival rates after cancer diagnosis is shown in Table 1.3.
After CRC diagnosis, surgery is usually the first treatment for tumors that have not spread yet to distant sites. Dependent on the tumor size and stage (0 and I), polypectomy (removing the polyps) or local excision through a colonoscopy is done in the most cases, while bigger tumors need to be removed by colon resection (colectomy). In contrast to later stages, further therapy at stages 0 and I is usually not necessary (Kudo et al., 1997).

In stage II, combinations of chemotherapeutic drugs and/or radiation therapy are often given after surgery (adjuvant chemo) in order to kill cancer cells that could not be removed (Benson et al., 2004). Commonly used drugs for chemotherapy are 5-Fluorouracil (5-FU, anti-metabolite) often combined with leucovorin (also known as folinic acid, a vitamin-like drug), Capecitabine (Xeloda®, an anti-metabolite), Irinotecan (Camptosar®, a topoisomerase inhibitor) and Oxaliplatin (Eloxatin®, an alkylating agent; platinum-compound chemotherapeutic drug) (Andre et al., 2004; Punt and Koopman, 2008). The different chemotherapeutic drugs may differ in their treatment duration and interval, and can be administer as a single agent or in combination, depending on the compatibility of the patient and genetic alterations (Evans and McLeod, 2003).

In general, adjuvant treatment is usually given for about 6 months. In some cases, chemotherapeutic drugs or radiation are administered before surgery in order to reduce the tumor size for an easier intervention (neoadjuvant chemo) (Kim, 2010; Lewis J Rose, 2015). However, chemotherapeutic drugs are very strong medications and target not only fast dividing cancer cells, but also tissue cells with a high dividing rate in the body can be affected. Examples are cells in the bone marrow, the lining of the mouth and intestines, and hair follicles. Subsequently, the side effects of chemotherapy can be excessive, resulting in hair loss, mouth sores, low blood counts, increased infection rates (due to the lack of white blood cells), easy bruising or bleeding, fatigue, loss of appetite, nausea and vomiting (Coates et al., 1983; Dunbar et al., 2014). Beside the fact that chemotherapy is a very aggressive form of therapy, affecting not only cancer cells but also fast dividing cells in the whole organism, it is currently one of the major treatment options of CRC.

While surgical intervention and chemo- and radiation-therapy is used up to stage III with a ~50% 5-year survival rate, the therapeutic options in the metastatic stage IV are much more limited with a prognosis of 8.1 % survival within 5 years (Stintzing, 2014). In most cases, surgery is unlikely to cure CRC, which normally spread from the colon to distant organs and tissues. Metastases can be found most frequently in the liver, but also lung, peritoneum or distant lymph nodes can be affected (Miguel A. Rodriguez-Bigas; Steup et al., 2002). Surgery simply relieves symptoms of the diseases and it is sometimes used when the tumor causes obstructions of the
colon. In this case, chemotherapy is administered before and after surgery. In the advanced stage, targeted therapy is given to the patients in addition to chemo- and radiation-therapy to control tumor growth (Gerber, 2008; Miguel A. Rodriguez-Bigas). The most common targeted drugs inhibit angiogenesis by targeting vascular endothelial growth factor (VEGF) or the epidermal growth factor receptor (EGFR) signaling pathway, which regulate cellular growth, survival, proliferation and differentiation (Frumovitz and Sood, 2007; Wieduwilt and Moasser, 2008).

In CRC increased expression levels of VEGF have been shown to be associated with advanced tumor stage, lymph node, liver metastases and overall survival. As VEGF promotes the formation of new blood vessels, tumor cells can be supplied with nutrients, which increased tumor cell survival and may support tumor growth (Rosen, 2002). Accordingly, inhibition of VEGF by Bevacizumab (Avastin®) a monoclonal antibody and ziv-aflibercept (Zaltrap®) a fusion protein, which acts as a decoy receptor, blocks the formation of new blood vessels, limiting nutrient supply in the tumor. However, compensatory effects in the VEGF pathway may lead to targeted drug resistance (Hurwitz et al., 2004; Patel and Sun, 2014; Willett et al., 2004).

Another targeted therapy is the use of the monoclonal antibodies Cetuximab (Erbitux®) and panitumumab (Vectibix®), which target the EGFR signaling pathway and prevent the EGFR-mediated activation of KRAS. However, this targeted drug therapy is limited to patients with wild type KRAS protein, since mutant KRAS protein is continuously activated independently of the EGFR signaling (see 1.2 Tumorigenesis) (Lievre et al., 2006; Peeters et al., 2010; Van Cutsem et al., 2009). Unfortunately, the side effects can be very strong resulting in high blood pressure, tiredness, bleeding, headache and, strong skin problems including infection and skin peeling (Fakih and Vincent, 2010).
Table 1.3 CRC 5-year survival rates after diagnosis. Source: (Stintzing, 2014)

<table>
<thead>
<tr>
<th>Standard</th>
<th>CRC Classification</th>
<th>**TNM classification (American Joint Commission on Cancer)</th>
<th>5-year survival rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>stage</td>
<td>*UICC stage</td>
<td>T stage</td>
<td>N stage</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>Tis</td>
<td>N0</td>
</tr>
<tr>
<td>I</td>
<td>I</td>
<td>T1/T2</td>
<td>N0</td>
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<tr>
<td>II</td>
<td>IIa</td>
<td>T3</td>
<td>N0</td>
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<tr>
<td></td>
<td>IIb</td>
<td>T4</td>
<td>N0</td>
</tr>
<tr>
<td>III</td>
<td>IIIa</td>
<td>T1, T2</td>
<td>N1</td>
</tr>
<tr>
<td></td>
<td>IIIb</td>
<td>T3, T4</td>
<td>N1</td>
</tr>
<tr>
<td></td>
<td>IIIc</td>
<td>Any T</td>
<td>N2</td>
</tr>
<tr>
<td>IV</td>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
</tr>
</tbody>
</table>

*UICC: Union Internationale Contre le Cancer. This classification system based on statistical investigations and show i.e. that the prognosis is dependent on the tumor size. The grading of malignant disease give an overview what the prognosis might be and what further therapy should be undertaken. **Key for TNM Staging: primary tumor (T), regional lymph nodes (N), and distant metastases (M). Tis: carcinoma in situ: intraepithelial or invasion of lamina propria. T1: tumor invades submucosa. T2: tumor invades muscularis propria. T3: tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues. T4: tumor directly invades other organs or structures and/or perforates visceral peritoneum. N0: no regional lymph node metastasis. N1: metastasis in one to three regional lymph nodes. N2: metastasis in four or more regional lymph nodes. M0: no distant metastasis. M1: distant metastasis.

Although chemotherapeutic drugs and targeted therapy show strong side effects and are only selectively applicable, these are currently the most frequently used medical options for the treatment of CRC. A major problem in anti-cancer therapy is the limited efficiency of anti-cancer drugs. Since, many tumor cells develop drug resistance mechanisms along the tumorigenesis, they contribute to the limiting efficiency of anti-cancer agents that can either arise at the time of drug presentation (innate drug resistance) and/or after an initial response (acquired drug resistance) (Holohan et al., 2013; Housman et al., 2014). As the aim of many anti-cancer drugs is to initiate the cell death of fast dividing cells, sophisticated resistance mechanisms in tumor cell prevent the cell death induction of such drugs (Johnstone et al., 2002). These effects can have various causes. In the next sections, the most relevant drug resistance mechanisms, such
as altering drug metabolite potency (see 2.0 Biotransformation) and the inhibition of the cell death-inducing pathway (see 4.0 Apoptosis), will be discuss in more detail.

2.0 Biotransformation

The biotransformation is a metabolic process of living organisms, in which hydrophobic substances are transformed in more water-soluble metabolites, so that they can be excreted by urine or stool. Thus, endogenous substrates and substrates foreign to the body (xenobiomics), including drugs, gets inactivated via the biotransformation process, which can be divided into phase I and phase II reactions (Liska, 1998). Since, phase II reactions are of major importance for this thesis, the phase I reaction will be discussed only briefly.

2.1 Phase I reaction

The main enzymes in the phase I reaction are cytochromes P450 (CYPs) enzymes, representing a superfamily of heme enzymes. They act as monooxygenases, dioxygenases and hydrolases and are responsible for the metabolism of xenobiomics and endobiomics (Anzenbacher and Anzenbacherova, 2001; Niwa et al., 2009). Thus, hydrophobic compounds are transformed to more polar metabolites by unmasking or by adding functional groups (e.g. –OH, -NH₂, -SH) by CYPs. The main reactions are: N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination (Guengerich, 1991; Jancova et al., 2010). If the transformed metabolites are sufficiently polar, they may be readily excreted, otherwise they are further metabolized in the phase II reaction.

2.2 Phase II reaction

The phase II enzymes play an important role in the biotransformation of endogenous substrates and xenobiomics, and have a particular relevance in the metabolic inactivation of pharmacologically active compounds. The most abundant detoxification enzymes in the cells are UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs), which catalyze the processes known as glucuronidation, sulfation and glutathione conjugations, respectively (Jancova et al., 2010). Therefore, endogenous substrates and xenobiomics are conjugated to their corresponding co-factors such as UDP-glucuronic acid, 3’phosphoadenosin-5’-phosphosulfate and glutathione (GSH) to form a more hydrophilic metabolite (Armstrong, 1991; Guillemette, 2003; Meech and Mackenzie, 1997; Nowell and Falany, 2006; Wilce and Parker, 1994). Specific binding sites of the endogenous substrates and xenobiomics enable the phase II reaction however, sometimes phase I reaction needs to occur
before. Finally, upon transformation the more water-soluble conjugates can be transported out of the cells by efflux pumps, and be excreted via bile or urine (Deeley et al., 2006).

GSTs are important enzymes in the detoxification of xenobiotics and relevant for this thesis. They will thus be discussed in more detail in the following sections.

2.3 Glutathione S-transferases

GSTs represent a superfamily of cellular phase II detoxification enzymes that are strongly involved in the metabolism of xenobiotics and oxidative stress products. GSTs catalyze the conjugation of the thiol-containing tripeptide glutathione (GSH, \(\gamma\)-L-glutamyl-L-cysteinylglycine) to a large number of nucleophilic aromatic substitutions, and hydrophobic and electrophilic compounds (Habig et al., 1974). Thereby, hazardous metabolic products, xenobiotics and oxidative stress products become rapidly neutralized by GSTs, protecting cells from potentially damaging substances and carcinogens (Hayes et al., 2005; Hayes and Pulford, 1995; Hayes and Strange, 1995, 2000; Salinas and Wong, 1999). Thus, GSTs play a critical role in the detoxification of cells and in the inactivation of pharmacologically active compounds (**Figure 2.3**) (Tew, 1994).

![Figure 2.3 Formation of glutathione conjugate. Source: (Jancova et al., 2010)](image)

GSTs can be subdivided into two distinct superfamilies. One of these superfamilies represents soluble dimeric enzymes, which are most frequently located in the cytoplasm. But they can also be found in the nucleus (Bennett et al., 1986; Sundberg et al., 1993), mitochondria (Raza et al., 2002) and peroxisomes (Morel et al., 2004). Soluble GSTs are subdivided into eight subclasses. Each subclass is named with the Greek letters (\(\alpha\) (alpha), \(\mu\) (mu), \(\pi\) (pi), \(\theta\) (theta), \(\sigma\) (sigma), \(\zeta\) (zeta), \(\omega\) omega, and \(k\) (kappa)) and abbreviated in Roman capitals (A, M, P, T, S, Z, O and K). Each subclass in turn includes several subunits, which are designated by an Arabic number (Mannervik et al., 1992; Mannervik et al., 2005). The subunit (ca. 199-244 amino acids in length, 22-29 kDa) contains a GSH-binding site in the amino-terminal domain and an H-site that binds
the hydrophobic substrates in the carboxy-terminal domain (Dirr et al., 1994; Reinemer et al., 1992; Reinemer et al., 1991). As the catalytic active enzymes are dimeric, they can compose of two identical subunits or of two different ones, e.g. GSTA1-2 composed of subunits 1 and 2 of the α class (Laborde, 2010). This gives rise to a large number of isoenzymes, showing a high variability of substrates. Interestingly, while most of the GST subclasses have a high degree of genetic polymorphism, some allelic forms have a stronger correlation with the development of certain diseases than others (Bohanec Grabar et al., 2009; Coles and Kadlubar, 2005; McIlwain et al., 2006).

The second superfamily of GSTs represents the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) protein family, which is mainly involved in the arachidonic acid metabolism (Jakobsson et al., 1999). Both, soluble GSTs and MAPEGs are widely distributed throughout the body. Their substrate specificities point towards a defined role of individual soluble GSTs and MAPEGs in the biotransformation of drugs and reactive compounds in diverse tissues (Faulder et al., 1987; Jancova et al., 2010; Rowe et al., 1997; Sherratt et al., 1997). Table 2.3 gives an overview about the soluble GSTs family members and their tissue distribution.

As GST of class P, subunit 1, GSTP1-1 is of major relevance for this thesis, I will next discuss the role of GSTP1-1 in the drug resistance mechanisms of cancer cells.

<table>
<thead>
<tr>
<th>Class</th>
<th>Protein</th>
<th>Organ</th>
</tr>
</thead>
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<td>testis ≈ liver &gt;&gt; kidney ≈ adrenal &gt; pancreas</td>
</tr>
<tr>
<td></td>
<td>GSTA2</td>
<td>liver = testis ≈ pancreas &gt; kidney &gt; adrenal &gt; brain</td>
</tr>
<tr>
<td></td>
<td>GSTA3</td>
<td>placenta</td>
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</tr>
<tr>
<td>Kappa</td>
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<td>liver</td>
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<tr>
<td>Mu</td>
<td>GSTM1</td>
<td>liver &gt; testis &gt; brain &gt; adrenal ≈ kidney &gt; lung</td>
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<tr>
<td></td>
<td>GSTM2</td>
<td>brain ≈ skeletal muscle ≈ testis &gt; heart &gt; kidney</td>
</tr>
<tr>
<td></td>
<td>GSTM3</td>
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</tr>
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<td></td>
<td>GSTM4</td>
<td>brain, heart, skeletal muscle</td>
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<tr>
<td></td>
<td>GSTM5</td>
<td>brain, heart, lung, testis</td>
</tr>
<tr>
<td>Pi</td>
<td>GSTP1</td>
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</tr>
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<td>GSTS1</td>
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</tr>
<tr>
<td>Theta</td>
<td>GSTT1</td>
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</tr>
<tr>
<td></td>
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</tr>
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<td>Zeta</td>
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</tr>
<tr>
<td>Omega</td>
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<td>liver = heart ≈ skeletal muscle &gt; pancreas &gt; kidney</td>
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2.4 The role of GSTP1-1 as detoxification enzyme in cancer cells

GSTP1-1 is an important phase II detoxification enzyme. In the catalytic active state, GSTP1-1 catalyzes the conjugation of GSH to a wide variety of endogenous substrates and xenobiotics, including chemotherapeutic drugs (Litwack et al., 1971; Tew, 1994). High expression levels of GSTP1-1 have been reported in a wide range of solid tumors, such as colon, breast, kidney, pancreas, lung, and ovarian cancer, and lymphomas. Along these lines, it has been shown to contribute to the development of drug resistances against chemotherapeutic drugs (Howells et al., 2004; Howie et al., 1990; Laborde, 2010; Moscow et al., 1989; Ranganathan and Tew, 1991; Tidefelt et al., 1992b). Especially for CRC, GSTP1-1 is overexpressed in all stages of tumorigenesis, from aberrant crypt foci to advanced carcinomas (Clapper et al., 1991a; Clapper et al., 1991b; Miyanishi et al., 2001; Ranganathan and Tew, 1991). It has been shown that GSTP1-1 is critical for the clonogenic survival and proliferation of HCT116 human colon cancer cells. Under stress condition, high GSTP1-1 expression levels protect cells from oxidative stress and associated apoptosis induction (Dang et al., 2005). Thus, GSTP1-1 overexpression contributes to sustain cell proliferation and tumor growth during tumorigenesis and can detoxify cells from anti-cancer drugs, such as alkylating agents, cisplatin, doxorubicin, or etoposide (Ban et al., 1996; Goto et al., 1999; Ishikawa and Ali-Osman, 1993). This results in the inactivation of such drugs and thereby limits the effect of chemotherapy (Townsend and Tew, 2003). However, it has been reported for the majority of anti-cancer drugs that the affinity to GSTP1-1 is too weak to bind and detoxified them, although a drug-resistant phenotype could be observed in GSTP1-1-overexpressing cancer cells. It might be possible that GSTP1-1 bind and inhibit cytosolic proteins, thereby affecting signaling pathways involved in cell proliferation and apoptosis (Laborde, 2010). The role of GSTP1-1 in the regulation of signaling pathways and associated drug resistance will be discussed next.

2.5 The role of GSTP1-1 as endogenous inhibitor in cancer cells

While high GSTP1-1 expression levels have been found to inactivate chemotherapeutic drugs in cancer cells, it has also been shown that GSTP1-1 binds and inhibits family members of the mitogen-activated protein kinase (MAPK) family, such as c-Jun N-terminal kinase (JNK) and to some extent also p38α (Adler et al., 1999; Wang et al., 2001; Wu et al., 2006). In this non-enzymatic role, GSTP1-1 sequestrates MAPKs, in particular JNK in a complex and preventing it to activate downstream targets, involved in cell survival and death signaling. MAPKs are serine/threonine kinases, which are strongly involved in stress responses, apoptosis, inflammation, as well as cellular differentiation and proliferation (Cargnello and Roux, 2011).
JNK and p38 get activated through the sequential phosphorylation of the MAPK cascade (Davis, 2000). Thus, MAP kinase kinases (MAP2Ks), such as MKK4 and MKK7, phosphorylate and activate JNK (Lin et al., 1995; Lu et al., 1997; Tournier et al., 2001), while MKK4 and MKK3/6 activate p38 (Lin et al., 1995; Remy et al., 2010; Zarubin and Han, 2005). MAP2Ks in turn are phosphorylated and activated by upstream MAP kinase kinase kinases (MAP3Ks) (Ichijo et al., 1997a; Johnson et al., 1996). Different stimuli can activate the MAPK pathway, such as growth factors, cytokines, UV radiation and other stress signals (Giehl et al., 2000; Kim et al., 2004; Nishitoh et al., 1998; Zanke et al., 1996). Once activated, JNK in turn can phosphorylate a large number of different substrates, such as c-Jun, a component of the activator protein-1 (AP-1) transcription factor that targets genes encoding proteins involved in cell proliferation and cell death (Hibi et al., 1993; Karin, 1995; Shaulian and Karin, 2001). In addition, pro- and anti-apoptotic Bcl-2 (B-cell CLL/lymphoma 2) family members can be directly regulated by active JNK and thereby mediate cell death or survival (Hubner et al., 2008; Kim et al., 2006a; Yamamoto et al., 1999; Yu et al., 2004). The function of JNK is dependent on the stimulus, cell type and signal intensity that may result in cell proliferation or cell death (Bode and Dong, 2007).

In non-stressed 3T3-4A mouse embryonic fibroblasts, it has been found that GSTP1-1 is associated with the JNK-c-Jun complex, inhibiting the phosphorylation of c-jun by JNK through direct protein-protein interaction. Based on previous data, it was assumed that GST π monomers interact with the JNK-c-Jun complex. However, further studies point rather to the dimeric form of GSTP1-1 that binds and inhibits JNK activation (Fabrini et al., 2009; Laborde, 2010). Under prolonged stress stimulation, for example by oxidative or chemical stress, GSTP1-1 dissociates from the JNK-c-Jun complex, undergoes oligomerization and mediates the activation of JNK, ultimately leading to the induction of apoptosis (Adler et al., 1999; Laborde, 2010; Wang et al., 2001). The interaction between GSTP1-1 and JNK could also be confirmed in vivo by using GSTP1/2-deleted mice. Thus, an increase in constitutive JNK activity was found in the liver and lung, compared to wild type mice. In addition, an increase in AP-1 DNA-binding activity could be observed along with an increased JNK activity in these knockout mice (Elsby et al., 2003). Accordingly, absence of the endogenous inhibitor GSTP1-1 leads to the activation of JNK, followed by the activation of downstream molecular targets of the JNK signaling pathway (Yin et al., 2000). Like JNK p38α, a member of the p38 family, can also be sequestrated by GSTP1-1, however the role of p38 sequestration is still unclear. While it has been reported that the interaction between GSTP1-1 and JNK decreases upon cellular activation, the interaction between GSTP1-1 and p38α was not affected in activated HeLa cells (Wu et al., 2006). In any
case, p38 can be activated via the MAPK cascade upon specific stimulation and can regulate cell growth, differentiation and apoptosis (Zarubin and Han, 2005).

In addition to JNK, TRAF2, a member of the TNF (tumor necrosis factor) receptor-associated factor (TRAF) protein family, has also been identified to be sequestrated by GSTP1-1 (Wu et al., 2006). TRAF2 is an important activator for the TNF receptor family-induced activation of the apoptosis signal-regulating kinase 1 (ASK1), which is a member of MAP3K (Liu et al., 2000a). Activated ASK1 in turn phosphorylates and activates the MKK4/7-JNK and MKK3/4/6-p38 (Ichijo et al., 1997b). Overexpression of GSTP1-1 inhibits TRAF2-induced activation of ASK1, and thereby the downstream activation of the MAPKs JNK and p38. Thus, GSTP1-1 may inhibit the TRAF2-ASK1-induced apoptosis by direct sequestration of TRAF2 (Figure 2.5) (Wu et al., 2006).

These reports highlight that GSTP1-1 does not only work as detoxification enzyme but also as endogenous inhibitor of MAPK activation, thereby modulating signaling pathways that control cell proliferation, cell differentiation, and cell death (Hayes et al., 2005; Laborde, 2010). Many anti-cancer drugs mediate cell death induction also via the activation of JNK and p38 (Boldt et al., 2002; Mansouri et al., 2003; Osborn and Chambers, 1996). GSTP1-1 overexpression in tumor cells in turn appears to modulate the activation of the MAPK signaling pathway by the direct interaction with GSTP1-1. Thus, increased GSTP1-1 levels in tumor cells alters the signaling pathways that control cell proliferation and apoptosis, and thereby rendering tumor cells resistant to chemotherapeutic drugs, not only via its detoxifying activities (Adler et al., 1999; Townsend and Tew, 2003; Wang et al., 2001; Wu et al., 2006). Since the affinity of GSTP1-1 to the majority of anti-cancer drugs is relatively weak (Mcllwain et al., 2006), this finding may provide an explanation for drug resistance in GSTP1-1-overexpressing tumor cells, even when anti-cancer drugs with low or no substrates specificity for GSTP1-1 were used.

The fact that GSTP1-1 has been found to be overexpressed in a number of cancer cells and has been associated with the development of tumor resistance to anti-cancer drugs, identifies GSTP1-1 as an interesting drug target in anti-cancer therapy.
2.5 GSTP1-1 in cell signaling pathways

In non-stressed cells GSTP1-1 binds and inhibits JNK and TRAF2, respectively, and keeps JNK activity at low level. Upon stress stimuli GSTP1-1 dissociates from the complexes leading to an increase activation of the MAPK pathway, and the MAPK JNK and p38. After prolonged stimulation, cells undergo apoptosis, while transient activation mediates differentiation and proliferation.

2.6 GSTP1-1 as a therapeutic target

GSTP1-1 has been identified as a promising drug target in cancer therapy due its role as a detoxification enzyme as well as an endogenous inhibitor of MAPK activation, both contributing to limit the effect of anti-cancer drugs. Thus, various GST inhibitors, such as ethacrynic acid (EA), were developed in the past in order to modulate GST-mediated drug resistance and thereby sensitizing tumors cells to anti-cancer drugs (Townsend and Tew, 2003). EA binds directly to the substrate binding site of GST-A, -M, and –P and potentiates the cytotoxic effects of chlorambucil in human colon carcinoma cell lines (Awasthi et al., 1993a; Petrini et al., 1993a; Tew et al., 1988b). The therapeutic value of EA as a chemosensitizer was tested in clinical trials in patients with advanced cancer, but the clinical utility of EA was limited due to its lack specificity for a specific GST isoform and its strong diuretic properties (O'Dwyer et al., 1991). An
inhibitor that interacts more specifically with GSTP1-1 is TLK199, a peptidomimetic glutathione analogue (Lyttle et al., 1994a; Townsend and Tew, 2003). However, the effect of TLK199 in sensitizing cancer cells to chemotherapeutic drugs, such as chlorambucil, adriamycin and mitomycin C, by inhibiting the GSTP1-1-mediated drug resistance were deemed not strong enough to further pursued in clinical trials (Laborde, 2010; Morgan et al., 1996b).

A much more promising molecule in anti-cancer therapy is the pro-drug TLK286 or γ-glutamyl-α-amino-β-(2-ethyl N,N,N',N'-tetrakis (2-chloroethyl) phosphorodiamidate)-sulfonylpropionyl-(R)-(−) phenylglycine), that targets GSTP1-1. TLK286 gets activated by GSTP1-1-dependent cleavage into a GSH analogue and nitrogen mustard, that can alkylate cellular nucleophiles, leading to an increase in DNA damage, followed by apoptosis (Lyttle et al., 1994b; Townsend and Tew, 2003). The cytotoxic effect of TLK286 has been investigated in vitro and in vivo, and correlates with the GSTP1-1 expression levels (Morgan et al., 1998; Rosario et al., 2000). Accordingly, GSTP1-1-overexpressing tumors are more sensitive to the cytotoxic effects of TLK286 compared to normal tissue. In an ex vivo clonogenic assay in human lung and breast tumors, 15 of 21 lung tumors and 11 of 20 breast tumors were sensitive to TLK286 (lzbicka et al., 1997). In addition, TLK286 acts in vitro synergistically with carboplatin, paclitaxel, doxorubicin, oxaliplatin, cisplatin, anthracyclines and other chemotherapeutic drugs (Hua Xu, 2004; Vergote et al., 2009). The drug-related side effects are minimal and well tolerated in patients. Currently, TLK286 is in phase III clinical trials, and it has been administered as a single agent or in combination with chemotherapeutic drugs in patients with advanced platinum-resistant cancer, such as ovarian cancer and non-small cell lung cancer (Sequist et al., 2009; Vergote et al., 2009; Zimmermann and Peters, 2015).

Although the pro-drug TLK286 is a promising anti-cancer drug, it shows only limited effects in certain types of tumors. For instance, it has been reported that HL60/TLK286 cells (clone from human promyelocytic leukemia cells) are resistant towards the active alkylating product of TLK286 (Rosario et al., 2000). This is associated with increased GSH levels, a mechanism commonly linked to the resistance to alkylating agents (Hayes and Pulford, 1995; Panasci et al., 2001; Tew, 1994). Accordingly, for such cells an alternative anti-cancer strategy needs to be developed. Here, we will next introduce thiazolides, small molecules, which have been found to induce apoptosis in CRC cells in a GSTP1-1-dependent manner (Muller et al., 2008b).
3.0 Thiazolide, a small molecule with lots of power

Thiazolides are small molecules, derived from the parent compound nitazoxanide (NTZ; 2-(acetyloxy-N-(5-nitro 2-thiazolyl) benzamide) and originally discovered by Jean-Francois Rossignol at the Pasteur Institute ~1975. First studies on NTZ showed anti-parasitic effects against tapworm infections. Thus, NTZ was initially developed as a veterinary anti-helminthic drug (Fox and Saravolatz, 2005). Later, Romark Laboratories launched NTZ (trade name Alinia) as an anti-microbial drug, frequently used for the treatment of patients with infectious diarrhea caused by Cryptosporidium parvum and Giardia lamblia (Fox and Saravolatz, 2005; Gargala et al., 2000; Rossignol et al., 2001a, b). Meanwhile, it is known that NTZ exhibits a broad spectrum of in vitro and in vivo activities against various intestinal parasites (Abaza et al., 1998; Adagu et al., 2002; Muller et al., 2006), anaerobe and aerobe bacteria (Dubreuil et al., 1996; Megraud et al., 1998) and even viruses, such as influenza A and B (Belardo et al., 2015; Rossignol, 2014), rotavirus (Rossignol et al., 2006), hepatitis B and C (Keeffe and Rossignol, 2009; Stachulski et al., 2011a; Stachulski et al., 2011c), among several others. For that reason, NTZ and NTZ-analogs are currently being tested in different clinical trials as an anti-viral agent (Rossignol, 2014).

