Non-Steroidal Anti-Inflammatory Drugs, DNA Repair and Cancer

Harpreet K. Dibra, Chris J.Perry and Iain D. Nicholl
University of Wolverhampton
UK

1. Introduction

Colorectal cancer is the third most common cancer in women and fourth in men, with respect to incidence, and 529,000 deaths occurred worldwide in 2002 (Parkin *et al.*, 2005). Approximately 2 - 5% of cases of colorectal cancer are due to a genetic predisposition of which the most common is hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is an autosomal dominant disorder with high penetrance and exhibits allelic and locus heterogeneity (Aarnio *et al.*, 1995; de la Chapelle, 2004; Dunlop *et al.*, 1997). In HNPCC there are heterozygous germline mutations in the DNA mismatch repair (MMR) genes MutS homologue 2 (*MSH2*), MutL homologue 1 (*MLH1*), MutS homologue 6 (*MSH6*), post-meiotic segregation increased 2 (*PMS2*) and post-meiotic segregation increased 1 (*PMS1*) (Bocker *et al.*, 1999; Buermeyer *et al.*, 1999; Jiricny, 1998; Jiricny & Marra, 2003; Lucci-Cordisco *et al.*, 2003; Mitchell *et al.*, 2002; Narayan & Roy, 2003; Nicolaides *et al.*, 1998; Plaschke *et al.*, 2004; Zabkiewicz & Clarke, 2004). Germline mutations in *hMLH1* and *hMSH2* are the most common with abnormalities in these genes found in more than 90% of HNPCC mutation carriers (Abdel-Rahman *et al.*, 2006; de la Chapelle, 2004; Hampel *et al.*, 2005; Lagerstedt Robinson *et al.*, 2007). The phenomenon of transmission of an epimutation in hMLH1 has also been reported (Hitchins *et al.*, 2007).

The DNA MMR system plays an essential role in identifying and correcting any replication errors and any additional errors which arise through physical or chemical damage. These errors may be base-base mismatches, short insertions/deletions and heteroduplexes, which can occur during DNA replication and recombination (Jiricny, 1998; Jiricny & Marra, 2003). The DNA MMR system therefore maintains genomic integrity and stability and in essence provides a tumour suppressor function. Deficiencies in DNA MMR lead to the accumulation of mutations in repetitive nucleotide regions, a phenomenon termed microsatellite instability (MSI) (Parsons *et al.*, 1993; Parsons *et al.*, 1995; Thibodeau *et al.*, 1993; Thibodeau *et al.*, 1998). Microsatellites are classically defined as simple tandem nucleotide sequence repeats of 1 – 6 base pairs in the genome (Hancock, 1999). Changes in the number of the repeat units due to defective DNA MMR are potentially cancer causing (Riccio *et al.*, 1999; Yamamoto *et al.*, 1998). The MSI phenotype or replication error positive (RER+) phenotype can be considered as an almost canonical feature of DNA MMR deficiency (Kinzler & Vogelstein, 1996; Parsons *et al.*, 1993). This MSI phenotype is observed in approximately 15% of all human colorectal cancer, gastric and endometrial carcinomas (Lothe *et al.*, 1993; Seruca *et al.*, 1995; Shibata, 1999; Umar *et al.*, 1994). Somatic inactivation of DNA MMR largely

arises as a consequence of epigenetic silencing of *hMLH*1 (through hypermethylation of promoter CpG islands) rather than via classic mutational inactivation (Herman *et al.*, 1998; Jacinto & Esteller, 2007; Jones & Laird, 1999; Peltomaki, 2001; H. Yamamoto *et al.*, 1998). Genes particularly prone to MSI include *Bax*, *TGF-β receptor II*, *hMSH3* and *hMSH6* (Yamamoto *et al., 1998);* other susceptible genes include the DNA glycosylase *MBD4* (Bader *et al.*, 2000; Bader *et al.*, 1999; Riccio *et al.*, 1999) and the epidermal growth factor receptor (EGFR) (Woerner *et al*., 2010). Additionally, as a consequence of MSI, inactivation of proteins in the Wnt signalling pathway (eg TCF-4) has been reported (Shimizu *et al*., 2002). Clinically defined MSI is where at least two of the loci tested in a panel out of five exhibit instability (Boland *et al*., 1998). Mutation frequencies in cells defective in DNA MMR can be increased 100-1000 fold (Parsons *et al.*, 1993; Shibata, 1999).
The protective role of the DNA MMR system in suppressing the mutator phenotype in a range of common cancers is thus well established. The hypothesis that aspirin may potentially modulate this pathway to prevent carcinogenesis (Goel *et al*., 2003) is of central interest, and has so far received relatively little attention and merits further investigation. The aim of this chapter will be to review the evidence that non-steroidal anti-inflammatory drugs (NSAIDS), including aspirin, celecoxib, sulindac and so on, affect DNA repair mechanisms and pathways and to further examine the consequences of this in relation to cancer development and progression. As NSAIDs are considered to be one of the most widely used over-the-counter drugs, we will also discuss the potential effect of NSAID use on cancer treatment. Aspirin and other NSAIDs may have the capacity to perturb DNA repair pathways and this may have important implications for the patient response to chemotherapeutic agents. It is also worth noting that inflammation – the 'seventh hallmark' of cancer (Colotta *et al*., 2009) - can possibly repress (by epigenetic mechanisms) DNA mismatch repair.

