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Epigenetics and chemoresistance in colorectal cancer: An opportunity for treatment tailoring and novel therapeutic strategies

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ABSTRACT

Colorectal cancer is the second leading cause of cancer-related deaths in the world. Despite many therapeutic opportunities, prognosis remains dismal for patients with metastatic disease, and a significant portion of early-stage patients develop recurrence after chemotherapy. Epigenetic gene regulation is a major mechanism of cancer initiation and progression, through the inactivation of several tumor suppressor genes. Emerging evidence indicates that epigenetics may also play a key role in the development of chemoresistance. In the present review, we summarize epigenetic mechanisms triggering resistance to three commonly used agents in colorectal cancer: 5-fluorouracil, irinotecan and oxaliplatin. Those epigenetic biomarkers may help stratify colorectal cancer patients and develop a tailored therapeutic approach. In addition, epigenetic modifications are reversible through specific drugs: histone-deacetylase and DNA-methyl-transferase inhibitors. Preclinical studies suggest that these drugs may reverse chemoresistance in colorectal tumors. In conclusion, an epigenetic approach to colorectal cancer chemoresistance may pave the way to personalized treatment and to innovative therapeutic strategies.

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1. Introduction

Worldwide, 1.2 million new colorectal cancer cases were detected in 2008, and 608,700 deaths have been estimated (Jemal et al., 2011). Colorectal cancer prognosis and treatment strategies are highly dependent on tumor stage. Stage I colorectal cancer, which is confined to sub-mucosa, shows a 5-year survival rate of approximately 90% (Kohne and Lenz, 2009). On the other hand, metastatic colorectal cancer patients (stage IV) treated with the best supportive care, have a median survival of just 6 months. For non-metastatic patients, surgical excision is the preferred option. Adjuvant chemotherapy is employed after surgical resection in stage III patients (localized tumor with lymph node invasion), while it is questionable for stage II patients (localized tumor without lymph node invasion) (Cunningham et al., 2010). Adjuvant treatment is based on 5-fluorouracil (5-FU) alone or in combination with oxaliplatin. Stage IV disease is treated with doublet chemotherapy, including 5-FU plus either oxaliplatin or irinotecan. Doublet chemotherapy plus biological agents (anti-angiogenic or anti-epidermal growth factor receptor (EGFR) molecules) increase stage IV patients' median survival to approximately 2 years (Kohne and Lenz, 2009).

Despite this plethora of therapeutic options, many questions remain open for the clinician. Should stage II patients be treated with chemotherapy? What is the best drug combination for metastatic disease? The answers differ according to each patient. Individual and tumor genetic background can significantly affect patient's response to a drug and thus the choice of treatment. In keeping with this hypothesis, it has been shown that only patients with wild-type KRAS tumors respond to anti-EGFR therapy (Qiao and Wong, 2009; Hollande et al., 2010; Dahabreh et al., 2011).

Even with some progress in molecular characterization of drug sensitivity, tailored therapy for colorectal cancer patients is still a challenge. Molecular determinants of response to chemotherapeutic agents, which are still the cornerstone of colorectal cancer therapy, are lacking (Fornaro et al., 2010).

In the present review, we explore a different mechanism of chemoresistance, which is not determined by DNA sequence alterations. Epigenetic modifications are defined as heritable changes, not determined by modification of the DNA primary structure (Dupont et al., 2009). Epigenetic modifications play a crucial role in colorectal cancer initiation and progression (Van Engeland et al., 2011). We will describe emerging mechanisms of

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chemoresistance induced by epi-mutations, suggesting how they could be employed as biomarkers of chemotherapy response in colorectal cancer patients. Unlike genetic alterations, epigenetic modifications are reversible, and can be targeted by specific drugs (Kelly et al., 2010). Thus, we think that the epigenetic perspective may broaden the biomarker panel in colorectal cancer and provide oncologists with novel molecules which may improve the outcome of chemotherapy.

2. Epigenetics and chemoresistance

2.1. Epigenetic mechanisms of gene silencing in cancer cells

A broad definition of epigenetics comes from developmental biology. This discipline was first defined as the sum of all those mechanisms necessary for the unfolding of the genetic program for development (Holliday, 2006). Epigenetic mechanisms of gene expression regulation are not only crucial to physiological tissue specification, but are also disrupted in many diseases, including colorectal cancer (Van Engeland et al., 2011). In the present review, we will refer to the two classical and most frequently investigated epigenetic mechanisms: DNA methylation and histone post-translational modifications. This choice is motivated by our translational purpose: both DNA methylation and histone modifications can be detected by validated diagnostic instruments (Weber, 2010), and can be targeted by specific drugs (Kelly et al., 2010).

Fig. 1 provides a schematic representation of some epigenetic modifications, occurring most frequently in the promoter region of target genes. DNA is not always accessible to RNA polymerase and transcription factors. Depending on local transcriptional activity, DNA-protein complexes may present as heterochromatin or euchromatin (Mathews et al., 2009). In the first case, DNA-protein complexes are tightly bound, and gene transcription is impossible. In the latter case, DNA-protein binding is loose, and gene transcription is allowed. Chromatin primary organization is represented by the nucleosome, a cylindrical structure composed of four couples of histones (H2a, H2b, H3, H4). Adjacent nucleosomes are linked by the H1 histone, thereby forming a chain. DNA

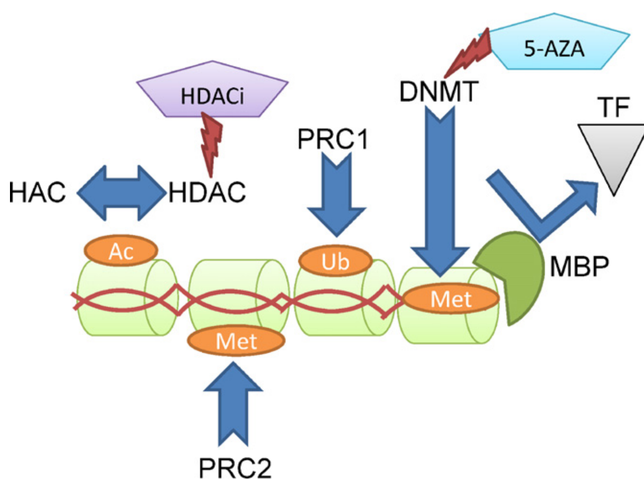

Fig. 1. Epigenetic mechanisms of gene expression regulation. The DNA helix (red) is wrapped around histones (light green cylinders). Histone acetylation (Ac) is regulated by the contrasting activities of HAC and deacetylase (HDAC). Polycomb repressive complex 1 and 2 (PRC) mediate histone H2a ubiquitination (Ub) and H3K27 methylation (met), respectively. DNA-methyl-transferases (DNMT) binds to pre-marked sites and methylates DNA. Methylated DNA is bound by methyl-DNA binding proteins (MBP), which hinder transcription factor (TF) binding, thereby causing gene silencing. Demethylating agents (5-AZA) and HDACI inhibitors (HDACIs) reactivate gene silencing.

is wrapped around each nucleosome, and is more or less tightly bound to these proteins, depending on post-translational modifications occurring in critical regions (histone tails). Several kinds of post-translational modifications have been described, including phopshorylation, methylation, acetylation, ubiquitination. Only a few of these have been functionally characterized, suggesting that a histone code regulates gene expression (Sawan and Herceg, 2010). Among characterized modifications, histone acetylation is always associated with reduced histone-DNA binding, and thus to gene activation. Histone acetylation is determined by the local balance between two classes of enzymes: histone acetylase (HAC) and histone deacetylases (HDACs). Some tumor suppressors (cell-cycle regulators, anti-apoptotic genes) are hypo-acetylated in colorectal cancer, resulting in gene silencing (Van Engeland et al., 2011). As we will see in Section 4, HDAC inhibitors (HDACIs) (e.g., vorinostat and romidepsin) are small molecules that may reactivate epigenetically silenced genes in cancer cells (Kelly et al., 2010).

Histone methylation may be associated with both gene activation and repression. In cancer cells, histone methylation at histone H3 Lys 27 is particularly important. It is a repressive mark mediated by Polycomb repressive complex 2 (PRC2), which is generally followed by histone H2a ubiquitination by PRC1 (Mathews et al., 2009). Polycomb complexes are crucial for tissue-specific gene silencing and stem cell self-renewal. In cancer cells, Polycomb genes silence several pro-apoptotic and anti-metastatic genes, thereby contributing to cancer progression (Piunti and Pasini, 2011). In colorectal cancer, the PRC2 member EHZ2 is over-expressed in poor prognosis patients (Wang et al., 2010).

PRCs and HDACs cooperate with DNA methyl-transferase (DNMT), a class of enzymes capable of adding a methyl group to cytosine residues (Mathews et al., 2009). DNA methylation generally occurs in CpG-rich in promoter regions (CpG island), and is associated with gene silencing. Approximately 70% of human genes harbor a CpG island at its promoter (Saxonov et al., 2006). In colorectal cancer, several anti-apoptotic, anti-metastatic and anti-angiogenic genes are silenced by DNA methylation (Van Engeland et al., 2011). DNMT inhibitors (DNMTIs) (e.g., 5-aza-cytidine, 5-aza-2′deoxycitidine) are able to reactivate methylated genes in cancer cells (Kelly et al., 2010).