3.1 Pharmacokinetic data of NTZ

Following oral administration (500 mg per dose), NTZ is absorbed from the gastrointestinal tract and to ~one-third excreted in urine of the oral dose and to ~two-thirds excreted in feces. NTZ as such cannot be found in blood, bile, urine or feces, because once absorbed by intestinal epithelial cells and transported to the circulation, NTZ is rapidly hydrolyzed by plasma esterases into its metabolic active form tizoxanide (TIZ) (desacetyl-nitazoxanide) (Figure 3.1) (Adagu et al., 2002; Broekhuysen et al., 2000; Stockis et al., 2002). TIZ is bound to plasma proteins and excreted after ~7 h via the kidneys. TIZ can be found in plasma, urine, bile, and feces. Once in the liver, TIZ is further transformed by glucoronidation to tizoxanide glucuronide (TIG) and is detectable in plasma, urine, and bile. It might be that TIZ and, to some degree, NTZ exhibit activity against intestinal pathogens (Fox and Saravolatz, 2005; Hadad et al., 2012; Stockis et al., 1996). The side effects of NTZ treatment are stomach pain, diarrhea, headache, and vomiting, however, NTZ is usually well tolerated in patients (Abaza et al., 1998).
3.2 The thiazolide mode-of-action is modulated by different substituents

Although, little is known about the thiazolide-induced anti-microbial mechanism-of-action, it could be shown that certain residues on the thiazole and benzene ring play a critical role in the regulation of its anti-pathogenic activity. For instance, it has been reported that the nitro-group (-NO$_2$) on position 5 of the thiazole ring of NTZ is important to exhibit activity against *G. lamblia* (Adagu et al., 2002; Esposito et al., 2005). Thus, the replacement of this nitro-group with a bromide atom, as in the bromo-thiazolidine RM4819 ($N$-(5-bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide), strongly attenuates this activity (Table 3.2) (Muller et al., 2006). Interestingly, studies on the protozoa *Neospora caninum* reported that both NTZ and bromo-thiazolides were active, suggesting a different mode-of-action and molecular target in *N. caninum* versus *G. lamblia* (Esposito et al., 2005). Thus, different substituents appear to modulate the thiazolidine mode-of-action in different parasites and may result in pathogen-specific variants.

Furthermore, it has been reported that thiazolides do not only exhibit activities against various intestinal parasites, anaerobe bacteria and viruses but also induce cell death in human colorectal cancer cells (Muller et al., 2008b).
3.3 Thiazolides exhibit activity against colorectal cancer cells

Müller et al. described for the first time an effect of thiazolides on mammalian cells, specifically on the colorectal adenocarcinoma cell line Caco2. It could be shown that the nitro-thiazolides NTZ, TIZ, and surprisingly also the bromo-thiazolide RM4819, which shows a different anti-parasitic activity than NTZ, induced significant cell death by apoptosis in Caco2 cells. Interestingly, the non-transformed human foreskin fibroblasts (HFF) were much less susceptible to thiazolides-induced apoptosis, indicating that the thiazolide drug target is differentially expressed in Caco2 vs. HFF cells. Further studies with RM4819 identified GSTP1-1 as a major interaction partner. Accordingly, GSTP1-1 mRNA expression was found to be 4-times higher in Caco2 cells than in HFF cells, explaining the reduced thiazolide sensitivity in HFF cells. Along this line, when GSTP1-1 is downregulated in Caco2 cells, RM4819-induced cell death is reduced, while it sensitizes the cells to doxorubicin, showing that a high GSTP1-1 expression level may render cells resistant to chemotherapeutic drugs but susceptible to thiazolides. Similarly, GSTP1-1 overexpression in HEK293T (human embryonic kidney cells), which shows low basal sensitivity to RM4819, strongly increases RM4819-induced cell death. Accordingly, the cytotoxic effect of RM4819 is strongly dependent on the GSTP1-1 expression levels. In addition, it has been reported that RM4819 acts predominantly on proliferating cells. Inhibition of cell cycle leads to reduced RM4819-induced cell death, indicating that RM4819 acts on dividing cells (Müller et al., 2008b).

The RM4819-mediated cell death in Caco2 cells exhibits typical features of apoptotic death, such as nuclear condensation, DNA fragmentation, and phosphatidylserine exposure (Müller et al., 2008b). However, the molecular mechanism of RM4819-induced apoptosis in Caco2 cells remains poorly understood. Since thiazolide activity against CRC cells seems to be dependent on the apoptotic cell death pathway, in this thesis we have focused on the analysis of the signal transduction pathways of thiazolide-induced apoptosis in CRC cells. Consequently, in the next sections the most relevant apoptosis signaling pathways will be discussed.

4.0 Mechanisms of programed cell death

The regulation of cell death is an important process in multicellular organisms, and is essential for tissue sculpting during embryogenesis, tissue homeostasis, and defense against pathogens. Deregulation of the cell death machinery can lead to cancer, and autoimmune and degenerative diseases (Hammond et al., 1997; Mattson, 2000; Reed, 1999; Salmaso et al., 2002). The best-characterized form of programmed cell death is the apoptotic cell death, which is executed by intracellular cysteine proteases called caspases (Cohen, 1997). But also necroptosis,
autophagic cell death and pyroptosis are forms of programmed cell death (Tait et al., 2014). As thiazolides were shown to induce the apoptosis in colorectal cancer cells, the major features of this programed cell death mechanism will be highlighted.

4.1 Apoptosis

Apoptosis is a highly dynamic cellular process characterized by typical morphological and biochemical hallmarks, including cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, cell membrane blebbing, phosphatidylserine (PS) exposure, and membrane-enclosed apoptotic bodies (Kerr et al., 1972; Saraste and Pulkki, 2000). This typical apoptotic morphology is mediated by intracellular cysteine proteases called caspases (Hirsch et al., 1997; Janicke et al., 1998; McIlwain et al., 2015; Woo et al., 1998). They cleave a large number of cellular proteins, and are responsible for the initiation of DNA fragmentation and the degradation of different cellular organelles (Fischer et al., 2003). Caspases represent a family of aspartic acid-specific cysteine proteases that are synthesized as inactive zymogens (Donepudi and Grutter, 2002; Thornberry and Lazebnik, 1998). During the apoptosis pathway, caspases get activated in a self-amplifying cascade by cleavage at specific internal aspartate residues. Activation of upstream apoptosis initiator caspases, such as 8 and 9, by pro-apoptotic stimuli leads to the proteolytic activation of downstream or effector (executioner) caspases, such as caspase 3, 6 and 7 (Donepudi and Grutter, 2002; Hirata et al., 1998; McIlwain et al., 2015; Slee et al., 1999b; Thornberry et al., 1997). As the effector caspase 3 cleaves the majority of the apoptotic substrates (Walsh et al., 2008), it is responsible for the morphological changes observed in apoptotic cells. For instance, catalytically active caspase 3 cleaves and inactivates the inhibitor of caspase-activated DNase (ICAD), leading to the activation of the DNase, DNA fragmentation and chromatin condensation during apoptosis execution (Enari et al., 1998). A further target protein of active caspase 3 is PARP-1 (poly(ADP-ribose) polymerase-1), a protein involved in DNA repair mechanisms (Nicholson et al., 1995). Cleavage of PARP-1 limits the recruitment of the enzyme to sites of DNA damage. However, although cleaved PARP-1 is used as an apoptosis marker, its function in the regulation of cell death is largely unknown (Boulares et al., 1999; Kaufmann et al., 1993; Los et al., 2002). The changes in cell shape during apoptosis are the result of the caspase-dependent cleavage of cytoskeletal proteins, such as actin or fodrin (Maravei et al., 1997; Mashima et al., 1999), while the proteolytic degradation of lamin A promotes nuclear shrinking (Fischer et al., 2003; Orth et al., 1996; Rao et al., 1996; Ruchaud et al., 2002). The initiation and activation of caspases can be found in two major
pathways that lead to apoptosis, called the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathways (Fulda and Debatin, 2006).

4.2 The extrinsic pathway of apoptosis

The activation of the extrinsic apoptosis pathway is triggered by extracellular signals that initiate the activation of death receptors (DRs) (Fulda and Debatin, 2006). DRs are members of the tumor necrosis factor (TNF) receptor superfamily that contain a conserved cytosolic death domain (DD). The specific binding of Fas ligand (FasL), TNFα, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to their cognate DR (CD95/Fas, TNF-R1, TRAIL-R1 and TRAIL-R2) leads to the receptors trimerization, clustering of DDs and recruitment of the adaptor molecule FADD (Fas-associated death domain) (Ashkenazi and Dixit, 1998; Chan et al., 2000; MacEwan, 2002; Siegel et al., 2000; Tartaglia et al., 1993; Thorburn, 2004; Walczak and Krammer, 2000; Wang and El-Deiry, 2003). FADD consists of a C-terminal death domain (DD) that interacts with the DRs by homotypic DD interaction (Chinnaiyan et al., 1995). The N-terminally located death effector domain (DED) of FADD in turn recruits pro-caspase 8 via its DED to form the death-inducing signaling complex (DISC) (Yan et al., 2013). Oligomerization of pro-caspase 8 upon DISC formation facilitates self-cleavage, increasing its enzymatic activity and promoting the release from the DISC (Salvesen and Dixit, 1999). Activated caspase 8 leads to the proteolytic activation of effector caspases (Hirata et al., 1998). However, two distinct types of cells have been identified with regard to how death receptors induce effector caspase activation (Scaffidi et al., 1998). On one hand, type I cells, such as lymphocytes and thymocytes, where active caspase 8 directly mediates the activation of effector caspases 3, 6 and 7 (Muzio et al., 1997; Ozoren and El-Deiry, 2002; Srinivasula et al., 1996). On the other hand, type II cells, such as hepatocytes and pancreatic β cells, where the amount of active caspase 8 is insufficient to fully activate downstream caspases, thus the activation of the mitochondrial apoptosis pathway (intrinsic pathway) is necessary to promote efficient caspase activation and apoptosis induction (Fig. 4.2) (Ozoren and El-Deiry, 2002; Scaffidi et al., 1998). This crosstalk between the extrinsic and the intrinsic pathway occurs through Bid, a pro-apoptotic Bcl-2 family member that is activated upon cleavage by caspase 8 (Li et al., 1998). Accordingly, the caspase-dependent cleavage of Bid leads to the truncated active from of Bid (tBid), which mediates the activation of the mitochondrial apoptosis pathway (Luo et al., 1998). To avoid spontaneous unscheduled cell death, both pathways are tightly controlled by proteins that inhibit the signaling cascade at multiple steps. Deregulation of the cell death machinery is frequently associated with an
impaired apoptosis and plays a critical role in cancer development. Since we are more focused on the intrinsic apoptosis pathway, the next section thus will be briefly discussed.

![Diagram of apoptosis pathways](image)

**Figure 4.2 Caspase cascade activation in type I and type II cells.** Source: (McIlwain et al., 2015)
The activated extrinsic apoptosis pathway leads to the induction of the caspase cascade (see text). In type I cells, activated caspase 8 is sufficient to induce apoptosis by mediating the activation of effector caspases, such as 3, 6, and 7, while in type II cells the amount of active caspase 8 is too low to allow activation of downstream caspases and induction of apoptosis. Thus, Bid is cleaved and activated by caspase 8, and amplifies the DR-mediated apoptosis though the activation of the intrinsic apoptosis pathway (see section 4.4), thereby promoting the activation of effector caspases.

### 4.3 Deregulation of the extrinsic pathway and its biological consequence

The regulation of cell fate after death receptor ligation is a complex process. As an example, it will be highlight the regulating events at the TNFR. Thus, the binding of the cytokine TNFα to its receptor, TNFR1, leads to the assembly of different signaling platforms, known as complex I and complex II, which can initiate cell survival or cell death signaling, respectively (Micheau and Tschopp, 2003). Following activation of TNFR1 and clustering of the DDs may recruit the
adaptor protein TNF receptor associated-protein with death domain (TRADD). This leads to the recruitment of further signaling proteins including TRAF2, receptor associated protein kinase 1 (RIPK1), and cellular inhibitor of apoptosis proteins cIAP1 and cIAP2, which form the signaling complex I (Chen and Goeddel, 2002; Muppidi et al., 2004). cIAP1 and cIAP2 can mediate the ubiquitination of RIPK1 via their E3 ligase activity and thus promote the activation of the transcription factor NF-κB (Bertrand et al., 2008; Varfolomeev et al., 2008). NF-κB in turn regulates the transcription of cytokines as well as anti-apoptotic proteins such as cIAP1/2 and cFLIP (Flice-like inhibitory protein), which prevent cell death and sustain inflammation (Chu et al., 1997; Kreuz et al., 2001; Micheau et al., 2001; Wang et al., 1998).

IAPs are often overexpressed in cancer cells and frequently correlate with a poor prognosis of cancer patients. It could be shown for cervical tumors and other types of cancer that an elevated cIAP levels lead to a drug-resistant phenotype (Imoto et al., 2002; Suzuki et al., 2000). The ability of IAPs to inhibit apoptosis, but also mediate pro-survival signals by activating the NF-κB pathway, contributes to tumor cell survival and suggests IAPs as interesting drug target for anti-cancer therapy (de Almagro and Vucic, 2012).

In a second step, the dissociation of TRADD, RIPK1 and TRAF2 from the TNFR1 leads to the recruitment of FADD and procaspase-8 and to the formation of the cytoplasmic complex II that promote cell death signaling (Micheau and Tschopp, 2003). The activation of complex II can be inhibited by c-FLIP, a caspase 8 homolog that lacks catalytic activity and competes with caspase 8 binding to FADD, thus regulating the activation of caspase 8 (Irmler et al., 1997; Tschopp et al., 1998). Accordingly, high expression levels of c-FLIP correlate with the resistance to death ligand-induce apoptosis and interestingly also to chemotherapy-induced apoptosis in numerous tumor cells, such as melanoma, hepatocellular carcinoma and colorectal cancer cells (Chen et al., 2010; Irmler et al., 1997; Wilson et al., 2007).

The activation of NF-κB may result in a positive feedback loop by expression of cIAP1/2 and cFLIP, thus preventing cell death induction. It has been shown in various cancer cells that the NF-κB activity is constitute and protects against apoptosis (Bargou et al., 1997; Sovak et al., 1997). Interestingly, ionizing irradiation and chemotherapeutic drugs have been shown to induce NF-κB, contributing to the development of drug resistances in cancer cells (Godwin et al., 2013; Pahl, 1999). Accordingly, the improper regulation of NF-κB might play a critical role in tumor development.
Many cancer cells are also resistant to TRAIL-induced apoptosis. Like TNFR1, TRAIL death receptors can also mediate the activation of the NF-kB pathway upon TRAIL binding, which may result in cell survival (Ehrhardt et al., 2003; Zhang and Fang, 2005). Thus, the improper regulation of the death receptor signaling pathway and the deregulated activation of the NF-kB pathway may prevent cell death and sustain cell survival in such tumor cells. Interestingly, it could be shown that TRAIL may sensitize tumor cells to other apoptosis triggers, which is currently being tested in clinical studies (Holoch and Griffith, 2009).

In general, the loss of the death ligand-induced activation of the death receptor signaling pathway and the aberrant expression of cytosolic components of the death receptor-mediated apoptosis pathway may inhibit apoptosis and is quite often associated with anti-cancer drug resistances (Plati et al., 2008)

4.4 The intrinsic apoptosis pathway and its regulatory mechanisms

Internal cytotoxic stimuli, such as DNA damage, lead to the activation of pro-apoptotic signal-transducing molecules that induce mitochondrial outer membrane permeabilization (MOMP) and effector caspase activation (Green and Kroemer, 2004). This process is strongly regulated by proteins from the Bcl-2 family whose activation leads to the initiation of pore formation in the outer membrane of the mitochondria (Brunelle and Letai, 2009; Fulda and Debatin, 2006). Since MOMP is an irreversible process, Bcl-2 family members thus tightly control the life/death balance in mammalian cells (Cory and Adams, 2002). They can be subdivided in pro- and anti-apoptotic Bcl-2 family members. The pro-apoptotic members can be in turn classified into two death-promoting families; the “killers” and the “regulators” (Giam et al., 2008; Westphal et al., 2011; Willis and Adams, 2005). Each of the three subfamilies harbors at least one or multiple Bcl-2 homology (BH) domain, which is required for their interaction with other Bcl-2 members. The pro-survival/anti-apoptotic Bcl-2 members, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 and Boo, contain four BH domains (BH1-BH4). The “killers” Bak, Bax and Bok show similar homology sequences (BH1-BH3) to pro-survival members (Moldoveanu et al., 2006; Suzuki et al., 2000; Westphal et al., 2011), while the “regulators” pro-apoptotic members such as Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa and Puma, contain only one BH domain (also named BH3-only proteins) (Willis and Adams, 2005). Each pro-survival Bcl-2 family member has a hydrophobic groove on its surface, to which a BH3 domain can bind. This interaction neutralizes the pro-survival members (Adams and Cory, 2007). In non-stressed cells, pro-survival Bcl-2 family members prevent Bax and Bak activation and thus the Bak- and Bax-induced pore formations in the outer mitochondrial membrane. In a response to pro-apoptotic stimuli, BH3-only proteins become
transcriptionally upregulated and/or post-translationally modified (or relocalized) to gain their full pro-apoptotic potential (Hubner et al., 2008; Ley et al., 2005; Lomonosova and Chinnadurai, 2008; Verma et al., 2001). Activated BH3-only proteins can either directly bind to and activate Bax and Bak, as in the case of Bim, Bid and Puma, or bind to and neutralize anti-apoptotic Bcl-2 homologs, thereby releasing primed Bax and Bak, and promoting MOMP (Adams and Cory, 2007; Shamas-Din et al., 2011).

While some BH3-only proteins, such as Bim, tBid and Puma, avidly bind to all the pro-survival Bcl-2 homologs, others like Bad and Noxa are more “selective binders” and interact only with distinct anti-apoptotic Bcl-2 homologs (Chen et al., 2005). Interestingly, such “promiscuous binders” that interact with a set of pro-survival Bcl-2 homologs are more potent killers than the others. However, it has been reported that co-expression of “selective binders”, showing complementary specificity (e.g. Noxa and Bad), kills cells as potently as the “promiscuous binders” (Chen et al., 2005). These findings indicate that the neutralization of pro-survival members by BH3-only proteins plays a central role for an efficient apoptosis induction by activating Bak and Bax (Adams and Cory, 2007).

It is well accepted that BH3-only proteins act upstream of Bak and Bax, because they cannot induce apoptosis in Bax/Bak-deficient cells (Lindsten et al., 2000; Wei et al., 2001; Zong et al., 2001). However, it is still controversial how BH3-only proteins activate Bax/Bak. Previously, two distinct models have been proposed, the direct activation model and the indirect activation model (Figure 4.4.1) (Willis and Adams, 2005). The direct activation model proposes that certain BH3-only proteins termed “activators” such as Bim, tBid and perhaps Puma, bind with low affinity to Bax and Bak in a “hit and run” fashion and promote their activation (Cartron et al., 2004; Gavathiotis et al., 2008; Kim et al., 2006b; Kuwana et al., 2005; Kuwana et al., 2002; Letai et al., 2002; Oh et al., 2006; Walensky et al., 2006). In addition and in accordance with this model, the other BH3-only proteins, termed “sensitizer”, interact only with pro-survival proteins (Kim et al., 2006b; Letai et al., 2002). Their binding to and neutralization of anti-apoptotic Bcl-2 homologs allows the liberation of any bound “activator” BH3-only proteins, promoting the direct activation of Bax and Bak, and already primed Bax and Bak (Kim et al., 2006b). On the contrary, the indirect model suggest that all BH3-only proteins bind primary to pro-survival Bcl-2 family members, thereby preventing them to bind and neutralize active Bax and Bak molecules (Chen et al., 2005; Willis et al., 2005; Willis et al., 2007). Thus, both models have in common that BH3-only proteins are required for the binding and neutralization of pro-survival Bcl-2 family members, and triggering the activation of Bax and Bak to induce apoptosis. Thus, it seems to be
that both death-promoting family members, the “killers” and the “regulators” need to be activated to induce the intrinsic apoptosis cascade (Adams and Cory, 2007; Giam et al., 2008; Lomonosova and Chinnadurai, 2008; Willis and Adams, 2005). Of note, more recently these two mutually exclusive models have been combined in an unifying model, taking in account direct Bax/Bak activation and neutralization of anti-apoptotic Bcl-2 homologs by BH3-only proteins (Shamas-Din et al., 2011).

Figure 4.4.1 Bax/Bak activation proposed by two distinct models (see text). Source: (Adams and Cory, 2007; Shamas-Din et al., 2011)

(A) Direct activation model: Activator BH3-only proteins bind to Bax and Bak and promote their activation. Pro-survival Bcl-2 family members inhibit the activator BH3-only proteins to suppress apoptosis, but do not interact with Bax and Bak. The sensitizer BH3-only proteins displace the bound activator BH3-only proteins from the pro-survival Bcl-2 family members, promoting apoptosis. (B) Indirect activation model: BH3-only proteins bind primary to pro-survival Bcl-2 family members, thereby preventing them to bind and neutralize active Bak and Bax molecules. Active Bak and Bax initiate pores in the mitochondrial outer membrane.

Once Bak and Bax are activated, they convert into pore-forming proteins by changing conformation and assembling into homo-oligomeric complexes in the mitochondrial outer
membrane (Westphal et al., 2011). Consequently, proteins from the mitochondrial intermembrane space (MIMS) such as cytochrome c, Smac/DIABLO and omi/HtrA2 are released into the cytoplasm (Figure 4.4.2) (Kluck et al., 1997; Saelens et al., 2004; Yang et al., 1997). Thereupon, cytoplasmic cytochrome c transiently binds to the apoptotic peptidase activating factor 1 (Apaf-1), a cytosolic protein with a caspase-recruitment domain (CARD), and facilitates the association of ATP/dATP (Zou et al., 1997). Subsequently, bound ATP/dATP triggers the oligomerization of Apaf-1 into a wheel-like heptamer that exposes its CARD on which procaspase 9 CARD binds to from the apoptosome (Adrain et al., 1999; Jiang and Wang, 2000; Li et al., 1997). This oligomerization leads to the auto-activation of procaspase 9, which in turn mediates the activation of the effector caspases to induce cell death (Bratton et al., 2001; Cullen and Martin, 2009; Renatus et al., 2001; Slee et al., 1999a; Twiddy et al., 2006). Thus, the formation of the apoptosome is an essential protein complex, mediating the activation of effector caspases in the intrinsic apoptosis pathway in response to various cell death stimuli (Cullen and Martin, 2009).

The X-linked IAP (XIAP) protein in turn is a known caspase inhibitor and a member of the inhibitor of apoptosis protein (IAP) family. It binds and inhibits caspase 3, 7 and 9 (Eckelman et al., 2006). Upon apoptotic stimuli and the releases of Smac/DIABLO and omi/HtrA2 from the MIMS into the cytoplasm directly bind XIAPs via their IAP-binding motif and antagonize the inhibitory effects of XIAPs (Du et al., 2000; Hegde et al., 2002; Liu et al., 2000b; Martins et al., 2002; Suzuki et al., 2001; van Loo et al., 2002). Accordingly, the releases of Smac/DIABLO and omi/HtrA2 indirectly promote caspase activation as a response of internal cytotoxic stimuli through neutralizing endogenous inhibitors of caspases and thus promote the apoptosis (Saelens et al., 2004).

Since Bcl-2 family members tightly control the intrinsic apoptosis pathway, their deregulation is strongly associated in cell death resistance and will be discussed next.
Figure 4.4.2 The intrinsic apoptosis pathway
Upon intracellular stress, activated pro-apoptotic "regulators" or also called BH3-only proteins bind and neutralize pro-survival Bcl-2 family members and/or may interact directly with pro-apoptotic "killers" Bak and Bax (see text). Active Bak and Bax form pores in the outer mitochondrial membrane, promoting permeabilisation and release of cytochrome c, Smac/DIABLO and omi/HtrA2. Cytochrome c binds to Apaf-1, triggering the recruitment and activation of caspase 9 in an apoptosome complex that leads to the activation of effector caspases, such as caspase 3. Endogenous inhibitors, such as XIAP inhibits caspases, which are neutralized by Smac/DIABLO and omi/HtrA2.

4.5 The deregulation of Bcl-2 family members in cell death resistance
Resistance of apoptosis induction is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). Thus, it is widely accepted that the impaired apoptosis is important for tumor cell survival, particularly during the late stage of tumorigenesis (Kirkin et al., 2004; Reed, 1999). In a large number of and in diverse malignant tumors, deregulation of Bcl-2 family members has been found, which generally correlates with a poor prognosis in many human cancers during the advanced tumor stage. For instance, Bcl-2 was the first described pro-survival family member of the Bcl-2 family that was found to be deregulated in human follicular B cell lymphoma. Through
the chromosome translocation t(14:18), the Bcl-2 gene was placed next to the immunoglobulin heavy chain enhancer, resulting in constitutive Bcl-2 gene expression (Tsujimoto et al., 1984). This chromosomal translocation is also found in CRC and chronic lymphocytic leukemia (CLL) as a mechanism for increased Bcl-2 expression (Adachi et al., 1990; Ayhan et al., 1994). While overexpression of Bcl-2 has been reported to protect cultured cells against several chemotherapeutic agents, downregulation of Bcl-2 in turn sensitizes tumor cell lines towards anti-cancer drugs (Miyashita and Reed, 1993; Simonian et al., 1997). Similar observations were made for Bcl-x<sub>L</sub>. High expression levels of Bcl-x<sub>L</sub> were detected in a numerous tumors, including a wide range of colorectal cancer cells, correlating with anti-cancer drug resistance (Amundson et al., 2000; Liu et al., 1999; Minn et al., 1995; Simonian et al., 1997; Violette et al., 2002). Also the pro-survival Bcl-2 family member Mcl-1 (myeloid cell leukemia 1) was identified by being up-regulated in tumor cells. Several types of cancer show the loss of endogenous microRNAs (miRs) that normally repress Bcl-2 family members. The loss of miR-29, a repressor of Mcl-1, was shown as potential reason for elevated Mcl-1 expression levels found in CLL and CRC (Calin et al., 2005; Cummins et al., 2006; Mott et al., 2007). Mcl-1 overexpression is associated with a poor prognosis and drug resistant phenotype in cancer patients (Michels et al., 2014; Palve et al., 2014).