2. Cancer and NSAIDs

From evidence adduced from epidemiological studies and clinical trials, it has been proposed that regular ingestion of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can promote colorectal tumour regression and reduce the relative risk of developing colorectal cancer (CRC) in the general population and in genetically susceptible individuals (for example see; (Baron *et al.*, 2003; Chan *et al.*, 2009; Cuzick *et al.*, 2009; Giovannucci, 1999; Imperiale, 2003; Logan *et al.*, 1993; Paganini-Hill, 1993; Sandler *et al.*, 2003; Thun *et al.*, 2002). NSAID use is also associated with a reduced risk of oesophageal adenocarcinoma particularly in patients with high risk molecular abnormalities, for example, with 17p LOH, 9p LOH, and DNA content abnormalities (Galipeau *et al.*, 2007). Recent meta-analyses of randomised clinical trials have strengthened the contention that aspirin has protective effects against CRC (Din *et al.*, 2010) and non-CRC related adenocarcinomas, including oesophageal and lung cancer (Rothwell *et al.*, 2011). Although such studies have now provided substantial evidence that regular use of aspirin based medication can reduce the risk of colorectal cancer (Bosetti *et al.*, 2002; Muscat *et al.*, 1994; Thun *et al.*, 1991) the molecular basis for the protective effect of aspirin *vis-à-vis* CRC and other cancers is rather controversial. A substantial number of theories are now in circulation. Whilst much of the focus in the recent past has understandably been on the intrinsic anti-inflammatory nature of the compounds in use, see for example, (Giovannucci, 1999; Keller & Giardiello, 2003), there

are a number of intriguing findings which suggest that NSAIDs can impact upon genetic stability and it is these aspects which we wish to highlight in this chapter. However, the optimism that arises from the findings that NSAIDs may offer protection against cancer should be tempered: NSAID use can result in serious side effects (gastrointestinal disturbances and cardiovascular events) in susceptible individuals (Cuzick *et al.*, 2009). This has lead to considerable debate amongst clinicians over recent years as to whether NSAIDs should be prescribed as chemopreventative agents, in particular, to those individuals at high risk of developing colorectal cancer, such as HNPCC or Familial Adenomatous Polyposis (FAP) patients.

Animal studies confirm that NSAIDs can protect against the development of colorectal neoplasia (Corpet & Pierre, 2003, 2005). For example, aspirin has been shown to suppress spontaneous intestinal adenoma formation and reduce incidence and volume of colon tumour induced by the carcinogen 1,2-dimethylhydrazine in rat models (Barnes & Lee, 1999). Continuous administration of a clinically relevant aspirin dosage was crucial in these studies in comparison to other studies where aspirin was administered at the start of carcinogenesis or one week after carcinogen exposure (Craven & DeRubertis, 1992). Taken together with findings from epidemiological studies in humans, there is thus the suggestion that long term, continuous usage of aspirin is required to gain any beneficial chemopreventative effects. Several studies carried out in the 1990s demonstrated inhibition of carcinogen induced tumour development by NSAIDs including aspirin and sulindac in rats (Rao *et al.*, 1995; Reddy *et al.*, 1993) and also in a murine model of FAP (Barnes & Lee, 1998; Chiu *et al.*, 1997; Jacoby *et al.*, 1996; Mahmoud *et al.*, 1998; Oshima *et al.*, 1996). Familial adenomatous polyposis (FAP) is a colorectal cancer syndrome inherited in humans in an autosomal dominant manner caused by an absence of a functional caretaker APC protein (Narayan & Roy, 2003). Lifetime administration of aspirin to a mouse model with germline defects in both the *APC* and *Msh*2 gens (APC $^{Min/+}$, Msh2 $^{-/-}$) suppresses intestinal and mammary neoplasia formation (Sansom *et al.*, 2001).

Epidemiological studies have identified environmental and dietary factors which alter the risk of developing colorectal cancer. Protective dietary factors include NSAIDs, fruit, vegetables, and folic acid and possibly calcium, whilst red and processed meat ingestion, alcohol use and obesity are perceived to increase risk (Forte *et al.*, 2008; Key, 2011; La Vecchia *et al.*, 2001; Ryan-Harshman & Aldoori, 2007; Scheier, 2001; Serrano *et al.*, 2004). An assessment of chemopreventative measures, such as NSAID and micronutrient intake, for the general population and individuals with an increased risk for colorectal cancer based on family history, has recently been published (Cooper *et al.*, 2010).