In colorectal cancer, epigenetic modifications play a crucial role at each step of carcinogenesis, and display a complex interaction with genetic alterations (Van Engeland et al., 2011). In general, global demethylation is found in many uncoding regions, leading to increased genomic instability. This is associated to hypermethylation of many tumor-suppressor genes. Approximately 20% of colorectal cancers display a CpG island methylation phenotype (CIMP). CIMP tumors display widespread methylation of tumor suppressor genes, and show a distinct pathological and molecular profile (Ogino and Goel, 2008). CIMP colorectal cancers are associated with proximal tumor location, female sex, poor differentiation, high BRAF and low TP53 mutation rates. Interestingly, epigenetic alterations seem to be strictly associated with colorectal cancer genetic features. A common genetic classification identifies two main groups of colorectal cancers: chromosomal instable (CIN) cancers, which are characterized by chromosomal aberrations, and microsatellite instable (MSI) cancers, which are characterized by abnormal expansion of short repetitive elements (Ogino and Goel, 2008). Both CIN and MSI tumors show distinct pathological and prognostic profiles. Recently, it has been shown that CIMP is generally associated with MSI, while CIN tumors are more frequently characterized by global DNA demethylation (Deng et al., 2006). As we will show in the next sections, genetic and epigenetic backgrounds are highly interactive in colorectal cancer, and need to be explored at the same time to identify markers of response to chemotherapy.

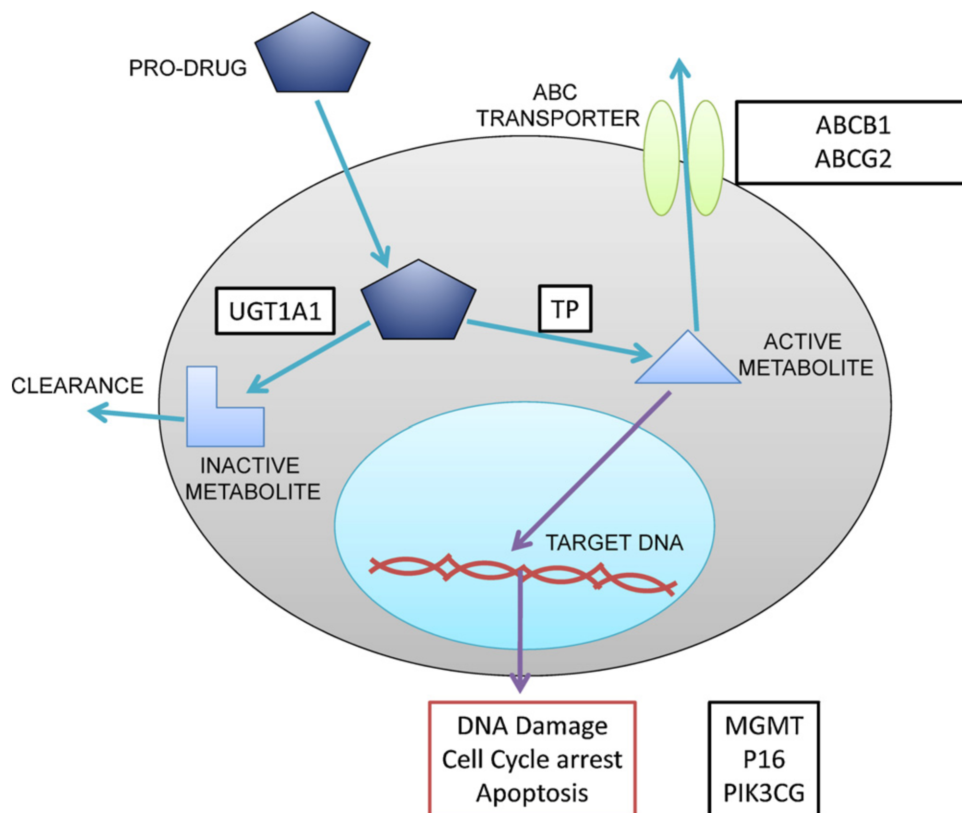

Fig. 2. Epigenetic mechanisms affecting cancer cell chemosensitivity. Chemotherapy agents are often pro-drugs, which can be activated or inactivated by specific cellular enzymes. Active metabolites penetrate the nucleus and induce DNA damage, cell cycle arrest and apoptosis. ABC transporters mediate drug efflux, thereby protecting cancer cells. ABCB1, ABCG2, UGT1A1, TP, MGMT, p16 and PI3KCG are examples of epigenetically regulated genes involved in each step of drug activity.

2.2. Epigenetic regulation of chemoresistance

In this section, we will briefly summarize some epigenetic mechanisms of chemoresistance and chemosensitivity. This will provide a molecular rationale for identifying epigenetic markers of response to chemotherapy in colorectal cancer patients.

As shown in Fig. 2, chemotherapy agents interact with many cellular targets. At each step, epigenetic gene silencing may enhance or hinder chemotherapy-induced cell death. First, many chemotherapy agents enter cancer cells as pro-drugs, which can be activated or transformed into harmless metabolites by specific enzymes. Some drug-metabolizing enzymes are regulated by epigenetic modifications. For example, capecitabine is an oral fluoropyrimidine pro-drug which is transformed into 5-FU by the thymidine phosphorylase (TP) enzyme (Malet-Martino et al., 2002). The *TYMP* gene, encoding for TP, is inactivated by DNA methylation in human mesothelioma cells, which are therefore resistant to capecitabine (Kosuri et al., 2010). This epigenetic inactivation is tumor-specific, since it is not found in normal pericardial tissue, and can be reversed by DNMTIs. On the other hand, some intracellular enzymes may inactivate chemotherapy agents. For example, UDP glucuronosyltransferase (UGT)-1A1 is the main enzyme for irinotecan detoxification (Kuhn, 1998). It is well known that genetic variants in the *UGT1A1* gene may affect both irinotecan toxicity and activity (Ramirez et al., 2010; Toffoli et al., 2010). Recently, it has been shown that *UGT1A1* expression is positively regulated by USF and HNF1-alpha transcription factors, and negatively regulated by DNA methylation, which hinders transcription factor binding (Belanger et al., 2010). Interestingly, these experiments have been performed on colorectal cancer cells, and may shed new light on personalized disease treatment.

Once the active drug penetrates the nucleus, the main mechanism of cancer cell killing is represented by DNA damage, which can be direct or mediated by a DNA-binding molecule (e.g., Topoisomerase I for camptothecins) (Engelmann and Pützer, 2010). Independently from molecular mechanisms, DNA damage activates several downstream pathways which may lead to DNA repair, apoptosis or cell-cycle arrest (Redmond et al., 2008). It is worth noting that several genes involved in these processes are epigenetically regulated in cancer cells (Van Engeland et al., 2011). For example, we showed that the cell cycle regulator p16, which is methylated in colorectal cancer, is a main mediator of irinotecan sensitivity (Crea et al., 2009). Cells harboring a methylated *p16* gene are more resistant to irinotecan-induced cell cycle arrest. DNMTIs reverse this resistant phenotype. Two apoptosis effectors, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and death-associated protein kinase (DAPK), are frequently methylated in gastrointestinal tumors, including colorectal cancer (Van Engeland et al., 2011). It has been shown that BNIP3 and DAPK methylation predicts lower response rates in gastric cancer patients treated with fluoropyrimidine-based chemotherapy (Sugita et al., 2011), probably due to increased resistance to chemotherapy-induced apoptosis.

Finally, two main mechanisms that counteract the chemotherapy activity are drug efflux and DNA repair. Both mechanisms are epigenetically regulated. ATP-binding cassette (ABC) transporters are a set of efflux membrane transporters involved in xenobiotic clearance. ABC transporters are also able to efflux some chemotherapy drugs, with partially overlapping substrate specificity (Sparreboom et al., 2003). Cancer cells may overexpress one or more ABC transporters, thereby displaying a multidrug resistance phenotype (Broxterman et al., 1996). Histone

deacetylation is a common mechanism of ABC transporter gene silencing. In this case, reversing the epigenetic mark by HDACIs may reactivate transporter expression, thereby causing chemoresistance (Huo et al., 2010).

The alkylating agent temozolomide is currently employed as first-line therapy for glioblastoma multiforme (Corsa et al., 2006). Temozolomide and related drugs cause cytotoxic DNA lesions such as O^6-methylguanine and N^7-methylguanine. O^6-methylguanine-DNA methyltransferase (MGMT) removes the O^6-methylguanine adduct and restores normal guanine (Kaina et al., 2001). The *MGMT* gene is inactivated by promoter methylation in approximately 30–50% glioblastoma multiforme samples (Dunn et al., 2009; Mellai et al., 2009). Thus, it is conceivable that MGMT silencing sensitizes cancer cells to temozolomide. *MGMT* methylation is associated with prolonged progression-free and overall survival in glioblastoma multiforme patients treated with temozolomide (Dunn et al., 2009).

In conclusion, DNA methylation and histone variants may affect cancer chemosensitivity through several mechanisms, some of which are still unexplored. In particular, histone post-translational modifications are an emerging area of research. Despite this, we already have a panel of putative epigenetic markers for colorectal cancer which will be discussed in the next section.

3. Colorectal cancer pharmaco-epigenetics

Knowledge of molecular mechanisms of resistance to chemotherapeutic agents in colorectal cancer has recently improved based on investigations both in experimental tumor models and in tumor explants from patients. The causes of colorectal cancer resistance to chemotherapy may comprise genetic as well as epigenetic mechanisms (Table 1).