Consequently, different genetic alterations during the tumorigenesis may contribute to the overexpression of pro-survival Bcl-2 members, which confer tumor cell death resistance and thus may reduce the efficiency of anti-cancer drugs (Johnstone et al., 2002; Kirkin et al., 2004; Reed, 1999; Yip and Reed, 2008). However, not only the overexpression of pro-survival factors provides resistance to the cell death induction, but also the inactivation of pro-apoptotic Bcl-2 homologs prevents apoptosis in cancer cells. For instance, acquired defects in the DNA mismatch repair system may lead to microsatellite instability and can thereby inactivate the “killer” pro-apoptotic member Bax through frameshift mutation. This occurs in up to ~ 50 % of cancer cells, including CRC (Grady and Carethers, 2008; Rampino et al., 1997). The loss of Bax leads to a resistant phenotype against chemotherapeutic drugs, such as 5-fluorouracil and doxorubicin, amongst others found in CRC and B cell chronic lymphocytic leukemia (B-CLL) (Bosanquet et al., 2002; Sturm et al., 1999; Zhang et al., 2000).

Defective expression of pro-apoptotic Bcl-2 family members also occurs once p53 is lost or mutated during tumorigenesis. p53 can directly promote the transcription of Bax, Bid, Puma and Noxa (Miyashita et al., 1994; Miyashita and Reed, 1995; Oda et al., 2000; Sax et al., 2002; Yu et
al., 2003). Thus, loss of function of p53 may result in a reduce expression of pro-apoptotic factors and thereby preventing cell death induction in tumor cells.

Consequently, defects in the apoptosis pathway may facilitate the clonal outgrowth of mutated cell populations that may result in tumor hyper-proliferation and malignant transformation (Kirkin et al., 2004) (Hanahan and Weinberg, 2000). In addition, the up-regulation of the pro-survival and down-regulation of the pro-apoptotic Bcl-2 family members may potentially attenuate the effect of anti-cancer agents during the advanced tumor cells. Thus, defects in the apoptosis signaling pathways may result in cell death resistance in tumor cells, sustaining cell proliferation and tumor growth. This observation strongly correlates with a drug resistant phenotype (Johnstone et al., 2002; Kirkin et al., 2004; Yip and Reed, 2008). This notion identifies Bcl-2 family members as potential drug targets in anti-cancer therapy. Thus, different therapeutic options have been developed based on these family members to sensitize tumor cells to apoptotic stimuli. One possibility is the development of BH-3 mimetics, such as ABT-737 and ABT-263 that bind and neutralize pro-survival Bcl-2 proteins and sensitize cells to other apoptosis triggers, such as carboplatin. Both BH-3 mimetics are already in clinical phase I and II trials for CLL, lymphoma and small lung cancer (Brunelle and Letai, 2009; Ni Chonghaile and Letai, 2008; Oltersdorf et al., 2005; Yip and Reed, 2008). Another potential strategy is the development of antisense oligonucleotides, targeting mRNAs of anti-apoptotic Bcl-2 members, which downregulates pro-survival Bcl-2 factors and thereby sensitizing cell to chemotherapeutic agents (Chi et al., 2000; Klasa et al., 2002; Waters et al., 2000).

Here, we like to highlight that the sophisticated drug resistant mechanisms in cancer cells are a prevalent problem in anti-cancer therapy. While the most anti-cancer drugs are linked to activate cell death singling pathway, such as the extrinsic and intrinsic apoptosis pathway, impaired apoptosis during the advanced tumor stage leads to cell death resistance and may thus limit the effect of such drugs (Pommier et al., 2004). In addition, overexpression of the detoxification enzyme GSTP1-1 in the course of the adenoma-carcinoma sequence facilitates the inactivation of chemotherapeutic drugs and thereby prevents apoptosis induction, resulting in sustained cell proliferation and tumor growth (Laborde, 2010; Tew, 1994). Current efforts focus on discovering novel therapeutic approaches to bypass the drug resistance mechanisms of cancer cells in order to make them more sensitive for cell death triggers.
OBJECTIVES

As GSTP1-1 is overexpressed in a number of solid tumors, including CRC cells, it has been proposed as a promising drug-target in anti-cancer therapy (Ranganathan and Tew, 1991). As a detoxification enzyme and inhibitor of important signaling pathways, it attenuates the effect of chemotherapeutic agents in CRC cells (Laborde, 2010; Tew, 1994). Previous studies of Müller et al. have identified thiazolides as inducers of cell death in CRC cells, such as Caco2 and LS174T. In particular, the bromo-thiazolide RM4819 directly targets GSTP1-1 and induces a GSTP1-1-dependent cell death upon treatment. However, the molecular mechanisms of thiazolides-mediated apoptosis in a GSTP1-1-dependent manner remain unclear. This thesis project thus further focusses on the underlying mechanisms of thiazolide-induced apoptosis in CRC cells.

Chapter I:
Analyzing the molecular structure-function relationship of RM4819-induced apoptosis induction in CRC cells: It still remains unclear which molecular structures of thiazolide are required for the interaction with GSTP1-1 and/or might be important for the conjugation to GSH in order to induce apoptosis in CRC cells. To clarify such, novel thiazolide derivatives were synthesized with different variations in their substituents, and their activities were tested in Caco2 and LS174T cells. The requirement of GSTP1-1 for cell death induction, caspase activation and DNA fragmentation by the different thiazolide derivatives were also examined in order to confirm the apoptotic cell death induction in a GSTP1-1-dependent process. In addition, since GSTP1-1 catalyzes the conjugation between GSH and substrates and thereby may promote drug resistances, its role in thiazolide-induced cell death was studied in more detail.

Chapter II and III:
Thiazolide-activated mitochondrial apoptosis pathway is regulated at multiple steps and sensitizes CRC to other apoptosis triggers upon thiazolide treatment: Currently, the mode of action of thiazolide-induced cell death in a GSTP1-1-dependent manner is poorly understood. In these chapters, we analyzed the GSTP1-1 and GSH expression levels in different CRC cell lines and tested their sensitivity to thiazolides. We further studied the requirement of GSTP1-1 enzymatic activity, and could confirm that both high GSTP1-1 expression level and enzymatic activity are necessary to induce cell death upon thiazolide treatment. The GSH levels were also found to play an important role in thiazolide-induced cell death in CRC cells. For a better understanding of the apoptosis signaling pathway activation, different apoptosis regulators were investigated. As the MAPKs JNK and p38 are sequestered by GSTP1-1, their respective role in
thiazolide-treated CRC cells was determined. Additionally, the regulation of pro- and anti-apoptotic Bcl-2 family members in the cell death signaling pathway was examined upon thiazolide treatment. Of special interest was whether thiazolides sensitize CRC cells to other apoptosis inducers through bypassing the GSTP1-1-mediated drug-resistance mechanism. Therefore, combined treatments of chemotherapeutic drugs or TRAIL with thiazolides were investigated in CRC cells.
Chapter I

Structure-Function Relationship of Thiazolide-Induced Apoptosis in Colorectal Tumor cells

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Abstract

Thiazolides are a novel class of anti-infectious agents against intestinal intracellular and extracellular protozoan parasites, bacteria and viruses. While the parent compound nitazoxanide (NTZ; 2-(acetolyloxy)-N-(5-nitro-2-thiazolyl)benzamide) has potent anti-microbial activity, the bromo-thiazolide RM4819 (N-(5-bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide) shows only reduced activity. Interestingly, both molecules are able to induce cell death in colon carcinoma cell lines, indicating that the molecular target in intestinal pathogens and in colon cancer cells is different. The detoxification enzyme glutathione S-transferase of class Pi 1 (GSTP1-1) is frequently overexpressed in various tumors, including colon carcinomas, and limits the efficacy of anti-tumor chemotherapeutic drugs due to its detoxifying activities. In colorectal tumor cells RM4819 has been shown to interact with GSTP1-1, and GSTP1-1 enzymatic activity is required for thiazolide-induced apoptosis. At present it is unclear which molecular structures of RM4819 are required to interact with GSTP1-1 and to induce cell death in colon carcinoma cell lines. Here, we demonstrate that novel thiazolide derivatives with variation in their substituents of the benzene ring do not significantly affect apoptosis induction in Caco2 cells whereas removal of the bromide atom on the thiazole ring leads to a strong reduction of cell death induction in colon cancer cells. We further show that active thiazolides require caspase activation and GSTP1-1 expression in order to induce apoptosis. We demonstrate that increased glutathione (GSH) levels sensitize colon cancer cells to thiazolides, indicating that both GSTP1-1 enzymatic activity as well as GSH levels are critical factors in thiazolid induced cell death.

Introduction

Thiazolides are a novel class of antibiotics frequently used for the treatment of intestinal parasitic, bacterial and viral infections (Fox and Saravolatz, 2005; Hemphill et al., 2006). The parent compound nitazoxanide (NTZ, 2-(acetolyloxy)-N-(5-nitro-2-thiazolyl)benzamide) has been shown to exhibit a broad spectrum of in vitro and in vivo activities against various parasites, bacteria and even viruses (Adagu et al., 2002; Fox and Saravolatz, 2005; Gardner and Hill, 2001; Muller et al., 2006). NTZ (trade name Alinia®, Romark Laboratories) is frequently used for the treatment of patients with infectious diarrhea caused by Cryptosporidium parvum (Rossignol et al., 2001a) and Giardia lamblia (Abboud et al., 2001b; Rossignol et al., 2001a). NTZ is a pro-drug and becomes rapidly hydrolyzed to the active metabolite tizoxanide (TIZ) after oral administration (Broekhuysen et al., 2000).
Previous studies in *Giardia lamblia* pointed out that the nitro group on the thiazole ring of NTZ is required for its anti-parasitic activity. Replacement of this nitro group with a bromide atom, e.g. as present in the bromo-thiazolide RM4819 (N-(5-bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide), strongly attenuates the anti-parasitic activity of these bromo-derivatives (Muller et al., 2006). Although thiazolides are best known for their anti-parasitic function, more recent studies revealed a surprising apoptosis-inducing activity in colorectal tumor cells. (Muller et al., 2008b) Interestingly, both NTZ as well as the bromo-thiazolide RM4819, lacking anti-parasitic activity against *Giardia lamblia* (Muller et al., 2006), showed significant apoptosis-inducing activity in colorectal tumor cells (Muller et al., 2008b). These findings indicate that the molecular targets differ in intestinal pathogens and colon cancer cells. Further studies in colorectal tumor cells (Caco2 and LS174T) identified glutathione S-transferase (GST) of the class Pi (GSTP1-1) as a RM4819 interaction partner and potential target protein (Muller et al., 2008b). GSTP1-1 is one of the 7 classes (Alpha, Mu, Pi, Theta, Sigma, Zeta, Omega) of mammalian cytosolic GSTs (Mannervik et al., 1992; Mannervik et al., 2005). In general, GSTs are phase II detoxification enzymes and catalyze the conjugation of the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl glycine) to electrophilic compounds (Goto et al., 1999; Ishikawa and Ali-Osman, 1993; Laborde, 2010; Talalay, 1989; Tew, 1994). GSTP1-1 is overexpressed in a wide range of tumors, such as colon, breast, kidney, lung and ovarian cancer cells (Howells et al., 2004; McIlwain et al., 2006; Tidefelt et al., 1992a). Inactivation of chemotherapeutic drugs via GSTP1-1-mediated conjugation to GSH may result in chemoresistance of these tumor cells (Goto et al., 1999; Ishikawa and Ali-Osman, 1993) and limit the therapeutic effect of different drugs. Interestingly, RM4819 does not seem to be an inhibitor of GSTP1-1, but rather a substrate as GSTP1-1 enzymatic activity was required for cell death induction after RM4819 treatment (Sidler et al., 2012) (see Chapter II). This compound appears to promote apoptosis in colorectal tumor cells via the GSTP1-1-mediated activation of c-Jun-terminal kinase (JNK) and subsequent activation of the pro-apoptotic Bcl-2 homolog Bim (Sidler et al., 2012). This identifies GSTP1-1 as an interesting target protein and as an Achilles' heel to induce cell death in chemotherapy-resistant colon carcinoma cells using thiazolides (Townsend and Tew, 2003).

At present it is known that GSTP1-1 activity is required for the thiazolide-induced activation of downstream effector molecules to initiate the mitochondrial apoptosis pathway. However, the structure-function relationship for thiazolide-induced apoptosis is not fully understood. Specifically, it is unclear which molecular structures of thiazolides interact with GSTP1-1 and/or might be conjugated to GSH to induce cell death in colon carcinoma cells.
In this study, RM4819 and several derivatives were synthesized for a subsequent structure-function analysis. The thiazolide basic structure consists of a thiazole ring and a benzene ring, linked by an amide bond. The lead compound RM4819 contains a bromide atom on position 5 of the thiazole ring, and a hydroxyl and methyl group on positions 2 and 3 of the benzene ring. Various modifications were made on positions 2 and 3 of the benzene ring of RM4819, where the hydroxyl and the methyl group of the different thiazolide derivatives were removed or replaced. In one of the thiazolide derivatives the bromo substituent was also replaced by a hydrogen atom.

Here, we show that modifications of the benzene ring of the different thiazolide derivatives alone did not affect their apoptosis-inducing activity in Caco2 and LS174T cells. Interestingly, removal of the bromo substituent led to a strong reduction of cell death-inducing activity indicating that this group is required for its activity. In contrast, the basic modules (thiazole ring or benzene ring alone) failed to promote cell death. Furthermore, we analyzed the requirement of GSTP1-1 for cell death induction by different thiazolide derivatives and confirmed that the tested apoptosis-inducing thiazolides required GSTP1-1 expression, and that the cell death induction can be enhanced by increasing GSH level in Caco2 cells. We also demonstrated that thiazolide-induced cell death was dependent on caspase activity, as caspase inhibition attenuated thiazolide-induced cell death.

**Results and Discussion**

**Cell death-inducing activity of thiazolide derivatives**

The bromo-thiazolide RM4819 has been previously shown to induce apoptosis in different colorectal tumor cell lines, i.e. Caco2, and LS174T (Muller et al., 2008b; Sidler et al., 2012). Furthermore, GSTP1-1 was found to physically interact with RM4819 and to be required for RM4819-induced cell death (Muller et al., 2008b). Of interest was the fact that overexpression of an enzymatically more active form of GSTP1-1 or an increase of intracellular GSH levels further enhanced RM4819-induced apoptosis (Sidler et al., 2012)(see Chapter II), suggesting that GSTP1-1 enzymatic activity is required for thiazolide-induced cell death, likely by conjugating GSH to RM4819 and thereby generating an active metabolite. However, at present it is not known which molecular structures of thiazolides interact with GSTP1-1 and/or might be conjugated to GSH to induce cell death in colon carcinoma cell lines. To characterize the structure-function relationship we synthesized novel derivatives of RM4819 with specific modifications at the benzene and thiazole ring (synthesis and analysis described in Supporting
Information). The variable groups R² and R³ are linked to the benzene ring, from which the hydroxyl and methyl group at position R², and R³ were removed and/or replaced in the different substituents. Furthermore, in one of the derivatives the bromide atom was additionally substituted by hydrogen on R¹ of the thiazole ring. The novel thiazolide derivatives are listed as compound 1-5 (Table 1A), whereas RM4819 and NTZ are conventionally used names.

Table 1: Thiazolide derivatives and basic modules. The thiazolide basic structure consists of the thiazole ring and a benzene ring, linked by an amide bond. A) Different modifications of RM4819 were done on position 5 of the thiazole ring (R¹) and position 2 and 3 of the benzene ring (R² and R³). B) The basic modules were subdivided in A and B with changes on their substituents at position R¹ and R².
We also analyzed the cell death induction of thiazolide basic modules. The benzene ring is labeled with A and the thiazole ring with B. Basic modules were also subdivided according to the variable groups in R\textsuperscript{1} and R\textsuperscript{2}. The basic modules are designated by numbers 6-11 (Table 1B).

The thiazolide derivatives and basic modules were initially tested for their cell death-inducing activity in Caco2 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) assay (Figure 1). As shown previously, the lead compound RM4819 dose-dependently induced cell death over a range of concentrations. In contrast, the basic module 5-bromothiazol-2-amine (6) failed to demonstrate a relevant cell death-inducing activity in Caco2 cells. Interestingly, all modifications of RM4819 in R\textsuperscript{2} and R\textsuperscript{3} of the benzene ring did not seem to compromise or enhance the cell death induction activity of the respective derivatives, which all induced cell death at similar rates as RM4819 (Figure 1A).

We next investigated the importance of the bromo substituent in R\textsuperscript{1} of the thiazole ring. Interestingly, although compound 1 (N-(5-bromothiazol-2-yl)-3-methylbenzamide) was found to be one of the most potent thiazolide derivatives, 3-methyl-N-(thiazol-2-yl)-benzamide (2), an analog of 1 lacking the bromide atom, showed a strongly reduced cell death-inducing activity (Figure 1B). As was expected from the results with the basic module 6, no cell death-promoting activity was detectable using any basic modules 7-11 alone (Figure 1C).

The results showed that the removal and/or replacement of the substituents on R\textsuperscript{2} and R\textsuperscript{3} of the benzene ring did not alter significantly the cell death-inducing activity of thiazolides (Figure 1A). However, removal of the bromide atom from the thiazole ring strongly attenuated cell death induction of this derivative (Figure 1B). This indicates that the bromide atom, but not the substituents on the benzene ring, are critical for the cell death-inducing activity of thiazolides in colorectal tumor cells. Furthermore, we found that different thiazolide basic modules were not active in Caco2 cells (Figure 1C), indicating that both thiazole and benzene ring, linked by an amide bond, are necessary to induce cell death in Caco2 cells.
Figure 1: Thiazolide-induced cell death in Caco2 cells. Caco2 cells were exposed to increasing concentrations of different thiazolide derivatives (A and B) and basic modules (C) for 40 h. Cell death induction was measured by MTT assay. Different concentrations of DMSO (%) were used as solvent control. Mean values of triplicates ± SD of a representative experiment are shown (n>3).

Thiazolide derivatives induce hallmarks of apoptosis

In order to study whether the cell death induction by the different thiazolides is also mediated by apoptosis, as previously shown for RM4819 (Muller et al., 2008b), we analyzed the induction of DNA fragmentation, a hallmark of apoptosis (Liu et al., 1996; Wyllie, 1980). The cellular DNA content was stained with propidium iodide (PI) and measured by flow cytometry. In agreement with the MTT assay, we observed that all thiazolide derivatives, with the exception of compound
and the basic module 6, were capable of inducing DNA fragmentation in Caco2 cells, resulting in reduced nuclear DNA (Figure 2A and B).

Figure 2: Thiazolides derivatives-induced DNA fragmentation. Caco2 cells were treated with different thiazolide derivatives (20 µM) or with DMSO as control (0.1%). DNA fragmentation was analyzed by PI staining. Representative PI staining from n=3 experiments are shown. Numbers indicate mean values of triplicates ± SD of cells with fragmented DNA. A) Results with active thiazolides. B) Results with inactive thiazolides, in comparison to RM4819.
To further confirm that all thiazolide derivatives induce cell death by apoptosis, we also analyzed caspase activity in Caco2 cells. Caspases are cysteine-aspartate proteases, which become activated during apoptosis and are critical for the execution of this form of cell death (Snigdha et al., 2012). Caspase 3 is one of the most critical effector caspases in this process. Caspase activity in thiazolide-treated Caco2 cells was assessed using Ac-DEVD-AFC as a fluorogenic substrate. The treatment with RM4819, and compound 1 and 3 lead an increase in caspase activity compare to control treated cells. In marked contrast and in agreement with the cell death assays, compound 2 (an analog of 1, with hydrogen in R1) failed to induce increased levels of caspase activity (Figure 3A). Caspase 3 activation was further confirmed by western blotting. Upon cleavage and activation by initiator caspases, caspase 3 further undergoes proteolytic self-processing (Harvey and Kumar, 1998; Kumar, 2007). The processed form of caspase 3 consists of large (17/19 kDa) and small (10/12 kDa) subunits, which dimerize to an active form of caspase 3. In agreement with the caspase activity data, western blot analysis demonstrate that the cell death inducing thiazolide derivatives, but not compound 2, were able to induce caspase 3 processing, as seen by the presence of the large subunit (17/19 kDa) (Figure 3B). Next to the 17/19 kDa bands, protein bands at 24 kDa were also detected, which represent an intermediate stage of cleaved caspase 3.

To assess the relevance of caspase activation for thiazolide-induced apoptosis, we used a specific caspase inhibitor, and measured DNA fragmentation after the treatment of cells with the thiazolides RM4819, compound 1, 2 and 3. Figure 3C shows that in the presence of the caspases inhibitor thiazolide-induced cell death is significantly reduced. The results of Figure 2 and 3 illustrate that the thiazolide derivatives induce the activation of the apoptosis pathway in colon carcinoma cells, which is caspase-dependent. We also found that the reduced cell death induction of compound 2 correlates with low caspase-inducing activity.
Figure 3: Thiazolide-induced cell death is caspase-dependent. A) Caco2 cells were treated with thiazolide derivatives (20 µM) for 40 h. Caspase activity was measured by a fluorimetric assay. Mean values of triplicates ± SD of a typical experiment out of n=3 are shown. B) Caco2 were treated with 20 µM thiazolides or 0.1% DMSO as a solvent control for 40 h. Cleaved caspase 3 and tubulin as loading control were detected by western blotting. C) Cells were pretreated with DMSO control or zVAD (80 µM), following stimulation with 20 µM thiazolides for 40 h. Apoptosis was assessed by PI staining. Mean values (% DNA fragmentation) of triplicates ± SD of a representative experiment are shown (n=3). Data were analyzed in an unpaired T-test (* P<0.001).

To investigate whether these results also apply to other colorectal tumor cells we investigated thiazolide-induced cell death in the colorectal tumor cell line L174T (Sidler et al., 2012). Comparable to Caco2 cells, the parent thiazolide RM4819, as well as the derivatives 1 and 3 efficiently induced cell death in L174T cells, whereas compound 2 only caused minimal cell death (Figure 4A). The sensitivity of L174T cells to active but not inactive thiazolides was confirmed when caspase 3 activation was used as a readout (Figure 4B).
Figure 4: LS174T cells are sensitive to active thiazolides. A) LS174T cells were treated with thiazolides for 40 h. Cell death was monitored by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n=3). B) Induction of cleaved caspase 3 by thiazolide treatment (20 µM) was detected by western blotting.

Thiazolide-induced cell death is GSTP1-1-dependent
We previously identified GSTP1-1 as a major thiazolide-binding protein in Caco2 cells, and GSTP1-1 activity as a prerequisite for thiazolide-induced apoptosis in colorectal tumor cells (Muller et al., 2008b; Sidler et al., 2012). Silencing of GSTP1-1 by RNA interference inhibited RM4819-induced apoptosis, whereas overexpression sensitized cells to RM4819-induced death, confirming that the RM4819-induced cell death is GSTP1-1-dependent. To assess the requirement of GSTP1-1 for cell death induction by the different thiazolide derivatives GSTP1-1 expression in thiazolide-sensitive Caco2 cells was downregulated using lentiviral small hairpin RNA (shRNA) constructs. Figure 5A illustrates that some of these small hairpin RNAs very efficiently downregulated GSTP1-1 (shGSTP1-1 #2 and #4) in comparison to control shRNA (shNone), whereas others were less efficient (GSTP1-1 #1). Control cells and cells transduced with shGSTP1-1 #4 were then used to assess the GSTP1-1 dependency of thiazolide derivative-induced cell death. Cell death induction by RM4819 as well as compound 1 and 3 was reduced in GSTP1-1-silenced cells, compared to control shRNA-transduced cells (Figure 5B).
Figure 5: Thiazolide cell death induction is dependent on the GSTP1-1 expression. A) Caco2 cells were transduced with GSTP1-1-targeting shRNA (shGSTP) #1, #2, or #4 or control shRNA (shNone). GSTP1-1 knockdown efficiency was monitored by western blot. Tubulin served as control for equal loading. B) Caco2 cells transduced with shNone or shGSTP1-1 #4 were treated with the different thiazolide derivatives (20 μM) or DMSO (0.1%) for 24 h. Cell death was analyzed by MTT assay. Mean values of triplicates ± SD of a typical experiment (n=3) are shown. C) HEK293T cells were transfected with myc/his-tagged-GSTP1-1 or with the empty control vector. GSTP1-1 expression was analyzed by western blotting after 24 h. D) and E) GSTP1-1-overexpressing HEK293T were treated with 10 μM thiazolides for 16 h. Cell death was analyzed by MTT assay. Mean values of triplicates ± SD are shown. F) Caco2 cells were pre-treated with 5 mM N-acetyl cystein (NAC) for 1 h to increase intracellular GSH levels, and then stimulated with 20 μM thiazolides, DMSO or cisplatin (10 μg mL⁻¹) for 40 h. Cell death was analyzed by MTT assay. Mean values of triplicates ± SD are shown (n=3). Data were analyzed in an unpaired T-test (* P<0.001; n.s., not significant).

We next analyzed whether overexpression of GSTP1-1 could sensitize HEK293T cells to cell death induction by the various thiazolide derivatives. HEK293T were efficiently transfected with myc-his-tagged GSTP1-1 expression plasmid and expressed high levels of GSTP1-1 (Figure
While control transfected HEK293T cells show minimal sensitivity to RM4819-induced cell death, overexpression of GSTP1-1 renders them more sensitive. (Muller et al., 2008b) In agreement with this finding we observed that control transfected HEK293T cells were only slightly sensitive to RM4819 and the novel thiazolide derivatives tested. However, overexpression of GSTP1-1 led to a strong sensitization to RM4819 and the compounds 1 and 3 (Figure 5D and E). These results confirm that thiazolide-induced cell death is GSTP1-1-dependent, and extends this finding to the novel derivatives 1 and 3 tested here. Furthermore, increase in intracellular GSH levels enhanced cell death induction after thiazolide treatment (Figure 5F). Cell death induction in Caco2 by RM4819, compound 1 and 3 was markedly increased upon pretreatment of cells with N-acetyl cysteine (NAC), leading to an increase in intracellular GSH levels. Interestingly, even cell death in response to compound 2, demonstrating minimal activity on its own, was enhanced by NAC pretreatment. The suppose conjugation of the thiazolide pro-drugs to GSH by GSTP1-1 enzymatic activity likely leads to a toxic metabolite and the activation of the apoptosis pathway. In contrast to the thiazolides tested, cisplatin induced cell death was reduced by NAC pretreatment due to the detoxifying properties of GSTP1-1 and GSH.

**Conclusion:** In this study, we investigated the structure-function relationship for thiazolide-induced cell death in Caco2 cells. We demonstrated that modifications of RM4819 on R² and R³ of the benzene ring do not significantly alter the cell death-inducing activity of the respective thiazolide derivatives. Thus, neither the hydroxyl group nor the methyl group of RM4819 are required for cell death induction, and likely also for the interaction with GSTP1-1, as both compound 3 and 1 efficiently promoted cell death in a GSTP1-1-dependent manner. Interestingly, changing the bromo substituent in R¹ of the thiazole ring to a hydrogen dramatically reduced cell death induction. The inability of this non-bromo-thiazolide compound 2, a derivative of compound 1, to induce cell death in Caco2 cells was further confirmed using DNA fragmentation and caspase activity assays. These results indicate that the bromide atom, but not the substituents on R² and R³ of the benzene ring, is critical for RM4819-induced apoptosis in colon carcinoma cells.

Since GSTP1-1 enzymatic activity is required for thiazolide-induced apoptosis (Sidler et al., 2012), it is plausible that the interaction between GSTP1-1 and thiazolide results in the formation of a toxic product, initiating the intrinsic apoptosis pathway. Furthermore, we assume that substitution of the bromide atom with a hydrogen might result in a reduced interaction between compound 2 and GSTP1-1, or a metabolite that lacks downstream apoptosis-inducing activity.
The observation that cell death in response to all thiazolide derivatives was enhanced by NAC suggests that GSTP1-1 couples thiazolides to GSH, likely leading an apoptosis-inducing metabolite.