3.1. 5-Fluorouracil

5-FU was developed more than 50 years ago (Heidelberger et al., 1957) and continues to be the backbone of treatment for patients with early or advanced colorectal cancer (Longley et al., 2003; NCCN Guidelines™ Colon Cancer, 2011). Resistance to 5-FU and the more recently developed oral fluoropyrimidines is, however, a major obstacle to successful therapy.

Studies undertaken to determine molecular mechanisms of intrinsic and acquired resistance to 5-FU have evidenced various alterations of drug target(s) and metabolism (Banerjee et al., 2002; Longley et al., 2003, 2006). Altered expression of apoptosis regulating genes also can lead to resistance to 5-FU (Wilson et al., 2009).

3.1.1. Thymidylate synthase expression as a mechanism of 5-FU resistance

Thymidylate synthase (TS), encoded by *TYMS*, is the main molecular target of 5-FU and related drugs and is the most widely studied biological marker of response to 5-FU chemotherapy. TS catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by using 5,10-methylene tetrahydrofolate as a cofactor. Thus, TS is the key enzyme in the *de novo* synthesis of thymidylate, an essential precursor for DNA replication and repair (Danenberg et al., 1974; Santi et al., 1974).

5-FU antitumor activity is mainly determined by inhibition of TS enzyme activity via the formation of a covalent ternary complex among the active 5-FU metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate, TS and the folate cofactor (Danenberg et al., 1974; Santi et al., 1974; Houghton et al., 1986). The stability of this complex is highly dependent on the different size and composition of cellular folate cofactor pools (Yin et al., 1983) while the formation of an unstable binary complex results in poor enzyme inhibition (Lockshin and Danenberg, 1981). The low availability of cofactor and its polyglutamates in tumors thus leads to intrinsic resistance to 5-FU (Houghton et al., 1981; Radparvar et al., 1989; Aschele et al., 1992). This has provided the rational basis for the use of combination treatment regimens of folinic acid and 5-FU for colorectal cancer (Mini et al., 1990).

Increased TS expression is widely accepted as a major molecular mechanism responsible for 5-FU resistance and has been suggested as a potential prognostic and predictive marker.

Johnston et al. (1994) first demonstrated a correlation between low TS levels and improved survival in rectal cancer patients receiving 5-FU adjuvant chemotherapy. A meta-analysis by Popat et al. (2004) showed that colorectal cancer patients with advanced disease treated with 5-FU had a significantly better overall survival if they had low TS expression in primary tumors or metastases.

In same cases, the increase in TS may be due to increased copy number. In *in vitro* human tumor models including colon cancer cell lines (H630) (Berger et al., 1985; Copur et al., 1995) and in one colon cancer patient (Clark et al., 1987), *TYMS* gene amplification associated with acquired resistance to 5-fluorodeoxyuridine or 5-FU has been reported. Thereafter, several reports have confirmed this finding (Wang et al., 2004; Yu et al., 2008; Watson et al., 2010).

Increased levels of TS as a result of translational upregulation have been shown to occur in tumor models following fluoropyrimidine treatment (Chu et al., 1996; Peters et al., 2002), based on an autoregulatory feedback pathway wherein the TS protein regulates its own translational efficiency (Chu et al., 1991). This may also contribute to the resistant phenotype, although the importance of this mechanism in cancer patients remains to be established.

Furthermore, *TS* transcription and translation are likely influenced by other genes, whose sequence (e.g., *p53*) (Nief et al., 2007) and expression (e.g., *E2F-1, AEG-1*) (Banerjee et al., 2000; Yoo et al., 2009) can be altered in tumor tissues. It has been suggested that *p53* status could play a role in *TS* expression in tumor cells, by altering transcription and/or translation levels (Nief et al., 2007). Increased expression of TS may also be a consequence of the overexpression of the transcription factor *E2F-1* and has been reported

Table 1
Examples of epigenetic modifications affecting chemosensitivity in colorectal cancer.

Gene	Epigenetic modification	Cellular effects	Effect on chemotherapy	Reference
ABC transporters	Histone deacetylation	ABC transporter silencing	Chemosensitivity	Sparreboom et al. (2003)
ASC	DNA methylation	ASC inactivation	Chemoresistance	Ohtsuka et al. (2006)
BNIP3, DAPK	DNA methylation	Reduced apoptosis	Chemoresistance	Sugita et al. (2011)
DPYD	DNA methylation	DPYD silencing	Capecitabine (5-FU) chemosensitivity	Noguchi et al. (2004)
DPYD	Histone deacetylation	DPYD silencing	Capecitabine (5-FU) chemosensitivity	Sato et al. (2006)
MGMT	DNA methylation	MGMT silencing	Temozolomide chemosensitivity	Dunn et al. (2009)
SPARC	DNA methylation	SPARC silencing	Chemoresistance	Cheetham et al. (2008)
TYMP	DNA methylation	TYMP silencing	Capecitabine (5-FU) chemoresistance	Kosuri et al. (2010)
TYMP	Histone acetylation	TYMS up-regulation	Capecitabine (5-FU) chemosensitivity	Glaser et al. (2003)
UGT1A1	DNA methylation	UGT1A1 silencing	Irinotecan chemosensitivity	Belanger et al. (2010)
UMPK	DNA methylation	UMPK silencing	Capecitabine (5-FU) chemoresistance	Humeniuk et al. (2009a,b)

Gene abbreviations are indicated in the text.

in pulmonary metastases from colorectal cancer patients (Banerjee et al., 2000). It has also been shown that astrocyte elevated gene-1 (*AEG-1*), known to augment invasion, metastasis and angiogenesis, directly contributes to 5-FU resistance, since it induces the expression of *LSF* (late SV40 factor), a transcription factor that regulates the expression of *TS* (Yoo et al., 2009).

Many studies have been conducted to investigate whether *TYMS* polymorphisms (Kaneda et al., 1987; Horie et al., 1995; Ulrich et al., 2000; Mandola et al., 2003; Lincz et al., 2007) might also explain differences in mRNA expression levels, but the results are heterogeneous and even controversial, especially in studies on samples from colorectal cancer patients (Pullarkat et al., 2001; Kawakami et al., 2001; Dotor et al., 2006; Fernández-Contreras et al., 2006; Gosens et al., 2008; Vignoli et al., 2011).

In addition, resistance to 5-FU has been associated with the production of altered structural forms of TS which has a reduced affinity for FdUMP in tumor cell culture models including human colon tumor cell line (HCT 116) as a consequence of *TYMS* gene mutations (Berger et al., 1988; Barbour et al., 1990; Tong et al., 1998; Landis and Loeb, 1998). However, no examples of mutant TS forms have been reported in clinical tumor samples (Sanguedolce et al., 2000; Calascibetta et al., 2010).

Based on the above evidence, a strategy for downregulating TS expression would be helpful in reversing 5-FU resistance. Since it has also been suggested that TS may function as an oncogene (Rahman et al., 2004; Bertino and Banerjee, 2004), biological aggressiveness of TS overexpressing cancer cells could also be controlled by such an approach. Targeting of proteins that act as TS expression regulators may affect the TS protein or mRNA expression level and consequently enhances 5-FU's cytotoxic effects.

HDACIs inhibit HDACs, leading to altered acetylation of histone and non-histone proteins. The *TYMS* gene has been demonstrated to be one of the most prominent genes to be down-regulated by HDACI treatment (Glaser et al., 2003). Lee et al. (2006) reported that the HDACI trichostatin A can reverse 5-FU resistance in human cancer cells, including colon cancer, by downregulating TS. Co-treatment with trichostatin A and cycloheximide (which inhibits the synthesis of new proteins), restored TS mRNA expression, suggesting that TS mRNA is repressed through induction of an unknown transcriptional repressor. Also, it was found that TS protein interacted with heat shock protein (Hsp) complex, and trichostatin A treatment induced chaperonic Hsp90 acetylation and subsequently enhanced Hsp70 binding to TS. This led to the proteasome degradation of TS protein.

Also other HDACIs (vorinostat – also named suberoylanilide hydroxamic acid, SAHA –, LBH589, MS275) have been shown to enhance the antiproliferative effects of 5-FU in *in vitro* and *in vivo* human colorectal cancer models and to aid in resensitizing 5-FU resistant cells (Tumber et al., 2007; Fazzone et al., 2009; Flis et al., 2010). Vorinostat and LBH589 caused a potent downregulation of *TYMS* gene expression through transcriptional repression both in colon cancer cell lines and in a mouse xenograft model (Tumber et al., 2007; Fazzone et al., 2009). This suggests that this effect may be achievable by these agents in tumors and that the combination of HIDACIs with 5-FU-based regimens represents a potential chemosensitization strategy to overcome TS-mediated resistance to 5-FU. It has also been shown that *p53* gene expression is upregulated by vorinostat in wild-type *p53* colorectal cancer cell lines and down-regulated in mutant *p53* cells, suggesting an additional mechanism of the antiproliferative synergistic interaction observed with 5-FU (Di Gennaro et al., 2009). MS275 also potentiated the 5-FU growth inhibitory effects on human colorectal cancer cell lines. Simultaneous exposure to 5-FU and MS275 influenced cell cycle progression and induced cell apoptosis (Flis et al., 2010).

HDACIs have also been shown to affect additional molecular pathways involved in colon cancer carcinogensis and growth. These include downregulation of Cyclin B1 in a p21WAF-1 and transcriptional dependent manner, suppression of Cox-activation and repression of Src family kinase members. It is therefore conceivable that molecular mechanisms of HDAC inhibition other than TS downregulation may be involved in their synergism with 5-FU (Tumber et al., 2007).