Of interest is the fact that bromo-thiazolides have only minimal anti-parasitic activity in *Giardia lamblia* (Muller et al., 2006), whereas the parent compound NTZ with a nitrogen group shows a broad spectrum of anti-microbial activities (Adagu et al., 2002; Fox and Saravolatz, 2005; Gardner and Hill, 2001; Muller et al., 2006). These opposite findings on the relative role of the bromo substituent in the thiazole ring on anti-parasitic versus anti-tumor functions suggests a different mode-of-action and molecular target in parasites versus tumor cells. Thus, specific modification of thiazolides may result in parasite-specific variants or derivatives with a highly tumor-specific activity pattern.

**Materials and Methods**

**Cell Lines and Reagents**
The thiazolides RM4819 and 1-6 were synthesized as described in supplementary information. Compounds were kept as 20 mM stock solutions in DMSO. The colon cancer cell lines Caco2 (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 (IMDM, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine and 50 µg mL⁻¹ gentamicin (all from PAA Laboratories) at 37°C and 5% CO₂. The human embryonic kidney cell line HEK293T was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplied with 10% FCS, 4 mM L-glutamine and 50 µg mL⁻¹ gentamicin. Cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere. Cisplatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromid (MTT) were obtained from Sigma-Aldrich and DMSO from Roth. The pan-caspase inhibitor (zVAD-fmk) and the caspase substrate Ac-DEVD-AFC were obtained from Enzo Life Science.

**MTT Assay**
Cell viability was measured by using MTT assay. Caco2 cells were seeded into 96-well plates at 5 x 10³ cells/well. After overnight attachment, cells were treated with thiazolides, or DMSO as a control for 40 h. In some experiments caspase activity was blocked by pretreating cells with 80 µM of the pan-caspase inhibitor z-VAD-fmk. Thereafter, cell culture medium was discarded and replaced with 0.5 mg mL⁻¹ MTT solution, dissolved in culture medium. Plates were incubated
under cell culture condition for an additional 2-3 h. After MTT reduction to purple formazan, the MTT solution was discarded and replaced with 100 µL DMSO to dissolve the formazan products. Plates were incubated for 15 min in a dark box at room temperature. After gently mixing of the plates, the intensity of the colored solution was quantified by measuring the absorbance at λ=562 nm on an ELISA reader (Tecan, Crailsheim, Germany). Cell death induction (%) was calculated as 100 * (1 - (OD exp. mean value (-substrate blank) / OD control mean value (-substrate blank)).

**Increase in intracellular GSH Levels**

Caco2 cells were seeded into 96-well plate at 5 x 10^3 cells/well. After attachment, cells were pre-treated with 5 mM N-acetyl cysteine (NAC) for 1 h, followed by stimulation with 20 µM thiazolides, 0.1% DMSO or 10 µg mL^-1 cisplatin for further 40 h. Cell viability was measured by MTT assay.

**DNA Fragmentation Assay**

Caco2 cells were seeded into 24-well plates at 2.5 x 10^4 cells/well. After overnight attachment, cells were treated with different thiazolides (20 µM) or with DMSO (0.1%) as a control for 40 h. Cells were harvested and pelleted at 500 x g for 5 min. The supernatant was discarded, cell pellets were resuspended in a hypotonic propidium iodide (PI) solution (50 µg mL^-1 PI, 0.5 x PBS pH 7.4, 0.1% Triton X-100) and incubated at 4°C for 15 min. The nuclear DNA content was analyzed by flow cytometry (FACS Calibur, BD Biosciences) using Cell Quest software.

**GSTP1-1 Overexpression in HEK293T Cells**

HEK293T cells were seeded into 10 cm cell culture dishes (1.8 x 10^6 cells total). After overnight attachment, cells were transfected with myc/his-tagged-GSTP1-1 in pcDNA3.1(A)+ or with the empty vector as a control, using the calcium-phosphate co-precipitation transfection method in unsupplemented DMEM. After 8 h, cells were moved to complete medium and further cultured for 24 h. Then, cells were transferred to 96-well plates (1 x 10^4 cells/well). After 8 h adherence, cells were treated with thiazolides (10 µM) or DMSO (0.05%) as a control for additional 16 h. Cell death analysis was performed by MTT assay. Transfection efficiency and GSTP1-1 overexpression was analyzed by western blotting.

**GSTP1-1 Knockdown in Caco2 Cells using Lentiviruses**

Lentiviral particles were produced in HEK293T cells using the MISSION® Lentiviral Packaging Mix (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, HEK293T cells were
co-transfected with the packaging plasmid pCMVdeltaR8.91, the envelope plasmid pMD2.G and pLKO-based plasmids containing shRNA against human GSTP1-1 (MISSION® shRNA, Sigma-Aldrich: clone 1 (NM_000852.2-332s1c1); clone 2 (NM_000852.2-331s1c1); clone 3 (NM_000852.2-209s1c1); clone 4 (NM_000852.2-482s1c1); clone 5 (NM_000852.2-158s1c1)). Plasmid SHC016 (Sigma-Aldrich) served as non-target control. Supernatants containing the viable virus particles were collected 48 to 72 h post transfection, and used for transduction of Caco2 cells. For GSTP1-1 lentiviral knockdown, Caco2 cells were infected with lentiviral supernatants in the presence of 8 µg/mL polybrene (Sigma-Aldrich) for 24 h. Stable clones were selected using 3 µg/ml puromycin. Silencing of GSTP1-1 was verified by qPCR and western blotting at day 9 post infection and confirmed in later passages.

**Western Blot**

Cells were lysed in NP40-lysis buffer containing 150 mM NaCl, 50 mM Tris pH 7.6, 1 mM EDTA and 1% NP-40. Cell lysates were diluted and denaturated in loading buffer, and separated on a denaturing 12-15% SDS-PAGE gel. After transfer to polyvinylidene difluoride membranes (Roche), GSTP1-1, cleaved caspase 3 or tubulin as loading control were detected using specific antibodies (anti-GSTP1-1 from Sigma-Aldrich Prestige, 1:1500; anti-cleaved caspase 3 from Cell Signaling Technologies, 1:1000; anti-tubulin from Sigma-Aldrich, 1:5000). Proteins were detected using horse radish-coupled secondary antibodies and enhanced chemiluminescence on an Image Quant LAS 4000 (GE Healthcare).

**DEVD Cleavage Assay**

Caco2 cells were seeded into 6-well plate (1.5 x 10^5 cells/well) and treated with 20 µM of thiazolides or with 0.1% DMSO as a control for 40 h. Following the treatment, cells were harvested and centrifuged at 500 x g for 5 min. The medium was removed and cells were lysed in 200 µL lysis buffer (100 mM potassium phosphate buffer pH 7.8, 0.2% Triton X-100, 1 mM DTT) for 15 min on ice. Cell lysates were pelleted for 15 min at 13,000 x g and 4°C, and cell-free supernatants were harvested. For caspase activity measurement, 50 µL of lysates were added to a non-transparent plate. Fifty µL of the substrate Ac-DEVD-AFC (200 µM in 40 mM HEPES pH 7.5, 20% glycerol and 6 mM DTT) were added to the lysates and incubated at 37°C for 210 min. The fluorescence intensity was measured on a Wallac Victor 1420 multilabel counter at excitation of λ = 400 nm/ emission of λ = 505 nm. Fluorescence intensity was normalized to protein concentrations in cell lysates. All measurements were done in triplicates.
Acknowledgments

A. Brockmann was supported by a fellowship from the Konstanz Research School Chemical Biology (supported by the DFG). The authors thank N. Schuster for assistance with the chemical synthesis, and the Brunner Lab for scientific and experimental support.
Supporting Information

Structure-Function Relationship of Thiazolide-Induced Apoptosis in Colorectal Tumor Cells
Compound Synthesis

General
All reagents are commercially available and were used without further purification. All reactions were performed under exclusion of air. Melting points are uncorrected and were determined on a Gallenkamp melting point apparatus. TLC was performed on Merck precoated plates (silica gel 60 F$_{254}$). $^1$H and $^{13}$C NMR spectra were recorded on Avance III 400 MHz spectrometer from Bruker at room temperature. Spectra were processed with the software MestReNova 6.1.1 from MestRelab Research and the chemical shifts are reported relative to the residual solvent peak. The ESI-IT and HRMS mass spectra were obtained with the Esquire 3000 plus and micrOTOF-Q II ESI-Qq-TOF from Bruker Daltonics. The vario MICRO Cube from Elementar was used for the CHN analysis. The reported yield refers to the analytically pure substance and is not optimized.

5-Bromothiazol-2-amine (6)

Synthesis: 5-Bromothiazol-2-amine was synthesized as described in literature (Andersen et al., 2008; Erlenmeyer et al., 1945).

Chemical structure:

Chemical formula: C$_3$H$_3$BrN$_2$S
Molecular weight: 179.04 g mol$^{-1}$
Yield: 69% (3.95 g, 22.1 mmol)
Visual nature: light brown crystals
TLC (n-hexane:ethyl acetate, 1:1): $R_f = 0.50$
M.p.: 93°C

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 7.17 (bs, 2H), 6.96 (s, 1H).

$^{13}$C NMR (101 MHz, DMSO-d$_6$) δ 169.23, 139.80, 91.57.
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**N-(5-Bromothiazol-2-yl)-2-hydroxybenzamide (4)**

**Synthesis:** N-(5-Bromothiazol-2-yl)-2-hydroxybenzamide was synthesized as described in literature. (Stachulski et al., 2011b)

**Starting material:** O-Acetylsalicyloyl chloride (Sigma Aldrich).

**Chemical structure:**

![Chemical structure](image)

**Chemical formula:** C$_{10}$H$_7$BrN$_2$O$_2$S

**Molecular weight:** 298.14 g mol$^{-1}$

**Yield:** 67% (1.50 g, 5.04 mmol)

**Visual nature:** white solid

**TLC** ($n$-hexane:ethyl acetate, 3:2): $R_f = 0.37$

**M.p.:** 198°C

**$^1$H NMR** (400 MHz, DMSO-$d_6$) $\delta$ 12.04 (bs, 1H), 11.77 (bs, 1H), 7.95 (dt, $J = 7.9$, 1.9 Hz, 1H), 7.66 (s, 1H), 7.53 – 7.40 (m, 1H), 7.04 (d, $J = 8.2$ Hz, 1H), 6.99 (t, $J = 7.6$ Hz, 1H).

**$^{13}$C NMR** (101 MHz, DMSO-$d_6$) $\delta$ 164.62, 158.25, 157.33, 138.56, 134.58, 130.30, 119.72, 117.13, 116.45, 102.10.

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3-Methyl-N-(thiazol-2-yl)benzamide (2)

Synthesis: 3-Methylbenzoyl chloride (1 eq, 5.7 mmol, 0.88g) was dissolved in 30 mL dry THF and cooled to -90°C. Then 1,3-thiazol-2-amine (1 eq, 5.7 mmol, 0.57 g) was added in one portion. TEA (1 eq, 5.7 mmol, 0.8 mL) was added to the suspension and the reaction mixture was stirred for 60 min at -90°C. Next, the mixture was stirred overnight at room temperature. The reaction was quenched with 30 mL H₂O and the mixture was extracted with ethyl acetate (3×20 mL). The organic layer was dried over MgSO₄, concentrated and purified by silica gel column chromatography using a n-hexane/ethyl acetate gradient to give the pure 3-methyl-N-(thiazol-2-yl)benzamide.

Chemical structure:

[Chemical structure image]

Chemical formula: C₁₁H₁₀N₂O₂S

Molecular weight: 218.27 g mol⁻¹

Yield: 73% (0.909 g; 4.2 mmol)

Visual nature: white solid

TLC (n-hexane:ethyl acetate, 3:2): Rₜ = 0.43

M.p.: 143°C

¹H NMR (400 MHz, DMSO-d₆) δ 12.57 (bs, 1H), 7.95 – 7.91 (m, 1H), 7.91 – 7.85 (m, 1H), 7.56 (d, J = 3.6 Hz, 1H), 7.47 – 7.38 (m, 2H), 7.28 (d, J = 3.6 Hz, 1H), 2.39 (s, 3H).

¹³C NMR (101 MHz, DMSO-d₆) δ 165.13, 158.69, 137.89, 137.59, 133.06, 132.10, 128.64, 128.43, 125.23, 113.76, 20.87.

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**General procedure for the coupling of 5-bromothiazol-2-amine with the corresponding benzoyl chloride**

![Chemical structure](image.png)

**Scheme S1: General reaction scheme for the coupling reaction.**

The appropriate benzoyl chloride (1 eq, 5.7 mmol) was dissolved in 30 mL dry THF and cooled to -90°C. Then 5-bromothiazol-2-amine (1 eq, 5.7 mmol, 1.02 g) was added in one portion. TEA (1 eq, 5.7 mmol, 0.8 mL) was added to the suspension and the reaction mixture was stirred for 60 min at -90°C. Next, the mixture was stirred over night at room temperature. The crude reaction mixture was dissolved in 60 mL ethyl acetate. The organic phase was washed with saturated NaHCO₃ (3×40 mL), HCl (1M, 3×40 mL) and brine (3×40 mL). The organic layer was dried over MgSO₄, concentrated and purified by silica gel column chromatography using an n-hexane/ethyl acetate gradient to give the product. The product was re-crystallised from ethyl acetate and washed with n-hexane to obtain the pure product in 12 to 67 % yields.
**N-(5-Bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide (RM4819)**

**Starting material:** 2-Hydroxy-3-methylbenzoyl chloride was synthesized as described in literature (Meyer, 1901).

**Chemical structure:**

![Chemical structure](image)

**Chemical formula:** \(C_{11}H_9BrN_2O_2S\)

**Molecular weight:** 313.37 g mol\(^{-1}\)

**Yield:** 12% (0.219 g; 0.7 mmol)

**Visual nature:** white solid

**TLC** (n-hexane:ethyl acetate, 3:1): \(R_f = 0.35\)

**M.p.:** 191°C

**\(^1\)H NMR** (400 MHz, DMSO-\(d_6\)) \(\delta\) 12.74 (bs, 1H), 11.97 (bs, 1H), 7.95 (d, \(J = 7.8\) Hz, 1H), 7.70 (s, 1H), 7.36 (d, \(J = 7.1\) Hz, 1H), 6.85 (t, \(J = 7.7\) Hz, 1H), 2.20 (s, 3H).

**\(^{13}\)C NMR** (101 MHz, DMSO-\(d_6\)) \(\delta\) 168.81, 159.43, 158.40, 137.65, 135.74, 126.57, 126.19, 118.61, 114.10, 102.11, 15.66.

**MS (ESI, negative ion mode):**

\[ [M(^{79}Br)-H]^- \]

- calculated: 311.0
- found: 310.9

\[ [M(^{81}Br)-H]^- \]

- calculated: 313.0
- found: 312.7

**HRMS (ESI, negative ion mode):**

\[ [M(^{79}Br)-H]^- \]

- calculated: 310.9477
- found: 310.9484

\[ [M(^{81}Br)-H]^- \]

- calculated: 312.9464
- found: 312.9457
**N-(5-Bromothiazol-2-yl)benzamide (3)**

**Starting material:** Benzoyl chloride (Sigma Aldrich).

**Chemical structure:**

![Chemical structure](image)

**Chemical formula:** C\textsubscript{10}H\textsubscript{7}BrN\textsubscript{2}OS

**Molecular weight:** 283.14 g mol\textsuperscript{-1}

**Yield:** 26% (0.424 g; 1.5 mmol)

**Visual nature:** white solid

**TLC** (n-hexane:ethyl acetate, 3:1): \( R_f = 0.30 \)

**M.p.:** 147°C

\(^1\text{H NMR}\) (400 MHz, DMSO-\textit{d6}) \( \delta 12.89\) (bs, 1H), 8.12 – 8.06 (m, 2H), 7.65 (s, 1H), 7.68 – 7.61 (m, 1H), 7.58 – 7.53 (m, 2H).

\(^{13}\text{C NMR}\) (101 MHz, DMSO-\textit{d6}) \( \delta 165.38, 159.00, 138.79, 132.81, 131.42, 128.59, 128.21, 102.20. \)

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**N-(5-Bromothiazol-2-yl)-2-chlorobenzamide (5)**

Starting material: 2-Chlorobenzoyl chloride (Sigma Aldrich).

Chemical structure:

![Chemical structure](image)

Chemical formula: $C_{10}H_6BrClN_2OS$

Molecular weight: 317.59 g mol$^{-1}$

Yield: 23% (0.412 g; 1.3 mmol)

Visual nature: light yellowish solid

TLC ($n$-hexane:ethyl acetate, 3:2): $R_f = 0.47$

M.p.: 188°C

$^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 13.02 (bs, 1H), 7.67 – 7.63 (m, 1H), 7.65 (s, 1H), 7.60 (dd, $J = 8.0, 1.4$ Hz, 1H), 7.58 – 7.53 (m, 1H), 7.47 (td, $J = 7.3, 1.6$ Hz, 1H).

$^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 165.23, 158.06, 139.06, 133.75, 132.24, 130.36, 129.90, 129.60, 127.34, 102.43.

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**N-(5-Bromothiazol-2-yl)-3-methylbenzamide (1)**

**Starting material:** 3-Methylbenzoyl chloride (Sigma Aldrich).

**Chemical structure:**

![Chemical structure of N-(5-Bromothiazol-2-yl)-3-methylbenzamide (1)](attachment)

**Chemical formula:** C₁₁H₉BrN₂OS

**Molecular weight:** 297.17 g mol⁻¹

**Yield:** 67% (1.106 g; 3.7 mmol)

**Visual nature:** white solid

**TLC (n-hexane:ethyl acetate, 2:1):** \( R_f = 0.58 \)

**M.p.:** 150°C

**¹H NMR** (400 MHz, DMSO-\(d_6\)) \( \delta \) 12.82 (bs, 1H), 7.95 – 7.91 (m, 1H), 7.91-7.85 (m, 1H), 7.65 (s, 1H), 7.52 – 7.35 (m, 2H), 2.39 (s, 3H).

**¹³C NMR** (101 MHz, DMSO-\(d_6\)) \( \delta \) 165.46, 158.99, 138.80, 138.00, 133.40, 131.36, 128.72, 128.49, 125.34, 102.15, 20.85.

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Thiazolide-induced apoptosis in colorectal cancer cells is mediated via the Jun kinase-Bim axis and reveals glutathione S-transferase P1 as Achilles’ heel

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Running title: Colon cancer, GSTP1 and thiazolide-induced apoptosis

Keywords: apoptosis, colon cancer, glutathione S-transferase, Bim, TRAIL, Jun kinase, chemotherapy, synergy
Abstract

Glutathione S-transferase of the Pi class (GSTP1-1) is frequently overexpressed in a variety of solid tumors and has been identified as a potential therapeutic target for cancer therapy. GSTP1-1 is a phase II detoxification enzyme and conjugates the tripeptide glutathione to endogenous metabolites and xenobiotics, thereby limiting the efficacy of anti-tumor chemotherapeutic treatments. In addition, GSTP1-1 regulates cellular stress responses and apoptosis by sequestering and inactivating c-Jun N-terminal kinase (JNK). Thiazolides are a novel class of antibiotics for the treatment of intestinal pathogens with no apparent side-effects on host cells and tissue. Here we show that thiazolides induce a GSTP1-1-dependent and glutathione-enhanced cell death in colorectal tumor cell lines. Downregulation of GSTP1-1 reduced the apoptotic activity of thiazolides whereas overexpression enhanced it. Thiazolidide treatment caused strong Jun kinase activation and Jun kinase-dependent apoptosis. As a critical downstream target of Jun kinase we identified the pro-apoptotic Bcl-2 homolog Bim. Thiazolides induced Bim expression and activation in a JNK-dependent manner. Downregulation of Bim in turn significantly blocked thiazolide-induced apoptosis. Whereas low concentrations of thiazolides failed to induce apoptosis directly, they potently sensitized colon cancer cells to TNF-related apoptosis-inducing ligand (TRAIL)- and chemotherapeutic drug-induced cell death. While GSTP1-1 overexpression generally limits chemotherapy and thus anti-tumor treatment, our study identifies GSTP1-1 as Achilles' heel and thiazolides as novel interesting apoptosis sensitizer for the treatment of colorectal tumors.

Introduction

Colorectal cancer is the third most common cancer in humans and the fourth most frequent cause of cancer death worldwide (Jemal et al., 2005). Despite extensive research, treatment of advanced colorectal cancer with anti-tumor agents is often limited by intolerable side effects or by the development of chemoresistance (Meyerhardt and Mayer, 2005; Weitz et al., 2005). Therefore, major scientific efforts have been invested to identify tumor-specific drug targets, i.e. molecules that are selectively expressed in cancerous tissues. Glutathione S-transferases (GSTs) were proposed as such promising targets, since they are overexpressed in a variety of solid tumors (Townsend and Tew, 2003). GSTs are phase II detoxification enzymes and conjugate the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl glycine) to a wide variety of endogenous metabolites, xenobiotics, and chemotherapeutics (Goto et al., 1999; Ishikawa and Ali-Osman, 1993; Ishimoto and Ali-Osman, 2002). Therefore, high levels of GST in tumor cells
may substantially limit the efficacy of anti-tumor chemotherapy. In addition to their enzymatic activity, several classes of GST have non-enzymatic functions in the regulation of relevant signal transduction pathways. Specifically, the glutathione S-transferase of the Pi class (GSTP1-1) has been described as an endogenous inhibitor of Jun kinase (JNK), critically involved in stress response and apoptosis (Adler et al., 1999; Villafania et al., 2000; Yin et al., 2000). Thus, overexpression of GSTP1-1 by solid tumors may limit the treatment of these tumors at different levels.

Small molecule inhibitors of GST have been developed in the past and validated in preclinical and clinical studies (Townsend and Tew, 2003). These include the diuretic agent ethacrynic acid, a competitive inhibitor of several GST classes. Ethacrynic acid effectively increases the sensitivity of tumor cells to alkylating chemotherapeutics (Tew et al., 1988b) and proved clinical effectiveness in a phase II trial for chronic lymphatic leukemia (Petrini et al., 1993b). However, the long-term utility of ethacrynic acid is compromised by dose-limiting toxicities related to diuresis and subsequent fluid imbalances (O’Dwyer et al., 1991). The second generation GST inhibitor TLK199 is an effective and specific inhibitor of GSTP1-1 and has been tested in phase I/II clinical trials in myelodysplastic syndrome (MDS) (Steensma, 2010), however, currently data on solid tumors are missing. Thus, efficient and specific targeting of GSTP1-1 in tumor cells requires further efforts.

Thiazolides are a novel class of anti-infective agents. Their parent compound nitazoxanide (NTZ) was initially designed as an anti-microbial drug and approved for the treatment of intestinal infections caused by Cryptosporidium, Giardia and anaerobic prokaryotes (Fox and Saravolatz, 2005; Muller et al., 2008a; Muller et al., 2006). Despite their effective anti-infective activity thiazolides show apparently little side-effects on host tissue. Interestingly, though, in a recent study we demonstrated that NTZ and several derivatives induce apoptosis in colon cancer cells in vitro (Muller et al., 2008b). Intriguingly, the pro-apoptotic effect was also evident in non-nitro-compounds lacking any anti-microbial activity (e.g. RM4819) (Muller et al., 2008a; Muller et al., 2006; Muller et al., 2008b) suggesting different molecular targets and mechanisms for the anti-microbial effect in prokaryotes and the pro-apoptotic effect in mammalian cells. Consequently, we identified GSTP1-1 as a major mammalian target of thiazolides (Muller et al., 2008b).

In this study, we investigated the underlying molecular mechanisms of thiazolide-induced apoptosis in colon cancer cells. We demonstrated that the thiazolide derivative RM4819 requires the expression and enzymatic activity of GSTP1-1. RM4819 caused a long-lasting activation of
JNK, resulting in transcriptional upregulation of the pro-apoptotic Bcl-2 homolog Bim and Bim-associated cell death. While RM4819 alone was a moderate inducer of apoptosis, it potently sensitized colon cancer cells for TNFα receptor apoptosis-inducing ligand (TRAIL) and chemotherapy-induced apoptosis. Thus, thiazolides are effective modulators of apoptosis signaling pathways in cancer cells and therefore promising candidates for single- or multi-agent therapy of colorectal cancer. Furthermore, while GSTP1-1 overexpression limits the efficacy of chemotherapy in tumor cells, it represents an Achilles’ heel in thiazolide induced apoptosis.

Results

RM4819 induces apoptosis in colon cancer cell lines

We have previously shown that thiazolides induce apoptosis in the colon cancer cell line Caco2, yet were relatively ineffective in a non-transformed human foreskin fibroblast cell line (Muller et al., 2008b). To further characterize the pro-apoptotic properties of thiazolides, we extended these studies to different colon cancer cell lines. Time- and dose-dependent DNA fragmentation was detected in Caco2 and LS174T cells upon RM4819 treatment (Figure 1A-B). Also other classical features of apoptosis were found upon thiazolide treatment, such phosphatidylserine exposure and caspase activation (data not shown). In contrast to these colon cancer cell lines, HT29 and Colo205 cells were rather resistant to RM4819-induced apoptosis (Figure 1C and D).

Figure 1: RM4819 induces cell death in colon cancer cell lines. Caco2 (A), LS174T (B), Colo205 (C) and HT29 (D) were treated with various concentrations of RM4819. After 16 h (O) and 40 h (●) DNA fragmentation was assessed by flow cytometry. Mean values of triplicates +/- SD of a representative experiment out of three are shown.
**Alteration of GSTP1-1 expression levels affects the sensitivity to thiazolides**

Our previous study identified GSTP1-1 as a major thiazolide-binding target in Caco2 cells (Muller et al., 2008b). Furthermore, we found that overexpression of GSTP1-1 sensitized HEK293T cells to thiazolide-induced apoptosis. To investigate if tumor cells’ sensitivity to thiazolides correlates with their GSTP1-1 levels, we determined the basal mRNA expression of GSTP1-1 in various colon cancer cell lines. GSTP1-1 was detected in all colon cancer cell lines tested. Intriguingly, the thiazolide-sensitive Caco2 cell line showed the highest expression of GSTP1-1, whereas significantly lower levels were detected in the resistant cell lines Colo205 and HT29 (Figure 2A). LS174T cells expressed intermediate levels of GSTP1-1, but were rather sensitive to thiazolides (Figure 1 B). A similar picture was seen when total levels of intracellular GSH were analyzed (Figure 2B).

To determine the exact role of GSTP1-1 in thiazolide sensitivity, we overexpressed GSTP1-1 in the RM4819-resistant cell line HT29, and assessed its sensitivity to RM4819. As predicted, almost no apoptosis induction upon treatment with RM4819 was observed when HT29 cells were transfected with a control vector. In marked contrast, a significant increase of cell death induction after RM4819 treatment was observed in GSTP1-1-overexpressing cells (Figure 2C), indicating that the GSTP1-1 expression level is a limiting factor for RM4819-induced apoptosis in these cells.

To assess the requirement of GSTP1-1 for thiazolide-induced apoptosis in the RM4819-sensitive cell line Caco2, GSTP1-1 expression was downregulated by RNA interference and sensitivity to RM4819 was analyzed. Caco2 cells constitutively expressed high levels of GSTP1-1 mRNA (Figure 2A and D), which was efficiently downregulated by GSTP1-1-specific siRNA (Figure 2D). Downregulation of GSTP1-1 in otherwise sensitive Caco2 cells resulted a significant protection from thiazolide-induced cell death over 16 h (Figure 2E) and 40 h (data not shown) at high doses of RM4819.
Figure 2: GSTP1-1 expression determines the sensitivity of colon cancer cells to thiazolides. A) Relative mRNA levels of GSTP1-1 were assessed in Caco2, LS174T, Colo205 and HT29 cells by quantitative RT-PCR. B) Glutathione (GSH) levels in these cell lines was detected by flow cytometry and presented as mean fluorescence intensity (MFI). C) HT29 cells were transfected with control vector or GSTP1-1, and GFP as transfection control. Cells were then treated with DMSO control or 20 μM RM4819, and Annexin V staining on GFP-positive cells was measured after 16 h by flow cytometry. D) Caco2 cells were treated with control (O) or GSTP1-1-targeting siRNA (●) for 72 h and GSTP1-1 mRNA levels were detected by quantitative RT-PCR. E) siRNA-treated cells were stimulated with DMSO control or 20 μM RM4819, and DNA fragmentation was measured after 16 h. Mean values of triplicates +/- SD are shown. *P<0.05.