3.1.2. Pyrimidine metabolism enzyme expression

Other potential molecular determinants of 5-FU resistance have been studied (Kidd et al., 2005; Nobili et al., 2011). In particular, a high expression of dihydropyrimidine dehydrogenase (DPD), the main enzyme responsible for the catabolism of 5-FU, encoded by the *DPYD* gene (Salonga et al., 2000) and a low expression of enzymes responsible for 5-FU activation (TP) (Metzger et al., 1998; Salonga et al., 2000), orotate phosphorybosyltransferase (OPRT) (Koopman et al., 2009a), and uridine monophosphate kinase (UMPK) (Humeniuk et al., 2009a), have been associated with colorectal cancer resistance to 5-FU.

3.1.2.1. Dihydropyrimidine dehydrogenase. A lack of DPD expression correlates with severe 5-FU toxicity in patients. Low levels of DPD in colorectal tumors have also been shown to add to the predictive role of low TS and to correlate with response to 5-FU-based chemotherapy (Salonga et al., 2000). This correlation is true for mRNA levels while controversial results have been reported for protein levels (Westra et al., 2005; Soong et al., 2008).

Because various reports have demonstrated that DPD activity is closely correlated with mRNA levels, attention has been focused on the regulatory mechanisms of *DPYD* gene expression. Noguchi et al. (2004) subcloned an approximately 3.0 Kb fragment of the 5′ region of the *DPYD* that contained a total of 60 CpG sites, suggesting that methylation status may affect the repression of *DPYD*. They found that aberrant methylation of the *DPYD* promoter region positively affected sensitivity to 5-FU in cancer cells such as HSC3 and HepG2 through transcriptional repression of DPD expression, thus decreasing DPD-mediated 5-FU degradation. Demethylation by the DNMTI 5-aza-cytidine caused a remarkable decrease in sensitivity to 5-FU along with a concurrent increase in DPD expression in a dose- and time-dependent manner.

Sato et al. (2006) provided evidence that DPD mRNA expression and DPD activity were correlated with the IC_{50} for 5-FU in biliary tract cancer cell lines, indicating transcriptional regulation of DPD expression. They also examined the epigenetic gene silencing of *DPYD* using a DPD-deficient cell line among those tested, KMG-C, which exhibited the highest sensitivity to 5-FU. They demonstrated restoration of DPD expression by another DNMTI, 5-aza-2′deoxycitidine, treatment in a time- and dose-dependent manner, suggesting gene suppression by promoter hypermethylation. However, methylated CpG sites in the 5′ flanking region and intron 1 of the *DPYD* gene were not identified and the restored DPD expression level was more strongly induced by the HDACI trichostatin A than 5-aza-2′deoxycitidine treatment. These findings suggest that histone deacetylation may be implicated in DPD suppression more than hypermethylation. Thus, epigenetic gene silencing appears to be an important mechanism of DPD suppression in cancer and this finding may aid in the selection of 5-FU chemotherapy.

3.1.2.2. Thymidine phosphorylase. TP, also known as the angiogenic, platelet-derived endothelial cell growth factor, catalyzes the reversible phosphorolysis of thymidine to thymine and deoxyribose-1-phosphate using inorganic phosphate as a substrate (Friedkin and Roberts, 1954; Iltzsch et al., 1985). TP also degrades the powerful TS-directed agent 5-fluorodeoxyuridine to the less potent 5-FU (Birnie et al., 1963). Conversely, the reverse reaction could be used to convert 5-FU to 5-fluorodeoxyuridine in the

presence of deoxyribosyl-donating compounds (Santelli and Valeriote, 1980). Intracellular TP levels can thus affect sensitivity of cancer cells to fluoropyrimidines. A higher TP level has been correlated with tumor growth, invasion, and metastasis in clinical studies and is an indicator of worse prognosis in several tumors including colorectal cancer (Metzger et al., 1998). At odds with the preclinical data (Schwartz et al., 1995; Patterson et al., 1995), Metzger et al. (1998) found that in colorectal cancer patients, tumors with the highest basal levels of TP mRNA expression were unresponsive to 5-FU-based chemotherapy, whereas the response rate in tumors with lower TP mRNA expression levels was greater than the overall response rate. This inverse association was attributed to the role of TP as an angiogenic factor. A confirmatory study was later published (Salonga et al., 2000).

It has recently been shown that promoter methylation of CpG dinucleotides within the *TYMP* gene leads to transcriptional silencing of TP in one mesothelioma cell line (H290) as well as in mesothelioma samples from patients (Kosuri et al., 2010). Pretreatment with 5-aza-2′-deoxycytidine of the H290 cell line led to increased TP mRNA and TP protein expression, and enhanced cytotoxic effects to capecitabine (Kosuri et al., 2010). This epigenetic mechanism may explain resistance to capecitabine in mesothelioma.

3.1.2.3. Orotate phosphoribosyltransferase. In mammalian cells, the last step of pyrimidine nucleotide synthesis involves the conversion of orotate to uridine monophosphate (UMP) that is catalyzed by OPRT (McClard et al., 1980). OPRT is also responsible for the conversion of 5-FU to the active metabolite fluorouridine monophosphate (FUMP). A high OPRT expression could therefore be a predictive factor for response to 5FU-based chemotherapy.

A decreased level of OPRT has been reported to cause resistance to 5-FU in experimental tumor models (Mulkins and Heidelberger, 1982). Controversial results have been reported so far in colorectal cancer from patients resistant to 5-FU (Tokunaga et al., 2007; Yanagisawa et al., 2007; Koopman et al., 2009b).

Epigenetic mechanisms of OPRT regulation are at present unknown. However, the presence of 31 CpG dinucleotides within the island that includes the ATG regulatory region of exon 1 might suggest possible epigenetic mechanisms of *OPRT* transcriptional modulation.

3.1.2.4. Uridine monophosphate/cytidine monophosphate kinase. UMP/cytidine monophosphate (CMP) kinase (UMPK) catalyzes the phosphoryl transfer from ATP to UMP, CMP and deoxy-CMP in the presence of magnesium, resulting in the formation of ADP and the corresponding nucleoside diphosphate (Hsu et al., 2005). This enzyme is crucial for the *de novo* and salvage synthesis of pyrimidine nucleotides required for cellular nucleic acid synthesis (van Rompay et al., 2000). Besides its physiological function, UMPK plays a very relevant role in the activation of 5-FU to 5-FUTP and its incorporation into RNA by converting FUMP into FUDP (Pasti et al., 2003).

Humeniuk et al. (2009a) showed both in the human 5-FU-resistant colorectal cancer cell line HCT-8 and in colorectal cancer patients treated with 5-FU, decreased expression of UMPK mRNA compared with the sensitive HCT-8 cell line and samples of patients not previously exposed to 5-FU, respectively.

Interestingly, exposure of 5-FU-resistant HCT-8 colon cancer cells characterized by decreased levels of UMPK to low dose 5-aza-2′deoxycytidine restored sensitivity to 5-FU. Moreover, the treatment of nude mice bearing a 5-FU-resistant HCT-8 cancer xenograft with 5-aza-2′deoxycytidine overcame resistance to bolus 5-FU. 5-aza-2′deoxycytidine-mediated restoration of 5-FU sensitivity was associated with increases in UMPK protein and mRNA levels both in cultured 5-FU-resistant HCT-8 cells and in mice bearing this tumor.

Sequencing and expression of the *UMPK* promoter region revealed no functional changes between the 5-FU-resistant and the parental cell line. Evaluation of 42 CpG sites within the island surrounding the putative transcription start site showed enrichment in DNA methylation across this region in 5-FU-resistant HCT-8 cells that reversed following 5-aza-2′deoxycytidine treatment. Due to the low percentage of *UMPK* methylation observed, it was not clear whether DNA methylation was the only factor responsible for lower expression of UMPK. Alternative transcriptional silencing pathways may exist.

In keeping with these observations, we have studied whether treatment with the DNA hypermethylation inhibitor 5-aza-2′deoxycytidine, might reverse acquired resistance to 5-FU in other models of HCT-8 colon carcinoma cell lines. 5-FU-resistant HCT-8 colon carcinoma cell lines developed in our laboratory were selected by both long-term and short-term drug exposure schedules (Tempestini et al., 2006). Both these *in vitro* treatment modes were similar but not identical to those described by Humeniuk et al. (2009a) or Aschele et al. (1992) and Pizzorno and Handschumacher (1995). Enhanced cell growth inhibitory effects were observed following pretreatment with long-term, low dose 5-aza-2′deoxycytidine, especially in the HCT-8/FUB/2R cell line selected by short-term (4 h) exposure to high dose 5-FU (2000 μM). Basal mRNA expression levels of genes involved in 5-FU anabolism was lower in the 5-FU bolus-resistant HCT-8/FUB/2R cell line as compared to sensitive parental cells. For some of these genes (e.g., thymidine kinase 1 and ribonucleotide reductase M1 and M2 polypeptides), increased expression levels were induced by 5-aza-2′deoxycytidine treatment and consequent partial restoration of 5-FU sensitivity was observed (unpublished data). We are currently investigating methylation patterns of these anabolic enzymes.

3.1.3. Other determinants

SPARC (osteonectin) is a matricellular protein to which several important biological functions have been attributed such as wound repair, cell migration and differentiation. There is also growing evidence for its role in malignancy as its expression level is variable and linked to cancer progression in a number of tumors (Tai et al., 2005).