RM4819 induces activation of mitogen-activated protein (MAP) kinases

As we had previously shown that thiazolides specifically target GSTP1-1, and GSTP1-1 regulates MAP kinase activation (Adler et al., 1999), we assessed whether treatment of colon carcinoma cells with RM4819 leads to MAP kinase activation. Remarkably, RM4819 treatment resulted in the phosphorylation and associated activation of the MAP kinases JNK, p38 kinase and ERK in RM4819-sensitive Caco2 and LS174T cells (Figure 3A). Interestingly, the activation of all three MAP kinases was observed over several hours. The induction of JNK activation by RM4819 was confirmed using an AP-1 (Jun/Fos) luciferase reporter system. Treatment of Caco2...
cells with RM4819 resulted in a substantial increase in AP-1 reporter activity, indicating JNK activation, whereas co-transfection of cells with a dominant negative form of JNK1 or treatment with JNK inhibitor completely abolished RM4819-induced AP-1 reporter activity (Figure 3B).

**Figure 3: RM4819 induces activation of mitogen activated protein (MAP) kinases.** A) Caco2 and LS174T cells were stimulated with 20 µM RM4819 for the indicated time points. Total protein lysates were analysed by western blot for the expression of phospho-JNK (p-JNK), phospho-p38 (p-p38) and phospho-ERK (p-ERK). Tubulin served as control for equal loading. B) Caco2 cells were transfected with 6xAP1-RE reporter construct and stimulated with 1 or 3 µM RM4819 for 8 h. In some experiments, cells were co-transfected with dominant negative forms of JNK I (DN JNK I) or treated with the JNK V Inhibitor (2 µM). Luciferase activity was measured and normalized to untreated cells. Mean values of triplicates +/- SD are shown. *P<0.05.

**RM4819-induced cell death is dependent on JNK and p38 kinase signaling**

MAP kinases are critical for various cellular functions, including differentiation, proliferation and apoptosis. In general, JNK and p38 kinase activities promote apoptosis, whereas ERK activity is important for cell survival (Chen et al., 2000; Davis, 2000; Wagner and Nebreda, 2009). We therefore investigated if MAP kinase activation is important for RM4819-induced cell death. Pharmacological inhibition of JNK by a specific inhibitor almost completely blocked RM4819-induced apoptosis in Caco2 cells (Figure 4A). Similarly, overexpression of dominant-negative JNK I protected cells from RM4819-induced death (Figure 4B). To assess the role of other MAP kinases in thiazolide-induced cell death, JNK, p38 and MEK1-ERK were blocked by specific inhibitors and RM4819-induced apoptosis in Caco2 (Figure 4C) and LS174T cells (Figure 4D) was analyzed. Interestingly, inhibition of p38 kinase desensitized the cells to a similar extent as JNK inhibition. In contrast, no such protection was observed for the MEK1-ERK inhibitor UO126.
Figure 4: RM4819-induced cell death is dependent on JNK and p38 kinase signaling. A) Caco2 cells were treated with buffer control or 2 μM JNK V inhibitor, and then stimulated with indicated concentrations of RM4819 for 16 h. Cell death was measured by DNA fragmentation. B) Caco2 cells were transfected with vector control or DN JNK I, and GFP as transfection control. Cells were treated with 20 μM R4819 for 16 h, and apoptosis on GFP-positive cells was detected by Annexin V staining. (C, D) Caco2 (C) and LS174T (D) cells were treated with DMSO control, 2 μM JNK V Inhibitor, 10 μM p38 Inhibitor (SB202190) or 2 μM MEKK1-ERK Inhibitor (UO126), and stimulated with 20 μM RM4819 for 16 h. Cell death induction was measured by detection of DNA fragmentation. Mean values of triplicates +/- SD are shown. * P<0.05.

Bim is a critical mediator of RM4819-mediated apoptosis

As JNK is an important regulator of apoptosis in cancer cells (Chen and Tan, 2000; Dhanasekaran and Reddy, 2008) and regulates the expression and activation of the BH3-only molecule Bim (Kuo et al., 2009; Lei and Davis, 2003; Putcha et al., 2003) we tested the role of JNK and Bim in thiazolide-induced apoptosis. In Caco2 cells, RM4819 induced a time-dependent increase in phospho-c-Jun levels, reflecting JNK activation, and in parallel of Bim protein levels. This induction was efficiently blocked upon treatment of cells with JNK inhibitor, indicating a major role of JNK in Bim induction (Figure 5A). Similar findings were made when RM4819-induced activation of the Bim promoter was analyzed in Caco2 (Figure 5B) and in LS174T cells (Figure 5C). RM4819 potentially induced the Bim reporter construct, whereas pretreatment of cells with JNK inhibitor significantly blocked Bim promoter induction. To verify whether thiazolide-mediated Bim induction engages the mitochondrial pathway, we investigated whether overexpression of anti-apoptotic Bcl-2 homologs could neutralize Bim (Youle and
Strasser, 2008) and prevent thiazolide-induced apoptosis. Overexpression of either Bcl-2 or Mcl-1 significantly protected Caco2 cells from RM4819-induced cell death (Figure 5D). Similarly, downregulation of Bim by RNA interference inhibited RM4819-induced apoptosis in Caco2 cells (Figure 5E). These experiments clearly show that thiazolides induce cell death via the mitochondrial pathway and critically involves the BH3-only molecule Bim.

**Figure 5: Role of Bim in RM4819-mediated apoptosis.** A) Caco2 cells were treated with buffer control or 2 μM JNK inhibitor V, and then stimulated with 20 μM RM4819 for the indicated time points. Protein lysates were analyzed by western blot for the expression of phospho-cJun (p-cJun) and Bim. Tubulin served as control for equal protein loading. B, C) Caco2 (B) or LS174T cells (C) were transfected with the Bim promoter reported construct, treated with buffer control or 2 μM JNK inhibitor V, and stimulated with indicated concentrations of RM4819 (μM). Luciferase activity was measured and normalized to untreated controls. D) Caco2 cells were transfected with control plasmid, Bcl-2 or Mcl-1, and GFP as transfection control. Cells were then treated with RM4819 for 16 h, and cell death on GFP-positive cells was detected by Annexin V staining. E) Caco2 cells were treated with control siRNA or Bim-specific siRNA for 72 h, and Bim levels were detected by western blot (inset). Cells were then exposed to increasing concentrations of RM4819 for 16 h, and apoptosis was detected by DNA fragmentation. Mean values of triplicates +/- SD are shown. *P<0.05.

**RM4819 sensitizes tumor cells to TRAIL and chemotherapeutic drugs**

While inhibition of GSTP1-1 by specific inhibitors can directly promote apoptosis or sensitize cancer cells to other pro-apoptotic agents due to limited detoxification (Townsend and Tew,
2003), thiazolid-induced apoptosis required GSTP1-1. We thus tested whether pretreatment of cells with RM4819 sensitizes or rather desensitizes cells to triggers of the death receptor and the mitochondrial apoptosis pathway. While low doses of TRAIL or RM4819 were rather weak inducers of apoptosis as single-agents, the concurrent stimulation with both drugs lead to rapid and profound cell death in all colon cancer cell lines tested (Figure 6A-D), even in the thiazolid-resistant cell line HT29. As we have shown above that thiazolid-induced apoptosis requires Bim (Figure 5A), and TRAIL has been reported to activate Bid (Li et al., 1998; Ozoren and El-Deiry, 2002), we silenced Bim and Bid in Caco2 cells using siRNA to determine their respective role in the synergistic induction of cell death by TRAIL and RM4819. Gene silencing led to a substantial reduction of Bim and Bid levels in Caco2 cells (Figure 6E). Intriguingly, both, Bim- and Bid siRNA-targeted cells were partially but significantly protected from RM4819 and TRAIL treatment, however, concomitant silencing of both molecules resulted in the strongest protection (Figure 6F).
We next investigated if thiazolides also sensitize cancer cells to chemotherapeutic drugs. This group of apoptosis triggers is of particular interest as xenobiotics are detoxified by GSTP1-1 (Ruzza et al., 2009). Low doses of cisplatin and RM4819 were rather weak inducers of apoptosis in the various colon cancer cell lines tested. However, as seen with TRAIL, concurrent treatment of cells with RM4819 and cisplatin caused a strong synergistic cell death (Figure 7A-E). As demonstrated in HT29 cells, the sensitizing effect of RM4819 was not restricted to cisplatin, but further extended to other agents such as CPT-11 and partially also doxorubicin (Figure 7E).
Figure 7: RM4819 sensitizes cancer cells to chemotherapeutic agents. A-D) Caco2 (A), LS174T (B), Colo205 (C) and HT29 (D) cells were treated with DMSO, 10 μM RM4819, cisplatin (10 μg/ml for Caco2, LS174T and Colo205, 20 μg/ml for HT29) or the combination thereof. DNA fragmentation was measured by flow cytometry after 16 h. (E) HT29 cells were stimulated with 50 μg/ml CPT-11, 1 μg/ml doxorubicin (Dox), and 20 μg/ml cisplatin (Cis) in the presence of DMSO (□) or 10 μM RM4819 (■) for 40 h. Then, DNA fragmentation was measured. Mean values of triplicates +/- SD are shown. *P<0.05.

To further assess whether the synergistic induction of cell death by RM4819 and TRAIL or cisplatin could be confirmed on a biochemical level, we analyzed the effect of thiazolides on TRAIL- and cisplatin-induced cytochrome C and SMAC release from mitochondria during apoptosis induction. Intriguingly, no or minimal release was observed after treatment with the single-agents RM4819, TRAIL or cisplatin at sub-optimal concentrations. However, combined treatment of cells with TRAIL and RM4819 caused an early release of cytochrome C at 8 h (Figure 8A). Later on, cytochrome C release was also detectable in cisplatin plus RM4819-treated cells, and SMAC release and degradation in both treatments (Figure 8B). This indicates
that the synergistic activity of thiazolides and other apoptosis triggers can efficiently activate the mitochondrial apoptosis pathway.

![Figure 8](image)[Figure 8: Synergistic induction of outer mitochondrial membrane permeabilization. A, B) LS174T cells were treated with DMSO, 10 μM RM4819, 12 ng/ml TRAIL, 10 μg/ml cisplatin or the combination thereof, as indicated. After 8 h (A) and 16 h (B) cytoplasmic and mitochondrial fractions were isolated and analyzed for the expression of tubulin, SMAC/Diablo and cytochrome C. Tubulin expression served as control for equal loading and purity of fractionations.]

**GSTP1-1 enzymatic activity is required for thiazolide-induced apoptosis**

As thiazolides required the expression of GSTP1-1, we assessed the role of its enzymatic activity in mediating thiazolide-induced apoptosis. A critical substrate for GST is GSH, which is used to capture and detoxify xenobiotics and radicals (Balendiran et al., 2004). When colon cancer cells were treated with N-acetylcysteine (NAC) a significant increase in total GSH levels was observed (Figure 9A). Interestingly, while cisplatin-induced apoptosis was efficiently blocked by NAC treatment, likely due to increased GST/GSH-mediated detoxification of the cell, significantly increased cell death was observed in RM4819-treated cells (Figure 9B). This indicates that increased GSTP1-1 activity enhances thiazolide-induced apoptosis or may even be a prerequisite.
Figure 9: N-Acetylcyesteine sensitizes colon cancer cells to RM4819. Caco2 cells were pre-stimulated with 5 mM NAC for 2 h, followed by treatment with 10 μM RM4819 and 10 μg/ml cisplatin for 16 h. Intracellular glutathione (GSH) levels were measured at time of cell harvesting by MCB staining (A), DNA fragmentation was determined by flow cytometry (B). Mean values of triplicates +/- SD are shown. *P<0.05.

We next assessed the role of GSTP1-1 enzymatic activity in sensitizing cells to thiazolides. Various polymorphisms in the GSTP1-1 allele have been described resulting in different enzymatic activities of the encoded proteins (Mcllwain et al., 2006; Watson et al., 1998). As predicted, recombinant wild type protein (GSTP1-1*A) showed an increased enzymatic activity than the mutant protein (GSTP1-1*B) when expressed and purified as recombinant proteins (Figure 10A and B). The wild type and mutant alleles were then cloned into mammalian expression vectors, transfected into HT29 cells and the response of GSTP1-1-expressing cells to RM4819 was assessed. In agreement with its increased enzymatic activity a higher sensitivity to RM4819 was seen with the wild type allele (GSTP1-1*A) than with the mutant allele (GSTP1-1*B) (Figure 10C).
Figure 10: GSTP1-1 enzymatic activity is required for RM4819-induced apoptosis.
Recombinant GSTP1-1*A and *B protein was purified from E. coli by affinity chromatography, and purity was assessed by gel electrophoresis and coomassie staining (A). Their enzymatic activity was determined in vitro as described previously (Muller et al., 2008b) (B). GSTP1-1*A or GSTP1-1*B expression vectors were co-transfected with GFP into HT29 cells. After 24 h, cells were treated with DMSO control or 20 μM RM4819 for 16 h. Apoptotic cell death in GFP-positive cells was measured by Annexin V staining (C). Mean values of triplicates +/− SD are shown. *P<0.05.

GSTP1-1 is overexpressed in colon cancer tissue
Given the important role of GSTP1-1 in thiazolide-induced apoptosis in colon cancer cell lines we aimed at assessing the relative expression of this enzyme in colon cancer tissue versus normal colonic tissue or tumor stroma. Tissue samples from 16 patients undergoing hemicolectomy for primary colorectal cancer were collected from the normal colonic mucosa, the tumor edge and the tumor center. When GSTP1-1 expression was analyzed by quantitative RT-PCR tumor tissue was found to significantly overexpress GSTP1-1 compared to normal colonic mucosa (Figure 11A and B). Similarly, when GSTP1-1 expression was analyzed by immunohistochemistry it was hardly detectable in normal colonic epithelial cells or hepatocytes, but expressed at high levels in cholangiocytes (bile duct cells) (Figure 12A and C). In marked contrast primary colorectal tumors as well as metastatic tumors in the liver strongly overexpressed GSTP1-1, while tumor stroma remained negative (Figure 12B and D).
Figure 11: GSTP1-1 expression in normal colonic mucosa and colorectal cancer tissue. GSTP1-1 expression in tissue specimen from the tumor center, the tumor edge and non-affected colonic mucosa from 16 patients was measured by quantitative RT-PCR. Values of individual patients are connected by lines. Data are presented as relative GSTP1-1 mRNA levels (A) and fold induction as normalized to the GSTP1-1 expression levels in the normal colonic mucosa of each individual patient (B). *P<0.05.

Figure 12: GSTP1-1 protein expression in primary and metastatic colon carcinoma. GSTP1-1 protein was detected by immunohistochemistry on normal colonic mucosa (A), primary colon carcinoma (B), normal liver tissue (C) and liver metastases of colorectal cancer (D). The sections for the colonic mucosa and the primary colon cancer, as well as for the hepatic parenchyma and the colon cancer metastasis were taken from adjacent regions of the same tissue section. Upper rows show specific GSTP1-1 staining at different magnifications, the lower row shows isotype control staining of the corresponding tissues. The bar indicates 50 μm.
Discussion

Resistance to cell death is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). Various types of tumors have developed different apoptosis resistance mechanisms, which permit their survival under harsh conditions. In particular, drug resistance of tumor cells by various mechanisms is a major problem in cancer therapy and severely limits the successful treatment of tumor patients with chemotherapeutic drugs. This phenomenon of drug resistance is also seen in colorectal tumors (Yang et al., 2009). Given the important role of drug and apoptosis resistance genes in the tumor development and survival, they not only represent difficult obstacles in the therapeutic treatment of cancer, but also offer novel opportunities to specifically target these tumors. Many “tumor-specific” markers are not tumor-restricted, but are also expressed by normal tissue cells or, on the other hand, are downregulated in certain subsets of tumor to escape targeted treatment. In contrast, drug and apoptosis resistance genes represent interesting therapeutic targets, as they are required by the tumor cells to survive and are thus unlikely to be lost or downregulated. For example, many tumors have overexpressed growth factor receptors, and their continuous stimulation promotes proliferation and survival. The epidermal growth factor receptor (EGFR), overexpressed in many colorectal tumors, is efficiently targeted with cetuximab, a monoclonal anti-EGFR antibody, leading to apoptosis of EGFR-expressing tumor cells (Garrett and Eng, 2011).

GSTs play a critical role in the detoxification of the cells from xenobiotics and radicals. Not surprisingly, different GST isoforms are overexpressed in various tumors (Ruzza et al., 2009; Townsend and Tew, 2003), where they severely limit the anti-tumor therapy by conventional chemotherapeutic drugs. In agreement with this notion we have seen here that GSTP1-1 is strongly overexpressed in colorectal tumors (Figure 11 and 12) and that overexpression of GSTP1-1 protects cells from chemotherapeutic drug-induced apoptosis (Muller et al., 2008b). Given the important role of GSTP1-1 in detoxifying and protecting tumor cells from chemotherapeutic drugs, different GST and more specifically GSTP1-1 inhibitors have been developed with the aim to reduce detoxification and increase the sensitivity of tumors to chemotherapeutic drugs. While ethacrynic acid has good GST inhibiting properties and sensitizes tumor cells to apoptosis, its clinical use is severely hampered by its strong diuretic action (O’Dwyer et al., 1991). A more specific GSTP1-1 inhibitor TLK199 is currently being tested in the clinics (Steensma, 2010), but data on solid tumors are missing. The basis of these therapies is, however, the same, i.e. inhibition of GSTP1-1 and increased sensitivity of tumor cells to apoptosis. The concept of thiazolide-induced apoptosis induction is substantially
different. Our present data clearly show that (high) expression of GSTP1-1 and induction of GSTP1-1 activity by increasing GSH levels is required for thiazolide-induced apoptosis. This indicates that thiazolides are prodrugs, which become activated by GSTP1-1 to induce a JNK and Bim-dependent apoptosis pathway. Thus, the higher the expression of GSTP1-1, the more resistant tumor cells become to chemotherapy and the more sensitive to thiazolides, revealing GSTP1-1 as an Achilles’ heel of thiazolide-induced apoptosis. Our data show a good correlation between GSTP1-1 expression and intracellular GSH levels and sensitivity to thiazolides. Furthermore, we also observed that, although RM4819 alone was a relatively weak and slow inducer of apoptosis in colorectal cancer cell lines (Figure 1), it strongly synergized with TRAIL and cisplatin in inducing tumor cell apoptosis (Figure 6 and 7). This is in as much surprising as thiazolide-induced apoptosis is promoted by GSTP1-1, whereas cisplatin-induced death is prevented by GSTP1-1. Furthermore, the combination of thiazolides and TRAIL or cisplatin promoted extensive cell death even in cell lines otherwise resistant to thiazolides, i.e. Colo205 and HT29. Thus, the simultaneous engagement of multiple apoptosis signaling pathway strongly sensitizes cells to apoptosis and offers an increased therapeutic window.

GSTP1-1 and also other GSTs have been reported to control apoptosis induction by at least two mechanisms. While their enzymatic activity is important for detoxification and thus limiting the effect of chemotherapeutic drugs, they also block apoptosis by sequestering critical signaling molecules. For example, GSTP1-1 has been shown to bind JNK, p38 and TRAF2 and limit their activity upon stimulation by specific signals (Adler et al., 1999; Townsend and Tew, 2003; Yin et al., 2000). Thus, GSTP1-1-deficient mice show increased basal JNK and TRAF2 activation (Elsby et al., 2003). Interestingly, this latter activity of GSTP1-1 seems not to be dependent on its enzymatic activity (Adler et al., 1999; Yin et al., 2000). Along these lines we observed strong thiazolide-induced activation of JNK and p38, and JNK- and p38-dependent cell death. This could mean that thiazolides inhibit GSTP1-1 and thereby liberate JNK, and possibly also p38, from the sequestration by GSTP1-1. Our results, however, suggest an alternative pathway leading to the activation of MAP kinases and subsequent cell death. Unlike its role in sequestering JNK, we found that thiazolide-induced apoptosis was dependent on the enzymatic activity of GSTP1-1. Reduced expression of GSTP1-1 resulted in reduced thiazolide-induced apoptosis, whereas an increase in GSH further boosted thiazolide-induced death. Similarly, overexpression of the enzymatically more active GSTP1-1 allele A* (Watson et al., 1998) sensitized cells to thiazolides, whereas the enzymatically less active allele B* did not. It is thus more likely that the product of GSTP1-1-converted thiazolides promotes the activation of the different MAP kinase pathways, rather than via inhibition of GSTP1-1. Along these lines is the
surprising observation that GSTP1-1-deficient mice develop increased numbers of intestinal tumors in the APC\textsuperscript{min/+} model (Ritchie et al., 2009), rather than having reduced tumor burden. This could indicate that also in vivo GSTP1-1 may enzymatically convert inactive endogenous or bacterial substances to tumoricidal products, which limit the growth and development of intestinal tumors. This hypothesis, however, needs to further investigated.

As a critical downstream effector pathway of thiazolide-induced apoptosis we identified a JNK-mediated induction and activation of the pro-apoptotic Bcl-2 homolog Bim. Thiazolide-induced apoptosis was efficiently blocked by downregulating Bim or overexpressing anti-apoptotic Bcl-2 homologs, which antagonize Bim. This finding shows interesting parallels to other studies from our lab and others. We have previously identified Bim as an important molecule involved in the crosstalk between the extrinsic and the intrinsic apoptosis pathway. JNK-mediated induction and activation of Bim played a critical role in Fas and TNF-induced liver damage (Corazza et al., 2006a; Kaufmann et al., 2009), chemotherapeutic drug-induced hepatocellular tumor cell apoptosis (Schneider-Jakob et al., 2010a), and paracetamol-induced liver damage (Badmann et al., 2011b). Thus, JNK-mediated activation of Bim and subsequent engagement of the mitochondrial apoptosis pathway seems to represent a common mechanism how primary and transformed cells are sensitized to apoptosis induction, and thus represent interesting targets of tumor therapy.

A similar mechanism of GSTP1-1-mediated activation of a pro-drug to its active compound has been reported for TLK286, a glutathionyl conjugate of nitrogen mustard pro-drug (Tew, 2005). Its activation by GSTP1-1 leads to the release of active nitrogen mustard. Though well tolerated in clinical studies no significant improvement over conventional chemotherapy alone has been observed so far. However, this study confirms the usefulness of the overall principle, i.e. that tumor-specific overexpression of a drug resistance gene can be employed to pharmacologically target tumors. Although thiazolides have not been tested yet in clinical studies for the treatment of colorectal tumors, its approved use in the treatment of intestinal infections has demonstrated its safety. Part of this good safety profile is also one of its potential limitations. Orally administered thiazolides, once absorbed by intestinal epithelial cells and distributed to the circulation, are rapidly inactivated in the liver and can thus not target anymore GSTP1-1-expressing cells elsewhere in the body. Primary intestinal epithelial cells, including the intestinal epithelial stem cells at the bottom of the crypts, show only low levels of GSTP1-1 expression, and are thus probably insensitive to thiazolides. However, it is very likely that orally administered thiazolides can efficiently target primary tumors of the intestine, which express very high levels
of GSTP1-1 and have direct access to luminal thiazolides. Of particular interest in this regard is our observation that thiazolides strongly synergize with TRAIL and chemotherapeutic drugs in inducing apoptosis in colorectal tumor cells lines. Oral treatments of tumor patients may sensitize colorectal tumor cells to systemically administered chemotherapeutic drugs or TRAIL, and thereby allow to treat patients with lower doses of these drugs with less side effects on uninvolved tissues.

In summary, we here demonstrate a novel role for GSTP1-1 in the induction of thiazolide-mediated apoptosis in colorectal tumor cells via a JNK- and Bim-dependent pathway. Our data further show that thiazolides strongly synergize with conventional anti-tumor drugs by sensitizing tumor cells. Given their minimal known side effects thiazolides are promising candidates for future therapeutic treatment of primary colorectal tumors.

Materials and Methods

Tissue culture reagents, biochemicals and pharmacological compounds
All chemicals were from Sigma Aldrich (Sigma-Aldrich, Buchs, Switzerland) unless stated otherwise. The thiazolide RM4819 was synthesized at the Department of Chemistry and Biochemistry, University of Berne and kept as 10 mM stock solution in DMSO at -80°C. The colon cancer cell lines Caco2 (ATCC HTB-37), HT29 (ATCC HTB-38), LS174T (ATCC CCL-188) and Colo205 (ATCC CCL-222) were from American Type Culture Collection ATCC (Manassas, VA). Cells were cultured in Iscove’s Modified Dulbecco’s Media supplied with 10% fetal calf serum, 50 µg/ml gentamicin at 37°C and 5% CO2. Recombinant TRAIL was kindly provided by S. Frese, Bern, cisplatin was obtained from Sandoz (Cham, Switzerland), doxorubicin and CPT-11 from Pfizer (New York, NY), JNK V Inhibitor and p38 inhibitor SB202190 from Calbiochem (Merck Chemical Ltd, Nottingham, UK), MEK inhibitor UO126 from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY).

Plasmids
The myc/his-tagged-GSTP1-1*A plasmid was generated by PCR-cloning human GSTP1-1 (GeneID: 2950) into a pcDNA3.1(A)+ vector (Invitrogen, Carlsbad, CA) using the BamHI and XhoI restriction sites and the primers: fw 5’-TTTGGATCCACCGCCATGCCGCCCTACACC-3’, rev, 5’-CCGCTCGAGCTTTTCGTTGCCATTGAT-3’. The expression plasmid for dominant-negative JNK 1 (DN JNK 1) was provided by Vicki Wätzig and Thomas Herdegen University of Kiel, Germany. The expression plasmid for Bcl-2 was from Cyrill Rentsch, Department for
Clinical Research, Berne, and the expression plasmid for Mcl-1 from Christoph Borner, Albert-Ludwigs-University, Freiburg, Germany.

The single nucleotide polymorphic variant GSTP1-1*B (Watson et al., 1998) was generated from the myc/his-GSTP1-1*A plasmid by mutating A313A to A313G using the QuikChange Site-directed Mutagenesis Kit from Stratagene using the following primers: fw 5'-CTCCGCTGCAAATACGTCTCCCTCATCTAC-3', rev 5'-GTAGATGAGGGAGACGTATTTF CAGCGGAG-3'. Positive clones of GSTP1-1*A and GSTP1-1*B were verified by sequencing. For purification of recombinant GSTP1-1 protein GSTP1-1*A and GSTP1-1*B cDNAs were subcloned into pET 151/D-TOPO expression vector (Invitrogen), transformed into E. coli BL21D3 and induced by 1 mM IPTG. Recombinant GSTP1-1 protein was purified by Ni²⁺-Sepharose affinity chromatography, and enzymatic activity was determined as described previously (Muller et al., 2008b).