Low levels of SPARC expression in colorectal cancers was correlated with decreased sensitivity to chemotherapy. Upregulation of SPARC expression or exogenous exposure to high levels of SPARC restored sensitivity to chemotherapy, including 5-FU and irinotecan either *in vitro* or *in vivo* (Tai et al., 2005; Tai and Tang, 2008). Cheetham et al. (2008) examined whether aberrant hypermethylation of the *SPARC* promoter was a potential mechanism for repressing SPARC in colorectal cancer and whether restoration of its expression with 5-aza-deoxycytidine could enhance chemosensitivity. They showed global hypermethylation of the *SPARC* promoter in colorectal cancers and identified specific CpG sites that were consistently methylated in colorectal cancers but not in the normal colon. Also SPARC repression in colorectal cancer cell lines could be reversed following exposure to 5-aza-deoxycytidine which resulted in increased SPARC expression leading to a significant reduction in cell viability and greater apoptosis when combined with 5-FU *in vitro* in comparison to 5-FU alone.

The tumor suppressor gene *p53* plays a crucial role in carcinogenesis and is also frequently mutated in the majority of malignant diseases, including colorectal cancer (Hollstein et al., 1991; Levine et al., 1994; Soussi et al., 2006). Its inactivation (Bunz et al., 1999; Lowe et al., 2004; McDermott et al., 2005; Adamsen et al., 2007) as well as that of several target genes (e.g., *bax*), (Zhang et al., 2000; Yu et al., 2003) is related with resistance to chemotherapeutic agents including 5-FU in colorectal cancer.

One of p53-target gene is apoptosis-associated speckle-like protein (*ASC*). It regulates p53-Bax mitochondrial apoptotic

pathway and it is also known to be a target of methylation-induced gene silencing. Inactivation of ASC may thus cause resistance to chemotherapy, and if this is the case, then the expression of ASC would restore chemosensitivity status. Ohtsuka et al. (2006) showed that *ASC* was methylated in 25% of specimens from colorectal cancer patients. They also investigated the role of ASC in p53 dependent-sensitivity to 5-FU. When expressed following exposure to 5-aza-2′deoxycytidine in colon cancer cells, in which ASC is absent due to methylation, ASC was found to enhance the sensitivity to 5-FU in a p53-dependent manner. In *p53* null cells, ASC increased p53-mediated cell death induced by p53 expressing adenovirus infection. Methylation-induced silencing of ASC might cause resistance to p53-mediated sensitivity to 5-FU in colorectal cancer. The role of p53 status and of that of p53 target genes as a marker of therapeutic activity in colorectal cancer requires additional investigation.

In the Colo-205 cell line, exposure to 5-aza-2′deoxycytidine combined with 5-FU increased mRNA levels of a series of apoptotic genes such as *p53, CCNE1*, *ATM*, and *CASP3* compared to 5-FU alone. In addition, change in the levels of genes specific for cell cycle progression such as increased levels of cyclin A1 and decreased levels of cyclin D1 and p21 were observed after exposure to combined 5-aza-2′deoxycytidine and 5-FU as compared with 5-FU alone (Flis et al., 2009a). The observed synergistic cell growth inhibitory effects of this combination were thus attributed to augmentation of apoptotic signaling when compared with 5-FU alone.

Loss of DNA mismatch repair (MMR) gene *MLH1* due to promoter methylation occurs in approximately 10–15% of colorectal cancer cases, provoking a characteristic molecular phenotype called MSI (Boland and Goel, 2010). It has been associated with resistance to chemotherapeutic agents, including 5-FU (Plumb et al., 2000; Arnold et al., 2003; Fujita et al., 2007). Arnold et al. (2003) showed that reactivation of *hMLH1* gene expression using 5-aza-2′deoxycytidine, reverses 5-FU resistance in colorectal cancer cell lines. Similar data have been obtained in human ovarian and colon tumor xenografts resistant to a number of clinically important anticancer drugs (Plumb et al., 2000).

Also it is now believed that as many as one-third to one-half of all colorectal cancers may be classified as having the CIMP phenotype (Jover et al., 2011). CIMP tumors with methylation-induced silencing of *MLH1* constitute the majority of sporadic MSI colorectal cancers (Kane et al., 1997). However, most CIMP positive tumors are associated with microsatellite stability (Weisenberger et al., 2006). These CIMP microsatellite stabile tumors share certain clinical and pathological features with MSI-colorectal cancers (Jass, 2007). Because a significant majority of CIMP tumors and sporadic MSI cancers share common characteristics, it would be suspected that they would have similar therapeutic responses. Recent results in a large population of colorectal cancer patients undergoing adjuvant 5-FU chemotherapy, suggest that CIMP-positive colorectal cancers (Jover et al., 2011), similarly to MSI tumors (Sargent et al., 2010), do not obtain a significant benefit from 5-FU-based adjuvant chemotherapy. A similar association between the CIMP phenotype and very poor prognosis has been reported in advanced colorectal cancer patients treated with 5-FU chemotherapy (Shen et al., 2007). These findings may have relevant implications for future selection of therapy in such patients.

Microarray analysis has identified a number of genes that are differentially expressed between CIMP-positive and CIMP-negative tumors (Ferracin et al., 2008). The metabolic activity responsible for converting 5-FU into its active metabolites may be different between these groups of tumors, resulting in differences in chemosensitivity. The apparent lack of response to 5-FU seems to be related to hypermethylation and not to the MMR status of the tumors, an important topic that should be addressed in future research.

3.2. Irinotecan

Irinotecan is a topoisomerase I-targeting camptothecin. Human carboxylesterases convert irinotecan into the active metabolite (SN38), which irreversibly binds topoisomerase I enzyme to DNA, thereby triggering genomic damage and apoptosis (Crea et al., 2009; Ramesh et al., 2010). Irinotecan is currently employed in combination with fluorouracil-leucovorin for the treatment of stage III and IV colorectal cancer. The FOLFIRI regimen plus the EGFR inhibitor cetuximab is an attractive therapeutic option for KRAS wild-type metastatic colorectal cancer patients (Van Cutsem et al., 2011). However, the FOLFOX regimen is generally the first choice, since it is associated with slightly longer survival (Nelson et al., 2011).

Irinotecan pharmacogenomics are mainly based on the UGT1A1 genetic profile (see paragraph 1.2). Both cancer cells and normal liver cells are able to inactivate SN38 through UGT1A1 (Iyer et al., 1998). In normal cells, SN38 induces severe side-effects such as diarrhea and neutropenia (Marsh and Hoskins, 2010). Thus, irinotecan could be particularly effective for patients expressing low levels of UGT1A1 in cancer cells, and high levels of the same enzyme in normal cells. Along with genetic variations, UGT1A1 mRNA is silenced in approximately 82% of primary colorectal cancer specimens (Gagnon et al., 2006). *In vitro* data indicate that UGT1A1 silencing occurs by DNA methylation, and that treatment with DNMTIs restores protein expression and enhances SN38 inactivation. Based on this evidence, we think that a combined genetic-epigenetic approach may improve therapy tailoring for patients to be treated with irinotecan. Especially patients bearing an active UGT1A1 gene in normal cells and patients with a silenced UGT1A1 gene in the primary tumor may derive particular benefit from the FOLFIRI regimen. Thus, germinal polymorphisms and cancer-specific DNA methylation should be tested to predict UGT1A1 status and irinotecan activity in colorectal cancer patients.

In addition to UGT1A1, emerging evidence indicates that other epigenetic factors may affect irinotecan sensitivity. For example, ABCB1, ABCC1 and ABCC2 polymorphic variants affect SN38 and irinotecan pharmacokinetic profile (Innocenti et al., 2009). Despite the evidence showing that histone deacetylation is a major player of ABC transporter gene silencing (Hauswald et al., 2009), we were unable to find studies on epigenetic profiling of ABC loci in colorectal cancer patients. We think that such studies would provide significant insight into the clinical mechanisms of irinotecan resistance. Another promising area of research is the relationship between MSI phenotype and irinotecan sensitivity. Approximately 15% of colorectal cancer display MSI, due to mismatch repair gene mutation or epigenetic silencing (Vilar and Gruber, 2010). As already shown, MSI predicts poor response to 5-FU in colorectal cancer patients (Vilar and Gruber, 2010). A clinical study tested the effects of 5-FU-leucovorin (FU/LV) or FU/LV plus irinotecan (IFL) regimens in stage III colorectal cancer patients, based on MSI phenotype (Bertagnolli et al., 2009). The authors found that MSI patients treated with the IFL regimen had a significantly longer disease-free survival, compared to microsatellite stable patients. The MSI phenotype did not predict response to the FU/LV regimen. Thus, it is conceivable that MSI status may specifically predict irinotecan sensitivity in colorectal cancer patients. Although these data are promising and in agreement with previous *in vitro* observations, they must be confirmed by independent studies. It has been shown that MSI colorectal cancer cells are more sensitive to SN38-induced apoptosis due to defective DNA repair processes (Vilar et al., 2008).

3.3. Oxaliplatin

Oxaliplatin is a diaminocyclohexane derivative of cisplatin (Kidani et al., 1978), characterized by higher water solubility, fewer

toxic side-effects, and lack of cross-resistance with cisplatin (Mathe et al., 1989; Di Francesco et al., 2002). Differently from cisplatin and carboplatin, this third-generation platinum compound showed *in vitro* and *in vivo* antitumor activity in colorectal cancer (Raymond et al., 2002). The combination of oxaliplatin with 5-FU and/or irinotecan has proven clinical efficacy with a good safety profile (Misset et al., 2000; Falcone et al., 2007).