The AP-1 reporter construct was generated by cloning six canonical AP-1 consensus sequences (TGAGTCA) (Strahl et al., 1997) into the pGL3 vector (Promega, Madison, WI) using the following primers: fw 5'-AAGCTTTGATGAGTCAGCCGCTGACTCATCATGATGAGTCAGCC AGG-3', rev 5'-AAGCCTGGCTGACTCATCATGATGAGTCAGCCGCTGACTCATCAAAG-3'. Self-annealing of the primers led to dsDNA fragments with two AP-1 consensus sequences, a canonical HindIII restriction site at the 5'-end and an incomplete HindIII restriction site at the 3'-end, respectively. The incomplete HindIII restriction site was competent for ligation, but incompetent for subsequent digestion. Three consecutive fragments were ligated into the multiple cloning region of a pGL3 luciferase vector leading to a 6xAP-1 reporter construct (later described as 6xAP1-RE-LUC). The 0.8 kb reporter construct for the human Bim promoter has been described previously (Badmann et al., 2011b).

**AP-1 and Bim promoter assays**

AP-1 reporter or Bim reporter constructs, and β-galactosidase expression vectors were co-transfected into Caco2 and LS174T cells using the lipofection method (MIRUS TransIT® LT-1, Mirus Bio LLC, Madison, USA). In some experiments cells were co-transfected with dominant negative JNK 1 expression vector. The amount of total transfected DNA was normalized with the corresponding empty vector plasmid. After 24 h, cells were cultured in 24-well plates and stimulated with various concentrations of RM4819 as indicated. In some experiments, cells were pre-incubated with 2 μM JNK V Inhibitor or DMSO for 30 minutes. After 8 h of stimulation, cells were lysed, and β-galactosidase and luciferase activity were measured as described previously (Mueller et al., 2007).
DNA fragmentation assay
Stimulated and unstimulated cells were collected from 24-well plates and stained with hypotonic propidium iodide solution (100 μg/ml propidium iodide in 0.5 x PBS pH7.4, 0.1% Triton-X100) for 30 min on ice (Nicoletti et al., 1991). Then, DNA content was analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA) and evaluated using FlowJo software (Ashland, OR). Nuclei in the Sub-G0 fraction were considered apoptotic.

Annexin staining assay
Caco2 and HT29 were transfected with expression plasmids such as GSTP1-1, Bcl-2 or Mcl-1 or the corresponding empty control vector as indicated. To track transfected cells, green fluorescence protein (GFP) was co-transfected. After 24 h, cells were stimulated for additional 16 h with RM4819 or DMSO. Then cells were stained with biotinylated Annexin V (1:400) and Streptavidin-PE/Cy5 (1:200, Biolegends, San Diego, CA) as previously described (Kasibhatla et al., 1998). Annexin V staining on GFP-positive cells was measured by flow cytometry and analysed by FlowJo software.

DEVD cleavage assay
Cells were cultured in 24-well plates and stimulated as described above. After 16 h, cells were collected and lysed in PBS, 1% Triton X-100. Caspase activity was determined using an Ac-DEVD-AFC protease assay (BD Pharmigen, San Jose, CA), as indicated by the supplier, and measured on a spectrofluorometer (Spectramax M2e, Sunnyvale, CA) at 400 nm excitation 505 nm emission wavelength.

Western blot
Cells were stimulated as indicated and lysed at indicated time points in PBS, 1% Triton X-100, and protease and phosphatase inhibitors (Roche, Basel, Switzerland). Lysates were separated on denaturing SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were probed with antibodies against phosphorylated JNK, phosphorylated p38 and phosphorylated ERK (all from Cell Signaling Technology, Davers, MA), Bid (kindly provided by Thomas Kaufmann, Berne, Switzerland), Bim (Sigma-Aldrich) and Tubulin (Sigma-Aldrich). Primary antibodies were detected with corresponding horseradish peroxidase-labeled secondary antibodies and visualized by chemiluminescence on a LAS4000 (Fujifilm, Dielsdorf, CH).
**RNA interference**

Caco2 cells were plated in 6-well plates and transfected with 15 nM siControl (Dharmacon, siGenome SMARTpool D-001820-01), siGSTP1-1 (L-011179-00), siBim (L-004383-02), siBid (L-004387-00) or the combination thereof using HiperFect transfection reagent (Qiagen, Valencia, CA) for 72 h. Cells were then cultured in 24-well plates and subsequently stimulated with RM4819 as indicated. The efficacy of knockdown was analyzed after 72 h by quantitative RT-PCR for GSTP1-1, and by Western blot for Bim and Bid.

**Mitochondrial isolation**

LS174T cells were cultured in 6-well plates, and treated with 12 ng/ml TRAIL or 10 μg/ml cisplatin in the presence or absence of 10 μM RM4819. After 8 and 16 h, cells were collected and fractionated for cytosolic or mitochondrial compartments, as described previously (Schneider-Jakob et al., 2010a). Cytosolic and mitochondrial lysates were then for cytochrome c, SMAC/Diablo and tubulin by Western Blot.

**Detection of GSTP1-1 in CRC cell lines and tumor samples by quantitative RT-PCR**

Tissue specimens were obtained from patients undergoing surgery for primary colorectal cancer at the Department of Visceral Surgery and Medicine, Inselspital, Bern University Hospital, University of Bern, Switzerland. All patients provided informed consent and experiments were reviewed by an institutional review board. Tissue samples from the tumor center, the tumor edge and non-cancerous normal mucosa, approx. 10 cm from the tumor edge, were collected. Total RNA was isolated and the expression of GSTP1-1 was analyzed by quantitative PCR on an Applied Biosystems Real-time PCR 7500 machine using SYBR green and Quantitect primer assays (Qiagen, Valencia, CA). Gene expression was normalized by tubulin RNA levels and plotted as relative mRNA levels.

**Immunohistochemistry for GSTP1-1**

Tissue sections from primary and metastatic colon cancer were deparaffinized and rehydrated. Antigen retrieval was performed by boiling sections for 10 min in 10 mM citrate pH 6.0 in a pressure cooker (Jakob et al., 2008). Then, endogenous peroxidase was blocked (Peroxidase Blocking Reagents, DakoCytomation) and GSTP1-1 expression was detected using an anti-human GSTP1-1 antibody (Sigma-Aldrich, Prestige Antibodies, 1:1000) and biotinylated secondary goat anti-rabbit IgG (Sigma Aldrich, 1:200). Bound antibodies were detected by the streptavidin-biotin method (ABC-kit, Vectastain, Vector Laboratories) and visualized by DAB
(Sigma). Sections were counterstained with Mayer’s hematoxylin. The negative controls were performed by incubation with equal concentrations of normal rabbit IgG.

**Detection of intracellular glutathione levels**
Endogenous glutathione (GSH) levels were measured by monochlorobimane (MCB) staining as described (Webb et al., 2006).

**Statistical analysis**
Statistical differences in data sets were analyzed by two-tailed students t test for experiments with two independent groups, and by ANOVA for experiments with three or more independent groups. Repetitive measures were respected using Bonferroni correction. $P$ values of <0.05 were considered as significant.

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Supplementary Information

Thiazolide-induced apoptosis in colorectal cancer cells is mediated via the Jun kinase-Bim axis and reveals glutathione S-transferase P1 as Achilles’ heel
Supplementary Finding to Fig01.
RM4819 induces Phosphatidylinerse exposure in Caco2 Cells
(Investigate 3 activation)

Supplementary Finding to Fig03
RM4819 induces AP1-transactivation in LS174T cells.
Supplementary Finding RM4819 Project

A
Supplementary Finding to Fig66.
RM4819 pretreatment further sensitizes Caco2 cells to TRAIL
(TRAIL only, vs. 0h pretreatment, 2h pretreatment and 4h pretreatment
with 10 μM RM4819)

B
Supplementary Finding to Fig66
Some cell lines such as JOM2 are not sensitized to TRAIL by RM4819.
Maybe a Type II specific effect?
Thiazolides promote apoptosis in colorectal tumor cells via MAP kinase-induced Bim and Puma activation

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Running title: Thiazolide-induced apoptosis in colorectal tumor cells

Keywords: Thiazolides, apoptosis, GSTP1-1, Bcl-2, Jun kinase, colorectal tumor

Abbreviation: ctrl, control, GSTP1-1; glutathione S-transferase pi 1; GSH, glutathione; HC, heavy chain; IgG, Immunoglobulin G; LC, light chain; MAP kinases, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid; carboxy-H2DCFDA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; NAC, N-acetyl-L-cysteine; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction
Abstract

While many anti-cancer therapies aim to target the death of tumor cells, sophisticated resistance mechanisms in the tumor cells prevent cell death induction. In particular enzymes of the glutathione S-transferase (GST) family represent a well-known detoxification mechanism, which limit the effect of chemotherapeutic drugs in tumor cells. Specifically, GST of the class P1 (GSTP1-1) is overexpressed in colorectal tumor cells and renders them resistant to various drugs. Thus, GSTP1-1 has become an important therapeutic target. We have recently shown that thiazolides, a novel class of anti-infectious drugs, induce apoptosis in colorectal tumor cells in a GSTP1-1-dependent manner, thereby bypassing this GSTP1-1-mediated drug resistance. In this study, we investigated in detail the underlying mechanism of thiazolide-induced apoptosis induction in colorectal tumor cells. Thiazolides induce the activation of p38 and Jun kinase, which is required for thiazolide-induced cell death. Activation of these MAP kinases results in increased expression of the pro-apoptotic Bcl-2 homologs Bim and Puma, which inducibly bind and sequester Mcl-1 and Bcl-xL leading to the induction of the mitochondrial apoptosis pathway. Of interest, while an increase in intracellular glutathione levels resulted in increased resistance to cisplatin, it sensitized colorectal tumor cells to thiazolide-induced apoptosis by promoting increased Jun kinase activation and Bim induction. Thus, thiazolides may represent an interesting novel class of anti-tumor agents by specifically targeting tumor resistance mechanisms, such as GSTP1-1.

Introduction

Glutathione S-transferases (GSTs) represent a superfamily of cellular phase II detoxification enzymes. Specifically, GSTs catalyze the conjugation of electrophilic substances to the tripeptid glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine). Thereby, hazardous metabolic products, xenobiotics and oxidative stress products become rapidly neutralized by GSTs, protecting cells from potentially damaging substances and carcinogens. Consequently, GSTs play a critical role in the detoxification of cells and inactivation of drugs (Hayes et al., 2005; Hayes and Pulford, 1995). At the present, seven classes (alpha, mu, pi, theta, sigma, zeta, and omega) of mammalian cytosolic GSTs are known, whose expression is regulated in a tissue-specific manner (Luo et al., 2011; Mannervik et al., 1992; Mannervik et al., 2005). The variability of GST isoform distributions across different tissues and their substrate specificities point towards a
defined role of individual GSTs in the biotransformation of drugs and reactive compounds in diverse tissues (Hiley et al., 1989; Strange et al., 1989).

GSTs play a critical role in tumor therapy, as numerous tumors overexpress various GSTs, which contribute to the development of resistance to chemotherapeutic treatments (Tew, 1994; Townsend and Tew, 2003). In particular, high expression levels of GST class pi (GSTP1-1) have been reported in a wide range of solid tumors, such as colon, breast, kidney, pancreas, lung, and ovarian cancer cells, and lymphoma (Howells et al., 2004; Laborde, 2010; Mcllwain et al., 2006). The sensitivity of these tumors towards chemotherapeutic drugs, such as cisplatin, doxorubicin, and etoposide, is negatively affected by high expression levels of GSTP1-1 (Ban et al., 1996; Goto et al., 1999; Ishikawa and Ali-Osman, 1993; Jankova et al., 2012; Johansson et al., 2011). Thus, overexpression of GSTP1-1 in solid tumors limits the therapeutic effects of different chemotherapeutic drugs via their GSTP1-mediated inactivation.

This observation identifies GSTs in general and GSTP1-1 in particular as important therapeutic targets in the treatment of solid tumors. Consequently, small molecular inhibitors of GSTs have been developed in the past to modulate GST activities and drug resistance of tumor cells, thereby sensitizing tumor cells to anti-cancer drugs. The competitive inhibitors ethacrynic acid (EA) binds directly to the substrate-binding site of several GST classes and potentiates the cytotoxic effects of chemotherapeutic drugs (Awasthi et al., 1993b; Tew et al., 1988a). The therapeutic effect of EA as a chemosensitizer was proven in a clinical trial with patients suffering from chronic lymphatic leukemia (Petrini et al., 1993a). However, long-term utility of EA was limited by its lack of GST isoform specificity and its strong diuretic properties, resulting in disturbance of the water-salt homeostasis (Odwyer et al., 1991). A somewhat different approach includes the GSH analog TLK199. TLK199 is metabolized and subsequently inhibits GSTP1-1 activities, making it a more selective GST inhibitor. Also TLK199 potentiates the cytotoxic effects of chemotherapeutic agents in GSTP1-1-overexpressing cells in vitro and in vivo (Morgan et al., 1996b). However, thus far experimental and clinical data on solid tumors are missing.

Thiazolides are a novel class of anti-infectious drugs used for the treatment of intestinal infection, and show a broad activity against intestinal intracellular and extracellular protozoan parasites, bacteria, and viruses (Adagu et al., 2002; Anderson and Curran, 2007; Fox and Saravolatz, 2005; Hemphill et al., 2006). The parent compound nitazoxanide (NTZ; 2-(acetolyloxy)-N-(5-nitro-2-thiazolyl)benzamide) is successfully used for the treatment of *Giardia lamblia* and *Cryptosporidium parvum* infections (Abboud et al., 2001a; Gardner and Hill, 2001;
Muller et al., 2006; Rossignol et al., 2001a). Though thiazolides generally have minimal side effects on host tissue cells during therapeutic treatments (Broekhuysen et al., 2000), it was recently noticed that they promote apoptosis induction in colorectal tumor cells, however sparing non-transformed cells (Muller et al., 2008b). Of interest, while the bromo-thiazolide RM4819 (N-(5-bromothiazol-2-yl)2-hydroxy-3-methylbenzamide) shows only reduced anti-microbial activity, both NTZ and RM4819 promote cell death in colorectal tumor cells. This indicates that the therapeutic targets of thiazolides are substantially different in intestinal parasites and colorectal tumor cells. Subsequent studies identified GSTP1-1 as a major RM4819-binding partner in colorectal tumor cells (Muller et al., 2008b). While it was initially thought that thiazolides are inhibitors of GSTP1-1, knockdown and overexpressing experiments of GSTP1-1 in tumor and non-tumor cells revealed that GSTP1-1 is required for thiazolide-induced cell death induction. Interestingly, an N-acetyl-L-cysteine (NAC)-induced increase in cellular GSH levels enhanced thiazolide-induced cell death, whereas it lowered the sensitivity towards chemotherapeutic drugs by promoting their GSTP1-1-mediated inactivation (Sidler et al., 2012). Thus, thiazolides appear to represent a novel class of GSTP1-1-activated pro-drugs, activated likely by conjugation to GSH, rather than GSTP1-1 inhibitors. This makes thiazolides an interesting novel class of anti-tumor drugs specifically targeting tumors with elevated levels of GSTs, and GSTP1-1 an Achilles’ heel for the potential therapeutic action of thiazolides. While thiazolides alone are relatively slow and weak inducers of apoptosis in colorectal tumor cells, they profoundly synergize with inducers of the intrinsic apoptosis pathway, such as chemotherapeutic drugs, as well triggers of the extrinsic pathway, such as TRAIL (TNF-related apoptosis-inducing ligand) (Sidler et al., 2012).

The mechanism of thiazolide-induced apoptosis and sensitization of tumor cells to other apoptosis triggers is presently incompletely understood, although GSTP1-1, the activation of the MAP kinases and the Bcl-2-regulated mitochondrial apoptosis pathways appear to play a critical role in this process (Sidler et al., 2012). In this study we investigated in more detail the underlying molecular signaling pathways leading to thiazolide-induced cell death in colorectal tumor cells. We find that activity of both, the MAP kinases p38 and Jun kinase (JNK) is critical for mediating thiazolide-induced apoptosis, as their combined inhibition blocks cell death induction. In particular JNK was found to be important for the induction and activation of the downstream effectors of the Bcl-2 family, i.e. the BH3-only proteins Bim and Puma. Bim and Puma appear to activate the mitochondrial pathway by interacting with and neutralizing the anti-apoptotic Bcl-2 homolog Bcl-xL, and inhibition of JNK prevented Bim and Puma induction, interaction with Bcl-xL, and induction of apoptosis. Furthermore, thiazolides induced interaction
of Bim with Mcl-1 and promote the degradation of Mcl-1. While an increase in cellular GSH levels inhibited chemotherapy-induced apoptosis, it resulted in a more robust activation of JNK, Bim induction, Mcl-1 degradation, and associated thiazolide-induced cell death.

In summary, we here show that thiazolides are a novel group of GSTP1-1-activated pro-drugs, which activate the mitochondrial apoptosis pathway at different levels. Given that GSTs are highly overexpressed in numerous tumors and that GSTs contribute to therapy resistance of these tumors, thiazolides may become an interesting therapeutic option for the treatment of chemoresistant tumor cells.

Results

Thiazolides induce JNK- and p38-dependent cell death
The molecular structure of thiazolides consists of a thiazole ring and a benzene ring linked by an amide bond. We have previously performed a structure-function study and shown that changes of substituents in the benzene ring do not affect the cell death-promoting activity of thiazolides, whereas removal of the bromide atom from the thiazole ring, as in compound 2, strongly reduces the activity (Brockmann et al., 2014). In order to investigate the thiazolide-induced apoptosis signaling pathways we employed RM4819 as an active thiazolide, and compound 2 is inactive control substance to induce apoptosis in the colorectal tumor cell lines Caco2 and LS174T. Figure 1a (Caco2 cells) and suppl. Figure 1 (LS174T cells) show that RM4819 induced cell death in a dose-dependent manner, whereas cells were almost insensitive to compound 2. In contrast, the chemotherapeutic drug cisplatin promoted cell death in both colorectal tumor cell lines.

Next, the ability of RM4819 and compound 2 in inducing activation of the MAP kinases JNK and p38 was assessed. JNK activation was observed at around 2 h and p38 activation 4 h after stimulation with RM4819. As expected from its lack of apoptosis-promoting activity stimulation of cells with compound 2 did not result in a detectable increase in MAP kinase activation (Figure 1b). While RM4819-induced MAP kinase activation was specific, though relatively slow and weak, the control substance cisplatin induced a pronounced and sustained activation of both, JNK and p38 (Figure 1b).

To address the relevance of JNK and p38 activation in thiazolide-induced cell death, we employed pharmacological inhibitors of JNK and p38. Interestingly, while inhibition of either JNK
or p38 only partially blocked RM4819-induced apoptosis, only combined inhibition of both, JNK and p38 almost completely prevented cell death induction (Figure 1c – e). This indicates that both MAP kinases are involved in thiazolide-induced cell death in Caco2 cells.

Figure 1: Thiazolide-induced cell death is JNK and p38-dependent. a) Caco2 cells were treated with indicated concentrations of RM4819, compound 2, cisplatin or DMSO as solvent control for 40 h. Cell death induction was calculated by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3). b) Caco2 cells were control treated (ctrl), or with thiazolides (20 µM), cisplatin (10 µg/mL), or DMSO (0.1%) for indicated time intervals. Phospho-JNK (p-JNK), phospho-p38 (p-p38) and tubulin as a loading control were detected by western blotting. c - e) Caco2 cells were pretreated with JNK V inhibitor (2.5 µM) (c), p38 inhibitor (10 µM) (d) or the combination of both (e) 1 h before stimulation with 20 µM RM4819 for further 40 h. Cell death induction was assessed by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3). *P< 0.01.

Thiazolides modulate expression levels of Bcl-2 family members

We have previously shown that thiazolides induce apoptosis via the Bcl-2 family-orchestrated mitochondrial apoptosis pathway. Specifically, we have seen that thiazolides induce the expression of Bim, which participates in thiazolide-induced cell death (Sidler et al., 2012). But likely other components of the mitochondrial apoptosis pathway contribute to the execution of thiazolide-induced apoptosis. In order to understand whether and how thiazolides affect expression levels of different pro- and anti-apoptotic Bcl-2 homologs, cells were stimulated with
RM4819, compound 2 and cisplatin as control, and levels of Bim, Puma, Bcl-xL and Mcl-1 were assessed by western blotting. As found previously, we observed an RM4819-induced increase in Bim levels already 2h after stimulation. Of interest, after 4 and 6 h Bim levels were not further induced but showed an increase in higher molecular weight species of BimEL, indicative of Bim phosphorylation (Corazza et al., 2006b; Geissler et al., 2013; Schneider-Jakob et al., 2010b). In marked contrast, only a minimal increase in Bim expression over that of control treated cells was observed in response to compound 2, with no apparent induction of Bim phosphorylation. Cisplatin treatment resulted in a strong increase in Bim expression, but not Bim phosphorylation. RM4819, but not compound 2 and cisplatin, also induced a substantial increase in Puma expression (Figure 2a and Suppl. Figure 2). While Bcl-xL levels remained unaffected by the treatment of cells with the different substances, a decrease of Mcl-1 expression was noticed after stimulation of the cells with RM4819, but not with compound 2 or cisplatin (Figure 2b and c). This finding was also confirmed in other colorectal tumor cells (LS174T) and for later time points (16 h) (Suppl. Figure 2a and b). This RM4819-induced degradation of Mcl-1 is likely not a consequence of caspase activation, as the pan-caspase inhibitor zVAD failed to prevent Mcl-1 degradation, whereas the proteosome inhibitor MG132 stabilized it (suppl. Figure 2c).

![Western blot images showing changes in Bcl-2 family member expression](image)

**Figure 2: Changes in thiazolide-induced Bcl-2 family member expression.** a) and b) Caco2 cells were treated with 20 μM thiazolides, 10 μg/mL cisplatin or 0.1% DMSO for 2, 4 and 6 h, and Bim and Puma (a), Mcl-1 and Bcl-xL (b) expression were detected by western blot. c) Mcl-1 and Bcl-xL expression were detected 8 h after treatment with complete medium as control, 0.1% DMSO, 20 μM RM4819 or compound 2, or 10 μg/mL cisplatin. Tubulin served as a loading control.
**RM4819-induced apoptosis is dependent on Bim and Puma**

The profound increase in Bim and Puma expression suggested that these BH3-only proteins might represent critical components in the activation of the mitochondrial apoptosis pathway. To test this hypothesis Bim and Puma were knocked down using lentiviral small hairpin RNA constructs inducing RNA interference. Infection of both, Caco2 and LS174T cells with specific lentiviruses resulted in a profound decrease in Bim, resp. Puma expression ([Figure 3a and b](#)). Control cells and cells with reduced Bim, resp. Puma expression were then stimulated with the thiazolides RM4819 and compound 2, or cisplatin as control. As expected compound 2 failed to induce cell death, whereas RM4819 induced a strong increase in apoptosis. Knockdown of both, Bim and Puma induced a significant reduction of RM4819-induced cell death in Caco2 and LS174T cells. Similarly, cisplatin-induced cell death was also attenuated in cells with downregulated Bim and Puma expression ([Figure 3a and b](#)), in agreement with previous observations (Jiang et al., 2006).

Since neither downregulation of Bim nor Puma alone completely inhibited RM4819-induced cell death, and BH3-only proteins often act in concert, we aimed at investigating whether combined downregulation of Bim and Puma resulted in a more pronounced inhibition of cell death. Control shRNA- or Puma shRNA-infected Caco2 cells were transfected with control siRNA or siRNA against Bim. Knockdown efficiency for these two BH3-only proteins was confirmed by western blot and revealed efficient silencing of Bim and Puma ([Figure 3c](#)). When cells were stimulated with increasing concentrations of RM4819 a partial protection was seen in Bim and Puma single knockdown cells. Importantly, combined knockdown of both BH3-only further increased protection from RM4819-induced cell death ([Figure 3c](#)), indicating that Puma and Bim cooperate in inducing the mitochondrial apoptosis pathway.
Figure 3: Thiazolide-induced cell death is regulated by Bim and Puma. Caco2 (a) and LS174T (b) cells were transduced with Bim- or Puma-targeting shRNA (shBim, shPuma) or non-targeting shRNA (shControl). Bim and Puma knockdown efficiency was monitored by western blot (insets). Cells transduced with shControl, shBim or shPuma were treated with DMSO (0.1%), RM4819 (20 µM) or cisplatin (1 µg/mL) for 40 h. Cell death induction was calculated by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3). *P < 0.01. (c) Caco2 cells transduced with shControl and shPuma were transiently transfected with Bim-targeting small interfering RNA (siBim) or control siRNA (siNone). Silencing of both Bim and Puma was confirmed by western blotting. Cells were treated with 10-20 µM RM4819 or 0.1% DMSO for 40 h. Cell death induction was assessed by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3).
**Bim and Puma interact with pro-survival Bcl-2 members**

As at least one mechanism how BH3-only proteins promote apoptosis is the binding to and neutralization of anti-apoptotic Bcl-2 members (Kang and Reynolds, 2009), we addressed the question whether thiazolides promote Bim and Puma binding to Bcl-x<sub>L</sub> and Mcl-1. When Bcl-x<sub>L</sub> was immunoprecipitated a constitutive binding of Bim and Puma was observed. Treatment of cells with RM4819 and cisplatin seemed to further induce Bim binding to Bcl-x<sub>L</sub>, whereas compound 2 failed to do so (Figure 4a). More pronounced were the changes in the binding of Puma to Bcl-x<sub>L</sub>. While only low levels of Puma were bound to Bcl-x<sub>L</sub> in control, cisplatin- or compound 2-treated cells, stimulation of cells with RM4819 resulted in a strong increase in Puma binding to Bcl-x<sub>L</sub>. This is likely due to the RM4819-induced increase in Puma expression (Figure 2a, Figure 4a, suppl. Figure 2). When BH3-only binding to Mcl-1 was analyzed only minimal changes in Bim binding were observed. In RM4819-stimulated cells even a reduced co-immunoprecipitation of Bim was observed, likely due to the fact that already lower levels of Bim were observed in cell lysates at this time point after RM4819 stimulation (8 h), and Bim might already being consumed and degraded during the process of apoptosis induction. Furthermore, RM4819 also resulted in the degradation of Mcl-1. In contrast, while Puma was detectable in cell lysates and induced by RM4819, no Puma binding to Mcl-1 was detected in these co-immunoprecipitation experiments (Figure 4b). Thus, RM4819-induced Puma appears to interact predominantly with Bcl-x<sub>L</sub>.
Figure 4: RM4819-induced binding of Bim and Puma to Bcl-xL and Mcl-1. Caco2 cells were treated with 0.1% DMSO, 20 µM RM4819 or compound 2, or 10 µg/mL cisplatin for 8 h. (a and b) Bcl-xL (a) and Mcl-1 (b) were immunoprecipitated and interaction with Bim and Puma were confirmed by western blotting. In input controls 4% of the total lysates, resp. 8% of the precipitates were loaded. IgG, isotype control; HC, immunoglobulin heavy chain.

JNK mediates Bim and Puma activation

As the MAP kinases p38 and JNK have been found to be critically involved in thiazolide-induced apoptosis in colorectal tumor cells (Figure 1c-e) (Sidler et al., 2012), we next aimed at analyzing their role in Bim and Puma induction and activation. Cells were thus treated with RM4819 for different time points in the presence of absence of JNK inhibitor. The presence of JNK inhibitor appeared to slightly stabilize Mcl-1 levels in untreated cells, but only minimally prevent its thiazolide-induced degradation, whereas Bcl-xL levels remained unaffected (Figure 5a and suppl. Figure 3). It also had a profound effect on thiazolide-induced Bim and Puma levels. JNK inhibition resulted in decreased Bim levels in both control treated and RM4819-treated cells, and
inhibited RM4819-induced Puma induction (Figure 5a and suppl. Figure 3). Inhibition of p38 had less pronounced effects on Bim and Puma expression. Only minimally reduced Bim or Puma levels were seen in cells treated with p38 inhibitor and RM4819 (suppl. Figure 4). Thus, Bim and Puma expression appears to be regulated predominantly by JNK.

We next assessed how JNK inhibition would affect RM4819-induced activation and binding of Bim and Puma to Bcl-xL and Mcl-1. Even more pronounced than its effect on Bim expression was the JNK inhibitor-induced inhibition of Bim binding to Bcl-xL, in both control and RM4819-stimulated cells. Whereas Puma inducibly bound to Bcl-xL inhibition of JNK strongly attenuated Puma binding. As seen before no Puma binding to Mcl-1 could be detected, whereas Bim binding to Mcl-1 was also reduced upon inhibition of JNK (Figure 5c). This indicates that JNK has an important role in the regulation of Bim and Puma expression, and their activation and binding to anti-apoptotic Bcl-2 family members.
Figure 5: JNK mediates Bim and Puma induction and activation. a) Caco2 cells were pretreated with buffer control or JNK V inhibitor (2.5 µM) for 1h before treatment with 0.1% DMSO or 20 µM RM4819 for 2, 4 and 8 h. Mcl-1, Bcl-xL, Bim and Puma were detected by western blot. Tubulin served as loading control. (b and c) Caco2 cells were pretreated with JNK V inhibitor for 1 h, before stimulation with RM4819 or DMSO for 8 h. Bcl-xL (b) and Mcl-1 (c) were immunoprecipitated, and Puma and Bim were detected by western blotting. IgG, isotype control; HC, immunoglobulin heavy chain; LC, immunoglobulin light chain.
Increase in intracellular GSH levels enhances thiazolide-induced JNK activation and apoptosis induction

GSH is an important anti-oxidant in cells. GSTs couple GSH to xenobiotics and drugs, and thereby inactivate them (Hayes et al., 2005; Hayes and Pulford, 1995). NAC is a precursor of GSH, and treatment of cells with NAC leads to increased intracellular GSH levels (Sidler et al., 2012). We have been previously suggesting that GSTP1-1 may also couple GSH to the pro-drug thiazolides, thereby generating an bioactive and pro-apoptotic product, since treatment of Caco2 cells with NAC sensitized them to thiazolides, yet reduced the sensitivity to cisplatin (Sidler et al., 2012). This observation was confirmed in Figure 6a, demonstrating enhanced thiazolide-induced cell death in NAC-treated cells. We thus set to analyze the effect of NAC on JNK activation, and Bim and Puma induction and activation. When cells were treated with NAC and stimulated with RM4819 an even more pronounced degradation of Mcl-1 was observed whereas levels of Bcl-xL remained unaffected (Figure 6b). Along these lines a strongly enhanced and sustained activation of JNK was observed. In contrast, only a minimal increase in p38 activation was observed (Suppl. Figure 5b). Next the effect of NAC on Bim expression and binding to Bcl-xL was assessed. NAC treatment appeared to induce an increase in Bim protein expression, and contribute also to the stabilization of Bim protein expression after RM4819 treatment (Figure 6b and c). Along these lines, a profound increase in Bim binding to Bcl-xL was observed (Figure 6c), suggesting also an increase in Bim activation. The relative NAC-mediated increase in Bim expression was also confirmed on mRNA level, where co-treatment with NAC and RM4819 lead to a 3-fold increase in Bim expression (Figure 6d).

In contrast to Bim, no increase or rather a slight decrease in Puma expression was seen, although JNK activation was drastically increased (Suppl. Figure 5b and c), and a contribution of JNK activity in the RM4819-induced Puma expression has been shown above (Figure 5a). This may be possibly explained by the anti-oxidant activity of NAC. Treatment of cells with RM4819 leads to increase in intracellular reactive oxygen species (ROS) (Suppl. Figure 5a), which may also contribute to Puma induction, possible via induction of DNA damage. ROS are however efficiently scavenged by NAC and NAC-induced increased levels of GSH. In line with this hypothesis, cisplatin-induced JNK and p38 activation, known to be induced via ROS (Benhar et al., 2001), was also attenuated by NAC (Suppl. Figure 5c).
Figure 6: Increased GSH levels promote thiazolide-induced apoptosis. a) Caco2 cells were pretreated with 10 mM NAC for 1 h, prior to stimulation with RM4819 (10 μM), cisplatin (10 μg/mL) or DMSO (0.1%) for 40 h. Cell death induction was monitored by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3). *P < 0.0001. b) Caco2 cells were pretreated with 10 mM NAC for 1 h, followed by stimulation with 0.1% DMSO or 20 μM RM4819 for times indicated. Phosphorylated JNK (p-JNK), Bim, Mcl-1, Bcl-xL and tubulin were detected by western blot. c) Caco2 cells were pretreated with 10 mM NAC for 1 h, and stimulated with 20 μM RM4819 or 0.1% DMSO for 8 h. Interaction of Bim with Bcl-xL was assessed by immunoprecipitation and western blotting. IgG, isotype control; HC, immunoglobulin heavy chain. d) Caco2 cells were pre-treated with 10 mM NAC for 1 h, and stimulated with DMSO (0.1%) or RM4919 (20 μM) for 8 h. Bim mRNA expression was assessed by quantitative RT-PCR. Mean values of experimental triplicates ± SD of a representative experiment are shown (n > 3). *P < 0.01. RQ, relative quantification.
Discussion

Sustaining cell survival and avoiding apoptosis are amongst the important hallmarks of cancer (Hanahan and Weinberg, 2000), and thus critical aspects in the development of a tumor. Current cancer therapy often targets specifically these survival- and cell death-regulating processes, for example by using chemotherapeutic drugs. Clearly, mechanisms, which limit the accumulation of such drugs in the tumor cells may lead to better survival and growth of the tumor. GSTs have an important function in the detoxification of cells in general, and the detoxification of tumor cells from such chemotherapeutic drugs in particular (Hayes et al., 2005; Hayes and Pulford, 1995; Tew, 1994; Townsend and Tew, 2003). Coupling of drugs to GSH inactivates and targets them for rapid secretion via bile or kidneys. Furthermore, more recently GSTs have also been found to sequester MAP kinases and thereby affect cellular signaling processes, such as apoptosis induction (Adler et al., 1999). It is thus not surprising that GSTs are overexpressed in a large number of tumors. Specifically, GSTP1-1 levels are increased in ovarian, lung, breast, kidney, pancreas and colon cancer, and lymphomas (Howells et al., 2004; Laborde, 2010; McIlwain et al., 2006), and limit chemotherapy (Ban et al., 1996; Goto et al., 1999; Ishikawa and Ali-Osman, 1993; Jankova et al., 2012; Johansson et al., 2011).

Given the important function of GSTs in detoxification and tumor cell survival, GSTs have become important therapeutic drug targets. EA is a potent GST inhibitor (Awasthi et al., 1993b; Petrini et al., 1993a; Tew et al., 1988a). However, clinical trials have not been successful due to its massive diuretic effects (Odwyer et al., 1991). TLK199 is a GSH analog and inhibits GSTP1-1, which is currently in different clinical trials for the treatment of myelodysplastic syndrome, with a similar mode of action, i.e. as a GSH-activated GST inhibitor (Morgan et al., 1996b). Various aspects indicate that the here described thiazolides act differently. Although we have initially seen that thiazolides bind to GSTP1-1 and inhibit the enzymatic activity of GSTP1-1 in vitro, very likely this observation was based on substrate competition. Indeed, we have seen later that enzymatic GSTP1-1 activity is required for thiazolides to kill colorectal tumor cells, and that increased GSH levels enhance thiazolide activity (Brockmann et al., 2014; Sidler et al., 2012) (Figure 6a). Thus, very likely GSTP1-1 couples GSH to thiazolides, and thereby generates active apoptosis-promoting products. While thiazolides may not be the most potent apoptosis-promoting drugs, with effective concentrations in the micromolar range, they have three interesting features. On one hand they appear to sensitize tumor cells to other drugs and potently synergize with triggers of the mitochondrial (chemotherapeutic drugs) and the extrinsic pathway (TRAIL) (Sidler et al., 2012). Importantly, GSTP1-1 appears to be the Achilles heel in
the thiazolide-induced cell death in colorectal tumor cells. While high GSTP1-1 expression makes them very resistant to chemotherapeutic drugs, but also to other damaging drugs and metabolites, it renders the cells also more susceptible to thiazolides. Thus, thiazolides bypass an important tumor resistance mechanism (Sidler et al., 2012). Last but not least, while there is no clinical evidence available yet for use of thiazolides in the treatment of colorectal tumors, they are already successfully used in the clinic for the treatment of intestinal infections (Abboud et al., 2001a; Anderson and Curran, 2007; Gardner and Hill, 2001; Rossignol et al., 2001a). Importantly, they show very little side-effects on normal tissue cells, indicating that untransformed tissue cells as well as hematopoietic cells seem to tolerate thiazolides rather well. Given that thiazolides are well tolerated, and sensitize tumor cells to chemotherapeutic drugs, the combined use of thiazolides with other anti-cancer drugs may also allow to administer lower drug doses with similar anti-tumor efficacy but reduced side-effects on tissue cells.

Though it is clear that GSTP1-1 converts the pro-drug RM4819 to an active component, thus far the underlying molecular processes causing cell death were not understood. The bromide atom at the thiazole ring likely plays an important role in this GSTP1-1-induced activation as replacement of the bromide with a hydrogen inhibits the pro-apoptotic activity of thiazolides (Brockmann et al., 2014). Here we now show that exposure of cells to thiazolides induces MAPK activation, and that MAP kinase activation is important for the execution of thiazolide-induced cell death. Interestingly, not only JNK and p38 become induced by thiazolides, but also ERK (Sidler et al., 2012), yet seems not to be required for cell death induction. Combined activation of JNK and p38 appears to promote the transcriptional induction and possibly also activation of the BH3-only proteins Bim and Puma. These two proteins bind and neutralize Bcl-x<sub>L</sub> and Mcl-1, thereby promoting apoptosis. Thiazolides not only induce neutralization of Bcl-x<sub>L</sub> and Mcl-1, but also promote Mcl-1 degradation, which likely further lowers the apoptosis resistance of tumor cells. While Puma and Bim-induced direct activation of Bax and Bak has been demonstrated previously (Kang and Reynolds, 2009) and may also feasible in the context of thiazolide-induced apoptosis, it has not been investigated in this study.

The induction and phosphorylation-induced activation of Bim is likely directly mediated by JNK. JNK inhibition results in reduced expression of Bim and reduced binding to Mcl-1. When cells were stimulated with active thiazolides, but not with inactive thiazolides, a shift of Bim to higher molecular weight forms is observed, likely representing JNK-mediated Bim phosphorylation (Figure 2a). Thus, JNK appears to regulate Bim at the transcriptional and post-translational level. Also thiazolide-induced Puma expression is likely controlled by JNK, as inhibition of JNK
leads to reduced thiazolide-induced expression levels of Puma (Figure 5a) and reduced Puma binding to Bcl-xL (Figure 5b). Likely, Puma induction is additionally regulated by other mechanisms. Like chemotherapeutic drugs thiazolides also induced the generation of ROS (Suppl. Figure 5a), which may induce JNK activation and also DNA damage (Benhar et al., 2001). Puma is a BH3-only molecule known to be induced by ROS and DNA damage (Bernstein et al., 2011; Lee et al., 2009). Interestingly, while RM4819-induced JNK activation (Figure 6b, Suppl. Figure 5b and c) and Bim induction (Figure 6d) was strongly enhanced by treatment of the cells with the GSH precursor NAC, it rather resulted in a reduced induction of Puma expression (Suppl. Figure 5c). NAC may thus scavenge thiazolide-induced ROS formation, resulting in reduced Puma expression. But clearly, NAC-induced inhibition of Puma expression must have only a minimal impact on thiazolide-induced apoptosis, as overall NAC still strongly enhances RM4819-induced cell death in Caco2 cells, while it potently inhibits cisplatin-induced cell death (Figure 6a). Thus, very likely cisplatin-induced JNK induction is strongly dependent on ROS induction, whereas RM4819-induced JNK activation is not (Suppl. Figure 5b and c). At presence the mechanism of thiazolide-induced MAP kinase activation is unclear, however, the recently described pathway, in which GSTP1-1 sequesters and inactivates JNK (Adler et al., 1999), which may be reversed by thiazolides, has to be explored in further detail.

While thiazolides are not extremely potent pro-apoptotic drugs, they potently synergize with other apoptosis triggers, such as TRAIL and cisplatin (Sidler et al., 2012). They appear to bring cells into a sensitized stage where little activation by other triggers is required to cause massive apoptosis induction (Sidler et al., 2012). Thus, thiazolides may be useful for future combination therapy with conventional chemotherapeutic drugs. In addition, thiazolides alone are well tolerated by tissue cells, possibly because not all cells have high GSTP1-1 levels, promoting the activation of thiazolides. Furthermore, we have previously made the intriguing observation that thiazolides are only active in proliferating cells (Muller et al., 2008b), although the underlying mechanism is presently not understood. Yet, the combination of restricted GSTP1-1 expression and proliferation status may protect tissue cells and bone marrow cells from thiazolide-induced destruction, while it renders tumor cells, specifically colorectal tumor cells, highly sensitive to thiazolide-induced apoptosis. Though clinical trials have to prove this concept and the overall usefulness of thiazolides in the treatment of colorectal tumors, the present data suggest that thiazolides may represent an interesting novel therapeutic approach for the treatment of this tumor entity.
Materials and Methods

Cell Lines and Reagents

The colon cancer cell lines Caco2 (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 (IMDM) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine and 50 µg/mL gentamicin (all from Sigma-Aldrich, Steinheim, Germany). Cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere. Cisplatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) were obtained from Sigma-Aldrich and DMSO from ROTH (Karlsruhe, Germany). JNK V inhibitor and p38 inhibitor SB202190 were purchased from Calbiochem (Darmstadt, Germany), the dye 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) from Life Technologies (Darmstadt, Germany). Thiazolides RM4819 and compound 2 were synthesized in-house as described earlier (Brockmann et al., 2014). Compounds were kept as 20 mM stock solutions in DMSO.

MTT Assay

Cell viability was measured by using MTT assay. Caco2 and LS174T cells were seeded into 96-well plates at 5-8 x 10³ cells/well. After overnight attachment, cells were treated with thiazolides, cisplatin or DMSO as a control for 40 h. In some experiments JNK and/or p38 inhibitors were added 1 h before apoptosis induction. In some assays cells were treated with 10 mM N-acetyl-L-cysteine (NAC, Sigma-Aldrich) for 1 h to increase intracellular GSH levels. At the end of the experiment cell culture medium was discarded and replaced with 0.5 mg/mL MTT solution, dissolved in complete medium. Plates were incubated under cell culture condition for an additional 1-2 h. After MTT reduction to purple formazan, the MTT solution was discarded and replaced with 100 µL DMSO to dissolve the formazan products. Plates were incubated for 15 min in a dark box at room temperature. After gently mixing of the plates, the intensity of the colored solution was quantified by measuring the absorbance at λ=562 nm on an ELISA reader (Tecan, Crailsheim, Germany). Cell death induction (%) was calculated as 100 * (1- (OD exp. mean value (-substrate blank) / OD control mean value (- substrate blank)).

Bim and Puma knockdown in Caco2 and LS174T cells using lentiviruses

HEK293T cells were seeded into 10 cm petri-dish and co-transfected with the packaging plasmid pCMVdeltaR8.2, the envelope plasmid pMD2-VSV-G and pLKO-based plasmid containing shRNA against human Bim (NM_138621.X-541s1c1 and NM_138621.3-559s21c1) and Puma (NM_014417.2-1318s1c1 and NM_014417.2-785s1c1) (pMission, Sigma-Aldrich) using Roti®-
Fect Plus (ROTH). Plasmid SHC002 (Sigma-Aldrich) served as non-target control. After 24h, medium was changed with complete Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS and 50 µg/mL gentamicin for further 24h. In parallel, Caco2 and LS174T were seeded into 6-well plates (1.8-2.2 x 10^5 cells/well). Next day, viruses in the supernatant were harvested and filtered through a 0.45 µm filter and charged with 8 µg/mL polybrene (Sigma-Aldrich). Target cells were then transduced with the virus mixture in a 1:2 dilution for one day. Stable cells were selected using 3 µg/ml puromycin. Silencing of Bim and Puma was verified by western blotting.

**Double knockdown of Bim and Puma in Caco2 cells**

For double knockdown experiments, Bim expression in control shRNA or Puma shRNA transduced Caco2 cells was silenced cells using Bim siRNA (L-004383-02) or control siRNA (D-001820-01) (Dharmacon, siGenome SMARTpool). The cells were seeded into 6-well plates (1.8 x 10^6) and transfected with siRNAs for 24 h using Lipofectamin®2000 (Life Technologies, Carlsbad, CA) as transfection reagent, as done previously (Schneider-Jakob et al., 2010b; Sidler et al., 2012). Afterward, cells were transferred to a 96-well plate (5 x 10^3 cells/well) and used for cytotoxicity assays. In parallel, Bim and Puma knockdown was verified 48 h post transfection by Western Blot.

**Western Blot**

Caco2 or LS174T cells were seeded into 10 cm petri-dish (2 x 10^6) and treated with thiazolides (20 µM), cisplatin (10 µg/ml), DMSO (0.1%) or with complete medium as control for 0, 2, 4 and 6 h and additional 8 h. In some experiments, Caco2 cells were pretreated with JNK inhibitor (2.5 µM) or with p38 inhibitor (10 µM) or with NAC (10 mM) for 1 h. before treatment with RM4819, cisplatin or DMSO for up to 16 h. Cells were then lysed in NP40-lysis buffer (150 mM NaCl, 50 mM Tris pH 7.6, 1 mM EDTA and 1% NP-40) and cell lysates were separated on a denaturing 12% SDS-PAGE gel. After transfer to polyvinylidene difluoride membranes (PVDF) (Roche, Mannheim, Germany), Mcl-1, Bcl-xL, Bim, Puma, phospho-JNK, phospho-p38, or tubulin as loading control were detected using specific antibodies (anti-Mcl-1 from eBioscience (Frankfurt, Germany); anti-Bcl-xL (54H6), anti phospho-SAPK/JNK (Thr183/Tyr185) (81E11), anti-phospho-p38 MAPK (Thr180/Tyr182) (D3F9), anti-Puma (D30C10) from Cell Signaling Technology (Davers, MA); anti-Bim, anti-α-Tubulin from Sigma-Aldrich). Proteins were detected using horse radish-coupled secondary antibodies and enhanced chemiluminescence on an Image Quant LAS 4000 (GE Healthcare, Braunschweig, Germany).
Immunoprecipitation
Caco2 cells were seeded into 10 cm petri dishes (2 x 10^6 cell/well) and treated with thiazolides (20 µM), cisplatin (10 µg/ml) or DMSO (0.1%) for 8 h. In some experiments cells were additionally pretreated with 2.5 µM JNK inhibitor or 10 mM NAC for 1 h. Cells were then harvested by trypsinization, washed once with PBS and lysed using 500 µL NP-40 lysis buffer containing a protease inhibitor cocktail. Cell lysates were then precleared using Sepharose protein G beads (GE Healthcare, Freiburg, Germany), and Mcl-1 and Bcl-x<sub>L</sub> were immunoprecipitated using Sepharose protein G beads coupled with specific antibodies or isotype controls for 24 h at 4°C under constant agitation. After washing proteins were eluted by boiling in SDS-PAGE loading buffer and resolved on a 12 % SDS-PAGE. Proteins detected by western blot as described above.

Detection of mRNA expression
Caco2 cells were seeded into 10 cm petri dishes (2 x 10^6 cell/well). After attachment, cells were pretreated with 10 mM NAC for 1 h to increase the GSH level before treatment with RM4819 (20 µM) or DMSO (0.1%) for further 8 h. Cells were harvested, lysed in PeqGOLD TriFast (PeqLab, Erlangen, Germany) RNA isolation reagent and RNA was isolated according to the manufacturer’s protocol. One µg RNA was reverse transcribed and cDNAs were used for quantification of gene expression by quantitative PCR using FASTSYBR® Green Master Kit and a StepOnePlus Real time PCR system (Applied Biosystems, Foster City, CA.). The following primers were used: human Bim forward 5'-ATG AGA AGA TCC TCC CTG CT-3’ and reverse 5'-AAT GCA TTC TCC ACA CCA GG-3’, GAPDH forward 5'-ATG GAG AAG GCT GGG GCT CA-3’ and reverse 5'-AGT GAT GGC ATG GAC TGT GGT CAT-3’ to normalize the gene expression by GAPDH expression levels.

Reactive oxygen species (ROS) measurement
Caco2 cells were incubated with 10 µM H2DCFDA for 1 h, prior to stimulation with 20 µM thiazolides, 1 mM H<sub>2</sub>O<sub>2</sub> or 0.1% DMSO for 15 min. In the present of ROS, the H2DCFDA molecule becomes oxidized and fluorescents at Ex/Em: ~492–495/517–527 nm. The mean fluorescence intensity (MFI) was analyzed by flow cytometry on a LSR Fortessa Cell Analyzer using FACS Diva software (BD Biosciences, Heidelberg, Germany).
Statistics
Statistical differences were analyzed using Prism 5 software and a multiple t-test. For experiments described in supplementary figure 4a, a one-way ANOVA with Dunnett’s multiple comparison post-test was performed.

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Supplementary Information

Thiazolides promote apoptosis in colorectal tumor cells via MAP kinase-induced Bim and Puma activation
Supplementary Figure 1: Thiazolide-induced cell death in LS174T cells. LS174T cells were stimulated with 10-20 μM RM4819 or compound 2, or corresponding concentrations of DMSO, for 40 h. Cisplatin (10 μg/mL) was as a positive control. Cell death induction was monitored by MTT assay. Mean values of triplicates ± SD of a representative experiment are shown (n > 3).
Supplementary Figure 2: Thiazolide-induced changes in Mcl-1, Bim and Puma expression. Caco2 (a) and LS174T cells (b) were treated with complete medium, 0.1% DMSO, 20 \( \mu \text{M} \) RM4819 or compound 2 or 10 \( \mu \text{g/mL} \) cisplatin for 16 h. Mcl-1, Bim and Puma were monitored by western blotting. Tubulin served as loading control. (c) Caco2 cells were pretreated with buffer control or with zVAD (80 \( \mu \text{M} \)), MG132 (10 \( \mu \text{M} \)) or with JNK V inhibitor (2.5 \( \mu \text{M} \)) before treatment with 0.1% DMSO or 20 \( \mu \text{M} \) RM4819 for 8 h. Mcl-1, Bcl-x\( _L \), Bim and Puma were detected by western Blot. Tubulin served as loading control.
Supplementary Figure 3: Effect of JNK inhibition on Bcl-2 family protein levels in control and RM4819-treated cells over 4 and 8 h. Caco2 cells were pretreated with buffer control or JNK V inhibitor (2.5 µM) for 1 h. Afterward, 0.1% DMSO or 20 µM RM4819 were added to the cells for 4 h and 8 h. Mcl-1, Bcl-xL, Bim and Puma were detected by western blot. Tubulin served as loading control.

Supplementary Figure 4: Role of p38 in thiazolide-mediated Bim and Puma induction. Caco2 cells were pretreated with 10 µM p38 inhibitor for 1 h, prior to stimulation with solvent control (0.1% DMSO), or RM4819 (20 µM) for times indicated. Mcl-1, Bcl-xL, Bim and Puma were detected by western blotting. Tubulin served as loading control.
Supplementary Figure 5: Increase in intracellular GSH levels enhances thiazolide-induced JNK activation and apoptosis induction. a) Measurement of reactive oxygen species (ROS). Caco2 cells were loaded with the ROS-sensitive dye H2DCFDA (10 µM) for 1 h before stimulation with 0.1% DMSO, 20 µM RM4819 or compound 2, or 1 mM H2O2 for 15 min. The mean fluorescence intensity (MFI) was measured by flow cytometry. Mean values of triplicates ± SD of a typical experiment (n=3) are shown. Data were analyzed with a one-way ANOVA followed by Dunnett's multiple comparison post-test (*P < 0.0001; n.s., not significant) b) - c) Cells were pretreated with 10 mM NAC, and stimulated with DMSO (0.1%), RM4819 (20 µM) or cisplatin (10 µg/mL) for 4 h (b) and 8 h (c). Phosphorylated JNK (p-JNK), phosphorylated p38 (p-p38) and Puma were monitored by western blotting. Tubulin served as loading control.
MY CONTRIBUTION TO THE PAPERS

Chapter I:
Structure-function relationship of thiazolide-induced apoptosis in colorectal tumor cells. I designed the substitutions of thiazolides theoretically based on the lead compound RM4819. In cooperation, Tobias Strittmatter synthesized the corresponding thiazolide derivatives. I performed almost all experiments, which were necessary for this paper, including the revision part by myself. While the master student Sarah May tested the activity of the thiazolide derivatives as a response to an elevating intracellular GSH level in Caco2 cells, she made also important contributions to the establishment of the assays under my supervisions. In collaboration with Kerstin Stemmer, she initiated to perform stable GSTP1-1 knockdown Caco2 cell lines. I also wrote the paper with the very valuable help and comments from Prof. Dr. Thomas Brunner, who did the final polishing of this paper.

Chapter II:
Thiazolide-induced apoptosis in colorectal cancer cells is mediated via the Jun kinase-Bim axis and reveals glutathione-S-transferase P1 as Achilles’ heel. Most of this work was performed by former lab members, in particular Daniel Sidler in Prof. Dr. Thomas Brunner’s lab in Bern. When Prof. Dr. Thomas Brunner moved to the University of Konstanz, I had the opportunity and pleasure to prepare the revisions for this paper.