Oxaliplatin forms DNA adducts by which it exerts its anti-tumor effects (Kweekel et al., 2005; Raymond et al., 2002). Primary DNA-Pt lesions induce apoptosis and this event is possibly enhanced by a contribution of targets other than DNA (Faivre et al., 2003).

The intracellular concentration of oxaliplatin is determined by its cellular uptake and efflux. Inside the cell oxaliplatin undergoes complex biotransformations. The oxaliplatin prodrug is activated by the conversion to monochloro-, dichloro- and diaquo-compounds by non-enzymatic hydrolysis and displacement of the oxalate group, which leads to the formation of DNA adducts. Cellular defence mechanisms prevent adduct formation (e.g., glutathione-S-transferase) or remove DNA adducts (e.g., nucleotide excision repair—NER, base excision repair, and replicative bypass repair (Kweekel et al., 2005)). Several different ways for a cell to become resistant to platinum compounds have been proposed: a decrease in cellular uptake, an increase in cellular efflux, quenching of the DNA monoadducts through reaction with glutathione or other metallothioneins (e.g., L-methionine, L-cysteine), increase in the NER pathway (Di Francesco et al., 2002; Viguier et al., 2005). Influx transporters like organic cation transporters (OCT) 1, 2 and 3 (SLC22A1, SLC22A2 and SLC22A3) (Zhang et al., 2006; Yonezawa et al., 2006; Yokoo et al., 2008; Burger et al., 2010, 2011) and efflux transporters such as copper efflux transporters, P-type ATPases, ATP7A and ATP7B (Safaei and Howell, 2005; Holzer et al., 2006; Martinez-Balibrea et al., 2009; Howell et al., 2010) may also play an important role in determining tumor sensitivity.

There is little information on potential epigenetic mechanisms involved in resistance to platinum compounds. An *in vitro* study identified a series of hyper-methylated genes whose inactivation contributed to cisplatin resistance (e.g., SAT, C8orf4, LAMB3, TUBB, G0S2, MCAM) and that were reactivated by the use of 5-aza-2′-deoxycytidine (Chang et al., 2010).

A few *in vitro* studies report on the pharmacodynamic effects of combining epigenetic drugs with oxaliplatin in colorectal cancer (Flis et al., 2009a,b; Na et al., 2010). These studies concern the possible synergism between HDACIs (Flis et al., 2009b; Na et al., 2010) or DNMTIs (Flis et al., 2009a) and oxaliplatin but do not provide data on reversal of oxaliplatin resistance by these drugs.

Flis et al. (2009b) showed that MS275, a synthetic benzamid, and to a lesser extent, suberic bishydroxamic acid, a hydroxamic acid derivative inhibited in a dose-dependent manner the growth of SW48, HT-29, and Colo-205 human colorectal cancer cell lines and displayed potent synergism when combined with oxaliplatin. Both HDACIs combined with oxaliplatin intensified perturbations in the cell cycle progression and induced caspase-dependent apoptosis. The two HDACIs, either alone or in combination with oxaliplatin also induced the disruption of mitochondrial membrane depolarization. In addition to their multiple points of action resulting from histone and non-histone protein acetylation, HDACIs generate genotoxic reactive oxygen species (Eot-Houllier et al., 2009) similarly to oxaliplatin (Laurent et al., 2005). These effects may contribute, at least in part, to the observed drug synergism by augmentation of apoptotic signals as also reported by others (Ruefli et al., 2001; Carew et al., 2008).

The combination of CG2, a new HDACI, with oxaliplatin was also more effective than the agents alone in inhibiting the growth of HCT116 colon cancer cells. This synergism may rely on CG2 effects on proapoptotic protein expression (Na et al., 2010).

Flis et al. (2009a) investigated the effects of combining 5-aza-2′-deoxycytidine or another cytidine analog DNMTI, the 2-pyrimidone ribonucleoside zebularine, to oxaliplatin on Colo-205 colon cancer cells. While 5-aza-2′-deoxycytidine was highly effective in potentiating the cell growth inhibitory effects of oxaliplatin, zebularine did not show substantial synergism. Similarly to HDACIs inhibitors, the concomitant use of 5-aza-2′-deoxycytidine with oxaliplatin affected cell cycle and apoptotic proteins (e.g., increased mRNA level of *ATM*, increased protein level of cyclin A1, decreased protein level of cyclin D1, increased protein levels of specific caspases). Observed changes in mitochondrial membrane potential induced by this drug combination suggest a possible involvement of the mitochondrial pathway in apoptosis induction also for DNMTIs.

None of the above reported studies evaluated specific epigenetic changes such as DNA methylation or histone acetylation, so we do not know whether these changes occurred and whether the observed oxaliplatin potentiating cell growth inhibition activity may be, at least in part, due to them. One example is the different cytotoxic activity exerted by 5-aza-2′-deoxycytidine and zebularine and their different potentiating effects on oxaliplatin cell growth inhibitory effects which may rely on cytotoxic proprties rather than epigenetic properties (Flis et al., 2009a).

4. Therapeutic opportunities

Epigenetic alterations contribute significantly to the development and progression of cancer through inactivation of many growth-regulatory genes (Humeniuk et al., 2009b). A substantial number of genes with promoter hypermethylation has been identified in colorectal cancer (Deng et al., 2001, 2004; Aguilera et al., 2006; Liang et al., 1999; Jacinto et al., 2007). Increasing evidence supports the hypothesis that epigenetic changes may be a driving force behind the acquisition of drug resistance (Glasspool et al., 2006; Humeniuk et al., 2009b; Baylin, 2011). Such observations have been reported in many solid tumors, including colorectal cancer (Arnold et al., 2003).

Two classes of chemical compounds, inhibitors of epigenetic enzymes, DNMTIs and HDACIs, have undergone major preclinical investigation and clinical development to tackle mechanisms of tumor progression and resistance (Tables 2 and 3).

Nucleoside DNMTIs comprise 5-aza-cytidine, 5-aza-2′-deoxycytidine, 5-fluoro-2′-deoxycytidine and zebularine. Non-nucleoside DNMTIs comprise small molecule inhibitors such as RG108. A third class of DNMTIs are oligonucleotides such as MG98.

Nucleoside DNMTIs are incorporated into DNA and act by preventing the resolution of a covalent reaction intermediate which leads to DNMT being trapped and inactivated in the form of a covalent protein-DNA adduct. Thus, cellular DNMT is rapidly depleted and concomitantly genomic DNA is demethylated as a result of continued DNA replication (Lyko and Brown, 2005). Non-nucleoside DNMTIs directly block DNMT activity by binding to the catalytic region of DNMTs. MG98 is a specific oligonucleotide compound able to suppress DNMT expression by antisense mechanisms currently being investigated in clinical trials.

Seven classes of HDACIs have been so far developed. Four of them are currently investigated in the clinic: short-chain fatty acids, cyclic peptides, hydroxamic acids and benzamides. Despite their structural diversity, they all act by inhibiting HDACs identified in humans. The inhibition of these enzymes leads to the accumulation of acetylation in histones. This event will be then followed by changes in cellular processes that are defective in cancer.

Epigenetic therapies have shown relevant activity in the treatment of hematological malignancies leading to the approval of four drugs (i.e. 5-aza-cytidine, 5-aza-2′deoxycytidine, vorinostat,

Table 2
Status of development of DNMTIs in colorectal cancer (CRC).

Class	Drug	Development phase	Combined drugs	Outcome	Authors
Nucleoside DNMTIs	5-Aza-cytidine (azacitidine)	Clinical, phase I and II in solid tumors including CRC	None	Little clinical activity in solid tumors (occasional responses were observed including CRC and were associated with significant toxicity). In these early trials methylation status and gene expression were not reported.	Lomen et al. (1975), Shnider et al. (1976), Moertel et al. (1972), Weiss et al. (1977), Quagliana et al. (1977)
		Clinical, phase I in solid tumors including CRC	Valproic acid	5-aza-cytidine was administered s.c. daily for 10 days q 28. Significant decrease in global DNA methylation and induction of histone acetylation were observed in peripheral blood mononuclear cells. Disease stabilization were observed in 25% of patients. Treatment was safe at doses up to 75 mg/m2 for 5-aza-cytidine.	Braiteh et al. (2008)
		Clinical, phase I in solid tumors including CRC	Sodium phenylbutyrate	No conclusive statement can be made on targeted DNA methyltransferase activity and histone acetylation changes in tumors and peripheral blood mononuclear cells. The combination was well tolerated and safe, yet lacked any real evidence for clinical benefit.	Lin et al. (2009)
	5-Aza-2′-deoxycytidine (decitabine)	Clinical, phase I in solid tumors including one CRC	None	Changes in methylation but no objective responses	Aparicio et al. (2003)
		Clinical, phase I in solid tumors including CRC	Carboplatin	Induction of dose-dependent, reversible demethylation in peripheral-blood cells; one objective response and disease stabilizations observed but not in CRC	Appleton et al. (2007)
		Clinical, phase I in solid tumors including CRC	Cisplatin	One partial response and disease stabilizations observed in tumors other than CRC	Schwartsmann et al. (2000)
		Clinical, phase II in solid tumors including CRC	None	One single partial response observed in malignant melanoma	Abele et al. (1987)
	5-Fluoro-2′- deoxycytidine	Clinical, phase I in solid tumors including CRC	Tetrahydrouridine	Disease stabilization was observed in 35% of patients	Newman et al. (2002)
	Zebularine (1-(beta-D-ribofuranosyl)1,2-dihydropyrimidin-2-one)	Preclinical, *in vitro* study in CRC cell lines	Oxaliplatin	Lack of synergistic interactions	Flis et al. (2009a)
Non-Nucleoside DNMTIs	RG108 (2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1*H*-indol-3-yl)propanoic acid	Preclinical, *in vitro* study including one CRC cell line	None	Demethylation and reactivation of tumor suppressor genes at low micromolar concentrations without detectable toxicity	Brueckner et al. (2005)
Antisense oligonucleotides	MG98	Clinical, phase I in solid tumors including CRC	None	Suppression of DNMT1 expression was observed in most patients. Treatment (7-day continuous i.v. infusion every 14 days) was well tolerated with evidence of clinical activity (one single partial response and one disease stabilization in tumors other than CRC)	Plummer et al. (2009)
		Clinical, phase I in solid tumors including CRC	None	No evidence of antitumor activity. Treatment (21-day continuous i.v. infusion every 4 weeks) was poorly tolerated in the highest doses	Davis et al. (2003)
		Clinical, phase I in solid tumors including CRC	None	No consistent changes in DNMT1 expression were observed in peripheral blood mononuclear cells. No objective response but disease stabilization was observed (2 h infusion twice a week for 3 weeks out of every 4). Reversible transaminase elevation and fatigue were the dose-limiting toxicities.	Stewart et al. (2003)

Table 3
Status of development of HDACIs in colorectal cancer (CRC).

Class	Drug	Development phase	Combined drugs	Outcome	References
Aliphatic acids	Valproic acid	Clinical, phase I in solid tumors including CRC	None	Histone hyperacetylation and downmodulation of HDAC2 were observed in peripheral blood lymphocytes. No objective response was observed. Two patients, including one with CRC, had stable disease.	Atmaca et al. (2007)
		Clinical, phase I in solid tumors including CRC	Epirubicin	Total and free valproic acid plasma concentrations increased linearly with dose and correlated with histone deacetylation in peripheral blood mononuclear cells. Partial responses were seen in 22% of patients and stable disease/minor responses in 39% of patients across different tumor types. Antitumor activity was observed in anthracycline-resistant tumors	Münster et al. (2007)
	Sodium phenylbutyrate	Clinical, phase I in solid tumors including CRC	None	No objective responses were observed, but 25% of patients had disease stabilization. Phenylbutyrate was well tolerated and achieved the concentration *in vivo* that has been shown to have biological activity *in vitro*.	Gilbert et al. (2001)
		Clinical, phase I in solid tumors including CRC	None	The therapy was well tolerated. Some disease stabilizations were observed in tumors other than CRC.	Camacho et al. (2007)
		Clinical, phase I in CRC	5-FU	Weekly infusions of 5-FU followed by phenyl butyrate were fairly well tolerated with disease stabilization in 75% of patients	Sung and Waxman (2007)
	Pivaloyloxymethyl butyrate (AN-9)	Clinical, phase I in solid tumors including CRC	None	No consistent increase in fetal hemoglobin (differentiating effect) with AN-9 treatment was observed. Mild to moderate toxicities were observed. One single partial response was observed but disease stabilizations seen in 21% of patients	Patnaik et al. (2002)
	Vorinostat (suberylanilide hydroxamic acid, SAHA)	Clinical, phase I in solid tumors including CRC	Doxorubicin	Histone hyperacetylation changes in peripheral blood mononuclear and tumor cells were comparable. Histone hyperacetylation seemed to correlate with pre-treatment HDAC2 expression. Partial response and disease stabilizations were observed in tumors other than CRC.	Munster et al. (2009)
		Clinical, phase I in solid tumors including CRC	5-FU	Evidence of TS downregulation in tumor biopsies was lacking. One partial response and disease stabilizations in 55% of CRC patients were seen.	Fakih et al. (2010)
		Clinical, phase I in CRC	5-FU and oxaliplatin	Vorinostat-induced TS dowenregulation was not consistent. 52% of patients had stable disease, but no patients developed objective response.	Fakih et al. (2009)
		Clinical, phase I-II in CRC	5-FU	Acetylation of histone 3 was observed in peripheral blood mononuclear cells following vorinostat treatment. Consistent reduction in intratumoral TS expression was not observed. Two out 10 patients showed disease stabilizations.	Wilson et al. (2010)
		Clinical, early phase II in solid tumors including CRC	None	Vorinostat in a daily oral schedule for 14 days q 3 weeks was tolerable at 200 mg bid only, and no responses were observed. Disease stabilizations were observed in 50% of patients, including one with CRC	Vansteenkiste et al. (2008)
Cyclic peptides	Romidepsin (Depsipeptide FK-228)	Clinical, phase II in CRC	None	No objective responses were observed. Four patients (25%) had stable disease.	Whitehead et al. (2009)

Table 3 (*Continued*)

Class	Drug	Development phase	Combined drugs	Outcome	References
Hydroxamic Acids	Belinostat ((E)-N-hydroxy-3-[3-(phenylsulfamoyl)phenyl] prop-2-enamide, PXD101)	Clinical, phase I in solid tumors including CRC	None	The safety profile was favorable. 41% of patients had stable disease, including one with rectal cancer	Kelly et al. (2009)
		Clinical, phase I in solid tumors including CRC	None	I.v. belinostat exhibited dose-dependent pharmacodynamic effects (e.g., histone acetylation) in peripheral blood mononuclear cells, was well tolerated and obtained stable disease in 50% of patients other than CRC patients at maximum tolerated dose.	Steele et al. (2008)
		Clinical, phase I in solid tumors including CRC	5-FU	Tissue expression in tumor tissue was downregulated in 4 of 4 evaluable patients. Disease stabilizations were observed in 26% of patients.	Northfelt et al. (2009)
	Panobinostat ((E)-N-hydroxy-3-[4-[[2-(2-methyl-1H-indol-3-yl) ethylamino]methyl]phenyl] prop-2-enamide, LBH589)	Clinical, phase I in solid tumors including CRC	Epirubicin	Disease stabilizations were observed in tumors other than CRC	Munster et al. (2010)
	Dacinostat (((E)-N-hydroxy-3-[4-[[2-hydroxyethyl-[2-(1H-indol-3-yl)ethyl]amino]methyl]phenyl]prop-2-enamide, LAQ-824)	Clinical, phase I in solid tumors including CRC	None	Consistent accumulation of acetylated histones post-therapy was observed in peripheral blood mononuclear cell; high doses resulted in increased and longer duration of pharmacodynamic effect. Changes in HSP90 client protein and HSP72 levels consistent with HSP90 inhibition were observed at high doses. No objective response was documented; disease stabilization was observed in 16% of patients.	De Bono et al. (2008)
Benzamides	Entinostat (3-pyridylmethyl N-{4-[(2-aminophenyl) carbamoyl]benzyl}carbamate, MS 275, SNDX 275)	Clinical, phase I in solid tumors including CRC	None	Levels of histone H3 and H4 acetylation in peripheral blood mononuclear cells increased qualitatively but with a high degree of interpatient variation. Two partial response were observed in tumors other than CRC. Disease stabilizations observed in 22% of patients, including one rectal and one colon cancer	Gore et al. (2008)
	Mocetinostat (N-(2-Amino-phenyl)-4-[(4-pyridin-3-yl-pyrimidin-2-ylamino)-methyl] benzamide dihydrobromide, MGCD0103)	Clinical, phase I in solid tumors including CRC	None	Inhibition of HDAC activity and induction of acetylation of H3 histones in peripheral white blood cells were observed. Disease stabilization was obtained in 16% of patients	Siu et al. (2008)

romidepsin). Minor efficacy has been reported in solid tumors, despite the high number of clinical trials performed in the last 25 years.

It is today believed that the reason for the poor success in solid tumors was mainly due to the use of high doses and short term administration of epigenetic drugs. In fact, DNMTIs exert their epigenetic clinical activity in myelodysplastic syndromes when administered at low doses, for several successive days and for multiple cycles (Issa, 2005; Oki et al., 2007). These conditions allow the survival of the cells but determine changes in their gene expression profile, thus favoring cell differentiation, decrease in cell proliferation and increased apoptosis (Jones and Taylor, 1980). Instead, at high doses DNMTIs induce cytotoxic effects. Under these circumstances, the inhibition of cell growth is due to the arrest of DNA synthesis by antimetabolic activity rather than to epigenetic effects.

Most of the trials performed with epigenetic drugs as single-agents in solid tumors were aimed at investigating the maximum tolerated dose (MTD) in previously treated patients with advanced disease rather than investigating their epigenetic effects. Results of such trials usually showed high grade toxicity associated with low grade or lack of anticancer activity both for DNMTIs (van Groeningen et al., 1986; Abele et al., 1987; Newman et al., 2002) and HDACIs (Vansteenkiste et al., 2008).