Chapter III:
Thiazolides promote apoptosis in colorectal tumor cells via MAP kinase-induced Bim and Puma activation. I produced the stable Bim and Puma knockdowns in Caco2 and LS174T cell lines using the corresponding lentiviruses. Moreover, I contributed to the immunoprecipitation experiments performed by Dr. Andrej Bluwstein by harvesting and treating the Caco2 cells used for these experiments. I performed all other experiments shown in the results section, with the exception of those shown in Fig. 1e and 3c, which were done by the master students, Sarah May and Annika Kögel. Annika Kögel additionally made important contributions to the establishment of the assays in this paper. Both master students worked under my supervision. During the generation of this paper, I also wrote the introduction, results, materials and methods, figure legends and a small part of the discussion, while Prof. Dr. Thomas Brunner did the corrections and the final polishing of the paper. In addition, I performed most of the work required for the revisions.
DISCUSSION

CRC is one of the most frequently diagnosed cancers in Europe, with a low 5-year survival rate after diagnosis for advanced tumor stages (Brenner et al., 2014; Ferlay et al., 2010; Ferlay et al., 2013). The major problem is the often unsuccessful treatment of tumor patients due to the limited efficacy of chemotherapeutic agents against CRC cells (Housman et al., 2014). One of the main causes for such poor results are the strong genetic alterations acquired during tumor development, which frequently lead to the development of resistance mechanisms, thereby limiting the accumulation or activity of chemotherapeutic drugs in such tumor cells (Hanahan and Weinberg, 2000; Housman et al., 2014). In particular, high expression levels of the detoxification enzyme GSTP1-1 have been found in CRC (Clapper et al., 1991a; Clapper et al., 1991b; Miyanishi et al., 2001; Ranganathan and Tew, 1991), but also in other solid tumor cells, such as breast, kidney, pancreas, lung and ovarian cancer cells (Howells et al., 2004; Howie et al., 1990; Moscow et al., 1989). While the goal of many anti-cancer drugs is to initiate the death of tumor cells by targeting fast dividing cells, high GSTP1-1 expression contributes to inactivate pharmacological active compounds by forming non-toxic GS-conjugates and/or by direct sequestration of JNK, p38α and TRAF2, which affects cellular signaling processes, such as apoptosis induction (Adler et al., 1999; Laborde, 2010; Tew, 1994; Townsend and Tew, 2003; Wang et al., 2001; Wu et al., 2006). Consequently, GSTP1-1 bypasses the drug-induced cell death and thereby sustains cell proliferation and tumor growth in GSTP1-1-overexpressing cancer cells. This identifies GSTP1-1 as a promising drug-target for anti-cancer therapy. Different GST and/or specifically GSTP1-1 inhibitors have been developed, such as EA and TLK199, which aim is to sensitize tumor cells to chemotherapeutic drugs by inhibiting the GST detoxification mechanism (Lyttle et al., 1994a; Townsend and Tew, 2003). However, the clinical utility of EA was limited due to its strong side effects (O'Dwyer et al., 1991). In addition, the effect of TLK199 in sensitizing cancer cells to other anti-cancer drugs was not efficient enough to be further pursued in clinical trials (Laborde, 2010; Morgan et al., 1996a). Although GSTP1-1 inhibition may sensitize cells to chemotherapeutic drugs, it also leads to the accumulation of endogenous oxidative stress products and potential carcinogens. It has been reported that downregulation of GSTs, including GSTP1-1 are associated with asthma and neurodegenerative diseases, which may be caused by enhanced oxidative stress products (Mazzetti et al., 2015; Schroer et al., 2011). Furthermore, the absence of GSTP1-1 expression is a hallmark for human prostate cancer cells, which paradoxically seems to be important for tumor development (Donkena et al., 2010; Lee et al., 1994; Yang and Park, 2012). In the APC−/− mouse model of intestinal tumors, it could also be shown that GSTP1-1 deficiency leads to an increased number
of intestinal tumors (Ritchie et al., 2009). Thus, the inactivation of GSTP1-1 may render cells more susceptible to additional genome alterations due to the accumulation of carcinogens and therefore may also facilitate the progression of diseases (Lin et al., 2001; McIlwain et al., 2006). Consequently, due to the biological relevance of GSTP1-1 in cells, the development of a direct GSTP1-1 inhibitor should be used with discretion.

We here describe that the mode-of-action of thiazolide-induced cell death is substantially different. Instead of inhibiting GSTP1-1 enzymatic activity and thereby its detoxification properties, our data clearly show that the thiazolide-induced cell death is strongly dependent on a high GSTP1-1 expression level, intracellular GSH level and GSTP1-1 enzymatic activity. Previous studies described thiazolides as a GSTP1-1 inhibitor (Muller et al., 2008b). However, this observation was likely based on thiazolide-mediated substrate competition. In support of this idea, overexpression of the enzymatically more active GSTP1-1 allele A* (Watson et al., 1998) sensitized cells to thiazolides, whereas the enzymatically less active allele B* did not. This result indicates that the thiazolide-induced cell death is dependent on the GSTP1-1 enzymatic activity. Additionally, we identified that high GSTP1-1 expression levels correlate with the thiazolide sensitivity in different CRC cell lines, such as Caco2 and LS174T. This leads to the assumption that thiazolides as pro-drugs get activated by GSTP1-1 to induce the mitochondrial apoptosis pathway. Interestingly, while increased GSTP1-1 expression levels render tumor cells resistant to certain drugs (McIlwain et al., 2006; Tew, 1994; Townsend and Tew, 2003), it makes them more susceptible to thiazolides, thus identifying GSTP1-1 as a critical activator of thiazolide-induced apoptosis. Although thiazolides are relatively weak cell death inducers in CRC cells, showing activity only in a micromolar range, they strongly synergize with TRAIL and chemotherapeutic drugs, in particular cisplatin, in inducing apoptosis of tumor cells. Even tumor cells, which are rather RM4819 resistant, e.g. HT29 and Colo205, are susceptible to the combination treatment of thiazolide plus TRAIL or cisplatin. Thus, thiazolides appear to bypass the GSTP1-1 drug resistance mechanism and therefore sensitize tumor cells to other apoptosis inducers. It might be that the dual treatment of thiazolides plus other cell death triggers activates the apoptosis pathway at multiple steps, finally leading to a synergistic cell death induction in tumor cells. Similar effects have been observed for the pro-drug TLK286; It gets activated in a GSTP1-1-dependent process by cleaving TLK286 into a GSH analogue and nitrogen mustard, which alkylates DNA, RNA and proteins, and initiates the apoptosis (Lyttle et al., 1994b). Like thiazolides, the cytotoxic effect of TLK286 is also dependent on high GSTP1-1 expression levels, and the GSTP1-1 enzymatic activity is required to convert the pro-drug into toxic metabolites (Morgan et al., 1998; Rosario et al., 2000). It may also be observed that TLK286
sensitizes drug-resistance ovarian cancer, non-small cell lung cancer and breast cancer to chemotherapeutic agents, such as carboplatin, resulting in a synergistic cell death induction upon dual treatment (Hua Xu, 2004; Sequist et al., 2009). Despite the similarly activities of thiazolides and TLK286, there exists a remarkable difference between both GSTP1-1-targeting drugs. While increased GSH levels lead to a TLK286-resistant phenotype (Rosario et al., 2000), and also lower the sensitivity towards chemotherapeutic drugs (Hayes and Pulford, 1995; Panasci et al., 2001; Tew, 1994), in our model the thiazolide-mediated cell death induction is dramatically increased when CRC cells are pre-treated with the GSH precursor NAC. Thus, the intracellular GSH levels play, in addition to GSTP1-1 enzymatic activity, a critical role in converting thiazolide to an active product that might overcome the TLK286-resistant phenotype in such tumor cells with elevated GSH levels. Although it is not clear how thiazolides become activated in a GSTP1-1/GSH-dependent manner, there is evidence that points towards the requirement of the bromo-substituent of RM4819 to induce apoptosis in CRC cells. Several thiazolide derivatives were synthesized with different variations on the substituents of the benzene ring and thiazole ring, based on the lead compound RM4819. When modifying RM4819 on position 2 and 3 of the benzene ring, the thiazolide-induced cell death was not affected in CRC cells. Thus, neither the hydroxyl group nor the methyl group of RM4819 are required for cell death induction in a GSTP1-1-dependent manner. However, the substitution of the bromide atom on the thiazole ring to a hydrogen, considerably reduced the cell death-inducing activity, as shown for compound 2. Thus, we assume that the bromide atom on this position plays an important role in interacting with GSTP1-1 and/or in the formation of a toxic metabolite. Since GSTP1-1 enzymatic activity is required for thiazolide-induced apoptosis, and the fact that we observed an enhanced cell death induction for all synthesized thiazolide derivatives in response to elevated GSH levels, it is likely that GSTP1-1 couples thiazolides to GSH that results in the formation of a toxic product, initiating the mitochondrial apoptosis pathway. It might be that elevated GSH levels lead to an increased amount of pro-apoptotic metabolites, while TLK286 and chemotherapeutic drugs, such as melphalan and cisplatin, are inactivated (Goto et al., 1999; Rosario et al., 2000). This proposes thiazolides as a novel pro-drug able to overcome the drug resistance mechanisms in GSTP1-1-overexpressing cells, or cells with high GSH levels, and therefore offers a new therapeutic window in anti-cancer therapy.

While it is well established that GSTP1-1 enzymatic activity is necessary for detoxification and, thus inactivating chemotherapeutic drugs (Tew, 1994), it also modulates apoptosis by sequestering critical molecules in a non-enzymatic manner (Laborde, 2010; Townsend and Tew, 2003). For instance, GSTP1-1 binds and inhibits the activation of JNK, TRAF2 and, to
some extent, p38α (Adler et al., 1999; Wang et al., 2001; Wu et al., 2006). Thus, GSTP1-1-deficient mice show a basal increase in JNK activation (Elsby et al., 2003), indicating that GSTP1-1 controls important signaling pathway. While several anti-cancer drugs, such as cisplatin mediate cell death induction through the activation of JNK and p38 (Boldt et al., 2002; Mansouri et al., 2003; Osborn and Chambers, 1996), overexpression of GSTP1-1 in tumor cells may modulate the activation of the MAPK signaling pathway by the direct interaction of GSTP1-1, and thereby allows the tumor cells the ability to escape apoptosis induction during drug treatment (Laborde, 2010; Townsend and Tew, 2003). Along this line, we tested MAPKs activation in response to thiazolide treatment, and observed a thiazolide-induced phosphorylation and activation of JNK and p38, and a JNK- and p38-dependent cell death induction. However, since JNK activation and possible p38 are modulated by GSTP1-1 in a non-enzymatic way (Laborde, 2010), it is rather unlikely that thiazolide-mediated activation of JNK and p38 is regulated through the liberation from the GSTP1-1 complex. Because the thiazolide-induced cell death is strongly dependent on high GSTP1-1 expression levels and GSTP1-1 enzymatic activity, and we further recognized that the mode-of-action of thiazolide- vs chemotherapeutic drug-mediated activation of the MAPK signaling pathway can be differently regulated. Once GSH is increased, JNK activation is strongly enhanced in thiazolide-treated Caco2 cells, which correlates with enhanced cell death induction, whereas the treatment with cisplatin leads to a reduced JNK activation, and results thus in a chemotherapeutic drug-resistant phenotype. This suggests that the activation of JNK can be differently regulated by diverse apoptotic stimuli. Given that thiazolide- and cisplatin-mediated activation of JNK and JNK-mediated cell death induction is differently regulated, it might explain the synergistic cell death induction upon combined treatment of thiazolide and cisplatin in CRC cells, due to the different way to activate the apoptosis pathway.

Further studies on the underlying mechanism of thiazolides identified a JNK-mediated induction and activation of the BH3-only proteins Bim and Puma that can bind and neutralize anti-apoptotic Bcl-2 family members, such as Bcl-xL and Mcl-1, and thereby promote the mitochondrial apoptosis pathway. Thiazolides not only induce neutralization of Bcl-xL and Mcl-1, but also promote Mcl-1 degradation. Interestingly, acquired genetic alterations along the adenoma-carcinoma sequence may lead to an upregulation of Bcl-xL and Mcl-1, which results in an impaired apoptosis sensitivity (Liu et al., 1999; Michels et al., 2014; Minn et al., 1995; Mott et al., 2007). Thus, it is likely that the neutralization of Bcl-xL and Mcl-1, and Mcl-1 degradation by thiazolides, lowers the apoptosis resistance mechanisms of tumor cells and therefore sensitizes cells to apoptosis triggers.
In any case, in our model inhibition of JNK leads to a reduced Bim and Puma induction and therefore to a reduced binding and neutralization of Bcl-xL and Mcl-1, that prevent cell death induction. This identified JNK as a critical regulator of the downstream effector pathway in the presence of thiazolides. However, it is not clear how Bim and Puma are induced and activated by JNK. Since the gene promoters of both BH3-only proteins contain an AP-1-binding site (Ameyar et al., 2003; Cazanave et al., 2010; Cazanave et al., 2009; Whitfield et al., 2001; Zhao et al., 2012), it is theoretically possible that the JNK-initiated activation of AP-1 mediates the expression of Bim and Puma in thiazolide-treated CRC cells. In addition, it has been reported that Bim contains a post-translational modification site at Thr112, which is phosphorylated by JNK (Hubner et al., 2008). This phosphorylation site appears to activate Bim and to increase its binding affinities to Bcl-2-like survival factors (Akiyama et al., 2009; Hubner et al., 2008). In line with these reports, we observed a shift of Bim to a higher molecular weight form 4 h after thiazolide treatment. Therefore, it is very likely that JNK mediates Bim phosphorylation and activation at a post-translational level. Thus, active JNK possibly regulates Bim and Puma induction at a transcriptional level, while Bim can be additionally controlled post-translationally by JNK.

While we identified the MAPK JNK as a critical regulator of the downstream effector pathway in thiazolide-treated CRC cells, the role of p38 is unclear. Although we observed a thiazolide-mediated activation of p38, which is required for the cell death induction, inhibition of p38 only minimal reduced RM4819-induced Bim and Puma levels in Caco2 cells. Thus, Bim and Puma expression is likely regulated predominantly by JNK. Interestingly, not only JNK and p38 are activated by thiazolides, but also the MAPK ERK is induced. However, unlike JNK and p38, ERK seems not to be involved in the thiazolide-induced cell death. In general MAPKs regulate different cellular processes such as proliferation, differentiation and apoptosis. While JNK and p38 activities may promote apoptosis, ERK is mainly involved in the regulation of cellular survival processes that can be activated by the EGFR-Ras signaling pathway (Roberts and Der, 2007). Active ERK can regulate post-translationally the phosphorylation and degradation of FoxO3A and Bim (Akiyama et al., 2009; Yang et al., 2008). FoxO3A (forkhead box O3A) in turn is a transcription factor and can positively regulate the expression of Bim and Puma through binding to the corresponding promoter sequences of both BH3-only proteins, indicating that FoxO3A is an important regulator of pro-apoptotic factors (Akiyama et al., 2009; Essafi et al., 2005; You et al., 2006; Zhang et al., 2011). Since active ERK mediates the degradation of Bim and FoxO3A, and thereby suppresses FoxO3A-mediated expression of Bim and Puma, it plays a critical role in modulating survival pathways in cells. Thus, in our model, the thiazolide-
mediated activation of ERK appears to promote a permanent inactivation of Bim and FoxO3A, and consequently an inhibition of the FoxO3A-mediated Bim and Puma induction. However, active ERK seems to have a minimal impact on thiazolide-induced cell death, as overall Bim and Puma is induced, which promote the mitochondrial apoptosis induction after treatment.

Interestingly, although our data clearly show a JNK-dependent regulation of Bim and Puma upon thiazolide treatment, it might be possible that Bim and Puma are differently regulated by thiazolide-mediated JNK activation. Unlike Puma, the JNK-mediated Bim induction and binding to Bcl-x<sub>L</sub> and Mcl-1 degradation was strongly enhanced by combined treatment of the cells with NAC and RM4819, eventually potentiating cell death induction. Puma in turn showed a rather reduced expression in response to NAC plus RM4819 in Caco2 cells, although a strong JNK activation could be observed. Thus, it might be that Puma is regulated by an alternative pathway. Like chemotherapeutic drugs, thiazolides induce ROS formation, which may induce DNA damage and also JNK activation (Klaunig et al., 2010; Mizutani, 2007; Picco and Pages, 2013; Shi et al., 2014). Puma for instance is known to be induced by ROS and DNA damage (Bernstein et al., 2011; Lee et al., 2009; Yu et al., 2001). Thus, it is possible that the thiazolide-induced generation of ROS leads to the activation of JNK and JNK-mediated Puma induction. NAC know as important anti-oxidant may thus scavenge thiazolide-induced ROS formation that finally results in a reduced JNK activation and subsequently to a decreased Puma expression levels. While JNK is known to be sequestrated by GSTP1-1 in a non-enzymatic manner, it could be shown in the presence of oxidative or chemical stress that JNK liberates from the GSTP1-1:JNK complex that allows apoptosis induction (Laborde, 2010; Townsend and Tew, 2003). Thus, it might be that the ROS formation by thiazolides lead to the liberation of JNK and mediates the induction of Puma that can be blocked by NAC. Similar mechanisms could be observed for cisplatin. The cisplatin-mediated activation of JNK is also reduced in the presence of NAC, therefore preventing cell death induction. Thus, it is likely that the cisplatin-induced JNK activation is dependent on ROS generation, whereas JNK activation by RM4819 is not. Consequently, it seems to be that the thiazolide-mediated JNK activation is regulated by two distinct mechanisms, whereas the ROS-mediated activation of JNK and JNK-induced Puma expression has only a minimal impact on thiazolide-induced apoptosis, as overall NAC still strongly enhances RM4819-induced cell death in Caco2 cells. Accordingly, the NAC-induced increase in JNK activation and JNK-mediated Bim induction seems to be an important apoptosis regulating process in thiazolide-mediated cell death induction. Similar mechanisms could be observed in hepatocytes. Once treated with Fas, TRAIL or paracetamol, JNK gets activated and mediates the induction and activation of Bim that induce liver damage (Bermann et al., 2011a;
Corazza et al., 2006a; Kaufmann et al., 2009). Thus, it seems to be that the JNK-mediated regulation of Bim represents a common mechanism to sensitize cells to apoptosis. However, the mechanism regarding how JNK and Bim are activated by thiazolides remains unclear and has to be explored in further detail.

**Figure Discussion: Thiazolide-induced cell death is regulated at multiple steps (see text)**

Dependent on the GSTP1-1 expression levels, thiazolides induce the activation of the MAPK pathway, thereby mediating apoptosis. While both MAPKs, JNK and p38, are involved in the thiazolide-induced cell death, JNK appears to be regulated by two distinct mechanisms that induce and activate the BH3-only proteins Bim and Puma. It might be that Puma is induced via the thiazolide-induced generation of ROS that in turn activates JNK through the release of the GSTP1-1:JNK complex in a non-enzymatic way. This pathway can be inhibited by increased GSH levels. The induction and activation of Bim in turn is possibly regulated by an alternative pathway that can be increased by elevated GSH levels. Both BH3-only proteins bind and neutralize pro-survival proteins of the Bcl-2 family and thereby promote apoptosis.

Although thiazolides have not been tested yet in clinical studies for the treatments of CRC, we assume a good safety profile. Because thiazolides are already approved for the treatment of intestinal infections and well tolerated by patients (Abaza et al., 1998). After oral administration
of thiazolides, once absorbed by intestinal epithelial cells and distributed to the circulation, they can be inactivated in the liver and thus excreted by bile or urine, limiting their effects elsewhere in the body (Broekhuysen et al., 2000; Fox and Saravolatz, 2005; Stockis et al., 1996). While GSTP1-1 has been found to be overexpressed along the adenoma-carcinoma sequence of CRC (Clapper et al., 1991a; Clapper et al., 1991b; Miyanishi et al., 2001; Ranganathan and Tew, 1991), minimal expression of GSTP1-1 in primary intestinal epithelial cells, including the intestinal epithelial stem cells, was observed. Thus, it is possible that thiazolides efficiently target primary tumors of the intestine, which express high level of GSTP1-1, whereas the surrounding tissues are probably insensitive to thiazolides. Preliminary results in isolated crypt organoids from wild type mice confirm a low thiazolide sensitivity of primary intestinal epithelial cells (unpublished data). However, these experiments need conformation. To further support the good safety profile of thiazolide in normal tissue, previous studies made the observation that thiazolides act only in proliferating cells, although the underlying mechanism is still unclear (Muller et al., 2008b). Thus, the combination of restricted GSTP1-1 expression and proliferation status may protect tissue cells and bone marrow cells from thiazolide-induced destruction, while it renders tumor cells, specifically colorectal tumor cells, highly sensitive to thiazolide-induced apoptosis. Besides, TLK286 is already in phase III clinical trials and shows similar effects as thiazolides. Furthermore, the TLK286 drug-related side effects are minimal and well tolerated in patients (Sequist et al., 2009; Vergote et al., 2009). Thus, it is possible that thiazolides will also be well tolerated in CRC patients.

Of interest is our observation that thiazolides sensitize CRC cells to other apoptosis triggers, such as TRAIL and chemotherapeutic agents, which result in a synergistic cell death induction upon combined treatment, even in those CRC cells that are RM4819 resistant. This may offer an interesting therapeutic window. It is possible that after oral administration of thiazolides, tumor cells are sensitized to the systemical administration of chemotherapeutic drugs or TRAIL. The thiazolide-induced sensitization may allow to treat patients with lower dose of these drugs. Accordingly, the side effects on normal tissue cells may also be reduced in CRC patients. The currently most frequently used therapeutic approaches against CRC are chemotherapeutic drugs, irradiation and targeted therapy. However, these medications show strong side effects and have limited application (Holohan et al., 2013; Housman et al., 2014). Our studies propose GSTP1-1-activated thiazolides as a novel class of drugs to be used in the combination treatment of CRC.
Finally, we recognized that thiazolides are able to induce cell death in both p53 wild type (LS174T cells) and p53 mutant cells (Caco2 cells) (Djelloul et al., 1997; Liu and Bodmer, 2006). p53 is an important transcription factor that amongst others regulates apoptosis. Loss of p53 may result in the malignant transformation of tumor cells and occurs in up to 50% of CRC patients (Grady and Pritchard, 2014; Li et al., 2015; Rivlin et al., 2011). Since both Caco2 and LS174T cell lines are sensitive to thiazolides, it might be that the thiazolide activity is not restricted to the p53 status in CRC. Thus, it is possible that the effect and potential application of p53-mutated CRC cells is extended by thiazolides. However, it needs further experimentations to confirm this hypothesis..

**SUMMARY and CONCLUSIONS**

We here propose thiazolides as a pro-drug and novel therapeutic approach for the treatment of CRC. Their cell death inducing mechanism is strongly dependent on high GSTP1-1 expression levels and GSTP1-1 enzymatic activity. Furthermore, their cell death inducing effect can be increased by elevated GSH levels. While high GSTP1-1 expression makes many cancer cells resistant to chemotherapeutic drugs (Tew, 1994; Townsend and Tew, 2003), it renders tumor cells more susceptible to thiazolides. This study identifies GSTP1-1 overexpression as the Achilles heel in thiazolide-induced cell death in CRC cells. It is very likely that the thiazolide activity is not only restricted to CRC cells but also sensitizes other tumor cells with increased GSTP1-1 expression levels, such as breast, kidney, pancreas, lung, and ovarian cancer cells (Howells et al., 2004; Howie et al., 1990; Moscow et al., 1989). Our interesting finding that NAC increases the thiazolide-induced cell death induction dramatically indicates a strong correlation between GSTP1-1, intracellular GSH level and thiazolide sensitivity. Thus, it is very likely that GSTP1-1 couples GSH to thiazolide and thereby generates active apoptosis-promoting products, while the bromide atom on position 5 of the thiazole ring of RM4819 plays a critical role in this process. It would be of interest to know whether NAC together with thiazolides show a therapeutic effect in CRC patients. NAC is already used as a mucolytic agent, the treatment of paracetamol overdose-induced liver damage, for the treatment of HIV patients and show some effects in patients with Alzheimer diseases (Adair et al., 2001; Dodd et al., 2008; Scalley and Conner, 1978). The side effects of NAC are well tolerated in patients. Thus, the combined use of NAC and thiazolides could be an interesting approach for the treatment of CRC patients with GSTP1-1-overexpressing tumor cells.
Given that GSTP1-1 converts the pro-drug RM4819 to an active component, we identified the MAPKs JNK and p38 as critical regulators in thiazolide-induced cell death. In particular, JNK activation leads to the induction and activation of BH3-only proteins that bind and neutralize pro-survival Bcl-2 family members, and thereby induce apoptosis, while the role of p38 remains unclear. It might be that thiazolides overcome important apoptosis resistance mechanisms that have been reported for many tumors, and therefore sensitize cells to other apoptosis triggers, as we found for chemotherapeutic drugs and TRAIL. Although thiazolides may not be the most potent apoptosis-promoting drugs, minimal doses are already sufficient to sensitize cells to lower concentrations of cisplatin or TRAIL, leading to a synergistic cell death induction even in such cells that are RM4819 resistant. Although thiazolides have not been tested yet in clinical trials as anti-cancer drugs, we assume minimal side-effects on non-transformed tissue cells. The generally good safety profile of thiazolides as anti-microbial drugs and the combination of restricted GSTP1-1 expression and proliferation status may protect uninvolved tissue cells, but it renders tumor cells highly susceptible to thiazolide-induced apoptosis. While increased GSTP1-1 expression levels have been found during the early initiation stage of CRC up to metastatic carcinoma (Clapper et al., 1991a; Clapper et al., 1991b; Miyanishi et al., 2001; Ranganathan and Tew, 1991), it might be that thiazolides can be applied at multiple stages as a single agent or in combination of low doses of chemotherapeutic drugs or TRAIL. In addition, we observed that the thiazolide-induced cell death is probably not limited by the p53 status of CRC cells. This would increase the therapeutic window of thiazolides in the treatment of CRC. Altogether, it seems that thiazolides could have a strong therapeutic potential in the treatment of CRC patients.

FUTURE PERSPECTIVES

Thus far, our studies have been limited to the analysis of CRC cell lines in vitro, as a critical next step in the evaluation of thiazolides as anti-cancer drugs, I propose to study the tumoricidal activity of thiazolides in two mouse models of intestinal tumors, i.e. APCmin/+ mice and the AOM/DSS induced model of CRC. Furthermore, while our structure-function studies identified the critical residues in the thiazole lead compound RM4819, detailed analysis of the metabolites generated after the reaction of thiazolides with GSTP1-1 and GSH are required to identify the active thiazolide compound promoting cell death in CRC cells. The identification of this active compound will thus allow for the investigation of why thiazolides only induce cell death in proliferating cells.
ABBREVIATION

18qLOH: 18q loss of heterozygosity
ACF: aberrant crypt focus
AP-1: activator protein-1
Apaf-1: apoptotic peptidase activating factor 1
APC: adenomatous polyposis coli
Bcl-2: B-cell CLL/lymphoma 2
Caco2: colorectal adenocarcinoma cell line
CARD: caspase-recruitment domain
cFLIP: (Flice-like inhibitory protein)
cIAP: cellular inhibitor of apoptosis proteins
CIN: chromosome instability
CLL: chronic lymphocytic leukemia
CRC: colorectal cancer
CYPs: cytochromes P450
DD: death domain
DED: death effector domain
DISC: death-inducing signaling complex
DR: death receptor
EGFR: epidermal growth factor receptor
FADD: Fas-associated death domain
FAP: familial adenomatous polyposis
FoxO3A: forkhead box O3A
GSH: glutathione
GST: glutathione S-transferase
GSTP1-1: GST of class P, subunits 1
HEK293T: human embryonic kidney cells
HFF: human foreskin fibroblasts
HNPCC: hereditary non-polyposis colorectal cancer
IAP: inhibitor of apoptosis protein
ICAD: caspase-activated DNase
JNK: c-Jun N-terminal kinase
MAP2K: MAP kinase kinase
MAP3K: MAP kinase kinase kinases
MAPEG: membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK: mitogen activated protein kinase
MIMS: mitochondrial intermembrane space
MMR: mismatch repair
MOMP: mitochondrial outer membrane permeabilization
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NTZ: nitazoxanide, 2-(acetyloxy-N-(5-nitro 2-thiazolyl) benzamide
PARP-1: poly(ADP-ribose) polymerase-1
RIPK1: receptor associated protein kinase 1
RM4819: N-(5-bromothiazol-2-yl)-2-hydroxy-3-methylbenzamide
SULT: sulfotransferase
TGF-beta: transforming growth factor, beta
TNF: Tumor necrosis factor
TRADD: TNF receptor associated-protein with death domain
TRAF: TNF receptor-associated factor
TRAIL: TNF-alpha related apoptosis inducing ligand
UGT: UDP-glucuronosyltransferase
VEGF: vascular endothelial growth factor
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REFERENCES


de Almagro, M.C., and Vucic, D. (2012). The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Experimental oncology 34, 200-211.


Miguel A. Rodriguez-Bigas, M., Edward H. Lin, MD, and Christopher H. Crane, MD. Stage IV Colorectal Cancer (Holland-Frei Cancer Medicine. 6th edition.).


Sherratt, P.J., Pulford, D.J., Harrison, D.J., Green, T., and Hayes, J.D. (1997). Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse - Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human. Biochemical Journal 326, 837-846.