On the other hand, it has been shown that, the administration of low doses of DNMTIs may lead to the reactivation of methylated tumor suppressor genes also in solid tumors (Schrump et al., 2006). This effect is however transient and may be reversed when the administration of the drug(s) is suspended. This may hamper the use of these drugs as single agents in solid tumors. Similar observations have been reported for HDACIs (Wu et al., 2001; Kelly et al., 2005; Prince et al., 2009). Although activity of vorinostat has been demonstrated at 200 mg twice daily for 14 days followed by a 7-day rest in hematologic malignancies, no responses were observed in relapsed or refractory colorectal cancer using the same treatment schedule (Vansteenkiste et al., 2008). The number of patients treated in this study was small and thus no general conclusions are possible regarding efficacy for this tumor type. Treatment duration in this study was, however, shorter than that of the standard treatment schedule in cutaneous T cell lymphoma (400 mg daily, continuously). It should be noted that romidepsin at a dose of 13 mg/m^2 as a 4-h i.v. infusion on days 1, 8 and 15 of a 28-day cycle, i.e. similar to that administered as treatment of T cell lymphomas, was ineffective in the treatment of previously treated colorectal cancer patients with advanced disease (Whitehead et al., 2009). Since romidepsin has shown antiproliferative activity against several human solid tumor xenograft models, lack of single agent romidepsin clinical activity in colorectal cancer patients with advanced previously-treated disease may have occurred due to cellular factors (comprising intrinsic resistance to romidepsin) independently of the type of treatment schedule.

Epigenetic drugs may also play a role as potential reversal agents of tumor drug resistance due to epigenetic mechanisms in combination with cytotoxic drugs.

It has been shown that 5-aza-2′deoxycytidine resensitized *in vivo* drug-resistant colon SW-48 tumor xenografts that are hMLH1 negative because of gene promoter hypermethylation. Pretreatment of tumor-bearing mice with 5-aza-2′deoxycytidine at a non toxic dose, induced reexpression of hMLH1 associated with a decrease in *hMLH1* gene promoter methylation (Plumb et al., 2000). 5-aza-2′deoxycytidine alone had no effect on the growth rate of the tumors but greatly sensitized the xenografts to cytotoxic drugs (cisplatin, carboplatin, temozolomide) administered 6 days thereafter (Plumb et al., 2000). In keeping with these observations, Appleton et al. (2007) designed a phase I clinical and pharmacodynamic trial of 5-aza-2′-deoxycytidine in combination with carboplatin to determine the feasibility of administering 5-aza-2′-deoxycytidine at an equivalent dose and schedule in advanced solid tumors including colorectal cancer. This dose-finding trial used a series of doses/cycle of 5-aza-2′-deoxycytidine falling in the low dose range able to induce hypomethylation in preclinical studies. Two separate dose escalation of 5-aza-2′-deoxycytidine were conducted, the first with carboplatin fixed at area under the concentration-time curve (AUC) 5 and the second at AUC 6. The recommended phase II dose/schedule for this combination was 5-aza-2′-deoxycytidine 90 mg/m^2 on day 1 followed by carboplatin AUC 6 on day 8 every 28 days. Of the thirty patients evaluable for response, one with melanoma had a partial response and three other patients had stable disease. The majority of responses clustered at the recommended combination dose. 5-aza-2′-deoxycytidine induced dose-dependent reversible DNA demethylation in peripheral blood mononuclear cells (PBMCs), maximally at day 10. Furthermore the 5-aza-2′-deoxycytidine induced demethylation of the *MAGE1A* CpG island in PBMCs, buccal cells, and tumor biopsies, as well as elevation of HbF expression.

Exploiting gene reactivation by DNMTIs in combination with cytotoxic therapies may thus be a strategy that holds much clinical promise. A phase I-II trial using a demethylating dose of 5-aza-cytidine combined with capecitabine and oxaliplatin in advanced metastatic colorectal cancer is currently recruiting patients (www.clinicaltrials.gov, NCT01193517).

A phase I trial is evaluating the safety and feasibility of sequential 5-aza-2′-deoxycytidine with the anti-EGFR monoclonal antibody panitumumab in KRAS wild type metastatic colorectal cancer patients who underwent at least two lines of chemotherapy (www.clinicaltrials.gov, NCT00879385). A pharmacodynamic analysis investigating the re-expression or the reduction in promoter methylation of genes involved in tumor suppressor pathways known to be important in colorectal cancer or involved in EGFR signaling pathway is also planned.

Since *in vitro* and *in vivo* data demonstrated that vorinostat can downregulate TS expression at the transcription level (Glaser et al., 2003; Fazzone et al., 2009) and this resulted in synergistic antitumor activity when combined with 5-FU (see also previous paragraphs) (Ocker et al., 2005; Fazzone et al., 2009; Kim et al., 2009), clinical studies in refractory colorectal cancer to determine the MTD of vorinostat when combined with fixed doses of 5-FU and leucovorin (Fakih et al., 2010; Wilson et al., 2010) or 5-FU/leucovorin and oxaliplatin (Fakih et al., 2009) have been recently conducted.

In the study of Fakih et al. (2010), in refractory solid tumors, most of which were colorectal cancer, the MTD of vorinostat in combination with the sLV5FU2 regimen was 1700 mg orally daily $\times$ 3 or 600 mg orally twice daily $\times$ 3 days every 2 weeks. Twenty-one of 38 patients with 5-FU refractory colorectal cancer had stable disease and one had a partial response. Although vorinostat maximum serum concentrations at the MTD exceeded concentrations associated with TS downregulation *in vitro*, evidence of TS downregulation in tumor biopsies was lacking. It was thus hypothesized that the clinical benefit seen from the combination of vorinostat and 5-FU included induction of events other than TS modulation (e.g., cell cycle arrest, gene expression modulation, apoptosis, angiogenesis). An ongoing randomized phase II trial is currently evaluating the activity of low (800 mg) and high dose (1400 mg) vorinostat daily $\times$ 3 in combination with 5-FU/leucovorin in metastatic colorectal cancer patients refractory to 5-FU/leucovorin and to other standard therapies (www.clinicaltrials.gov, NCT00942266).

Wilson et al. (2010) studied an alternative dose schedule of vorinostat consisting of a 400 mg daily dose for 6 consecutive days prior to administration of a similar 5-FU and leucovorin

regimen in a limited number of refractory colorectal cancer patients. Dose-limiting toxicities were observed at the starting dose level which resulted in dose reduction. Two of ten patients achieved disease stabilization. Intratumoral TS downregulation was observed in one patient only. Although the presence of PBMC histone acetylation indicated biological activity of vorinostat, the toxicity and lack of reduction in intratumoral TS mRNA levels suggest that an alternate vorinostat dose schedule might be more effective.

Fakih et al. (2009) also conducted a phase I study to determine the MTD of vorinostat in combination with a fixed dose of 5-FU/leucovorin and oxaliplatin in patients with metastatic colorectal cancer who had failed at least two previou lines of treatment, including oxaliplatin, a fluoropyrimidine and irinotecan. The MTD of vorinostat in combination with the FOLFOX regimen was 300 mg twice daily for 1 week every 2 weeks. Vorinostat induced TS downregulation was not consistent. Eleven of 21 patients had stable disease but no patients developed an objective response. Alternative vorinostat dosing schedules may be needed.

Because of the close collaboration between DNA methylation and histone modification in gene silencing, another strategy is to combine a DNMTI with a HDACI (Bolden et al., 2006).

Two studies investigated escalating doses of 5-aza-cytidine in combination with valproic acid (Braiteh et al., 2008) and sodium phenylbutyrate (Lin et al., 2009), respectively, in patients with refractory solid tumors, including colorectal cancer. In the study of Braiteh et al. (2008) a significant decrease in global DNA methylation and induction of histone deacetylation were observed alomg with stable disease lasting 4–12 months in 14 patients (25%).

The study of Lin et al. (2009) showed individual cases of DNA methyltransferase activity and histone H3/H4 acetylation changes from paired biopsies or PBMCs within the limited number of samples available for analysis. The clinical response rate was disappointing: only one patient showed stable disease whereas 26 patients showed progressive disease.

A multicenter phase II trial with 5-aza-cytidine combined with entinostat, an HDACI benzamide, in pretreated metastatic colorectal cancer is currently ongoing and recruiting patients (www.clinicaltrials.gov, NCT01105377). A pharmacodynamic study focussing on changes in gene methylation and histone is being performed.

Exploitation of further epigenetic drugs is also needed. A relevant number of newer epigenetic drugs is available for this purpose (e.g., non-nucleoside DNMTIs such as the oligonucleotide MG98 and the HDACI hydroxamic acids such as belinostat (PXD-101), panobinostat (LBH589), dacinostat (LAQ824), trichostatin A).

5. Conclusions

Epigenetics may provide new strategies for treatment tailoring and chemoresistance reversal in metastatic colorectal cancer. The study of epigenetic variants in drug target- and drug metabolizing-genes could be added to estabilished genetic analyses. As shown in Section 3, evidence of 5-FU-related epigenetic variants is comprehensive, while more research is needed on irinotecan and oxaliplatin. However, it is already clear that for some genes (e.g., *UGT* or *DYPD*), adding epigenetic analyses to classic genetic discriminations would increase the sensitivity and specificity of the test.

Since epigenetic variants are reversible, they can be targeted by specific drugs. Despite some discouraging results, DNMT and HDACI are still being tested in metastatic colorectal cancer patients. As discussed in Section 4, we think that appropriate dosage and timing is crucial to explore the effectiveness of epigenetic drugs in this setting. In addition, trials should be designed to test the best combination with chemotherapy regimens. With these *caveats* in mind, we think that epigenetics may improve the quality of life and survival of patients in the near future.

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