2.1 Inflammation and cancer

Debate has arisen with regards to the molecular mechanism of action of aspirin and other NSAIDs in reducing the incidence of certain cancers. Based on the anti-inflammatory effects of these agents, one obvious explanation is that inflammation can drive cancer development. Indeed, inflammation is becoming increasingly recognised as being critical to cancer formation, and building on the framework proposed by Hannahan and Weinberg (Hanahan & Weinberg, 2000), it has been proposed that inflammation should be considered the seventh hallmark of genetic instability (Colotta *et al.*, 2009). It has been estimated that one in four cancers are linked to infection and chronic inflammation (Hussain & Harris, 2007). In an inflammatory microenvironment mutation frequency is increased (Bielas *et al.*, 2006). There is strong evidence for an increased risk of cancer in individuals with chronic

inflammatory states (inflammatory bowel disease, gastroesophageal reflux disease, asthma) with or without attendant bacterial or viral infection (eg *Helicobacter pylori*, hepatitis) (Grivennikov & Karin, 2010; Schetter *et al.*, 2010; Xie & Itzkowitz, 2008). The molecular drivers of cancer formation resulting from the interaction of pre-cancerous cells with activated immune cells and the surrounding stroma are complex. The host response to infection, injury and wound repair through production of reactive oxygen (ROS) and nitrogen oxide (RNOS) species, and pro-inflammatory cytokines (eg TNF-α, IL-1, IL-6) and chemokines (eg IL-8) (Wang *et al.*, 2009) is of central importance. Such 'micro-cytokine' storms can stimulate activation of transcriptions factors (eg Nuclear Factor-κB, AP-1, STAT3) fundamentally altering the expression profile of a cell and promoting cell survival, proliferation, angiogenesis, motility and invasion. It is clear that for individual transcription factors their activation can be context (eg tissue) dependent and they may not always promote tumour cell formation, and may indeed exhibit tumour suppressor activity (eg NF-κB in skin cancer) (Chaturvedi *et al.*, 2011; Ditsworth & Zong, 2004).

Microsatellite instability has been observed in non-neoplastic tissue in patients with chronic inflammatory conditions prior to the presence of dysplastic tissue, indicating that defects in DNA MMR can be an early event in inflammation-associated cancers (Brentnall *et al.*, 1995; Park *et al.*, 1998). There are tantalising findings which hint at the molecular basis for this MSI. Oxidative stress, in the guise of H_2O_2, increases frameshift mutations (Gasche *et al.*, 2001), and can inactivate the DNA MMR system (Chang *et al.*, 2002). Moreover, in a p53 and p21 dependent fashion, activated neutrophils induced replication errors and a G2/M arrest in colonic epithelial cells (Campregher *et al.*, 2008). In inflammatory bowel disease neoplasia, hypermethylation of the *hMLH1* gene and reduced hMLH1 protein expression occurs frequently (Fleisher *et al.*, 2000). In an animal model of colorectal cancer, inflammation and hypoxia were found to epigenetically silence hMLH1 expression: down-regulated expression of this DNA mismatch repair gene occurred as a consequence of decreased acetylation, which was reversible when the animals were treated with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) (Edwards *et a*l., 2009). Decreased expression of hMLH1 can alter hPMS2 stability and consequently, genetic integrity. Moreover, hMLH1 and hPMS2 can have a role in activating cell-cycle checkpoints and promoting apoptosis (Cejka *et al.*, 2003; Davis *et al.*, 1998; Ding *et al.*, 2009; McDaid *et al.*, 2009; Sansom *et al.*, 2003; Yanamadala & Ljungman, 2003; Zhang *et al.*, 1999). Thus, a functional DNA mismatch repair system prohibits expansion of cells containing DNA damage (Carethers *et al.*, 1996; Papouli *et al.*, 2004). Dysregulated hMLH1/hPMS2 expression could conceivably impact on the survival of cells containing damaged DNA and promote cancer progression. hMLH1 expression has been reported to be altered by tobacco usage and inflammatory state in the epithelium of the oral mucosa (Fernandes *et a*l., 2007). Increased tissue specific hMLH1 hypermethylation has been observed in the progression of oesophageal cancer (Vasavi *et al.*, 2006); the authors also reported that patients with gastroesophageal reflux disease (GERD) exhibited a very significant degree of hMLH1 hypermethylation prompting the suggestion that reflux can promote hypermethylation. The relationship between acid reflux and inflammation in GERD has been recently reviewed (Orlando, 2010). The phenomenon of elevated microsatellite instability at selected nucleotide repeats (EMAST) is also seen in 60% of sporadic colon cancers, is more common in individuals of African-American origin and has been linked to reduced expression of the hMSH3 protein. Moreover, EMAST is also more prevalent in rectal cancer with immune cell infiltration (Devaraj *et al.*, 2010; Lee *et al.*, 2010). As MSI in CRC can result in products with potentially increased immunogenicity, it is

possible that this further stimulates inflammation (Banerjea *et al*., 2004). Tumour infiltrating lymphocytes in CRC with MSI are activated and cytotoxic (Phillips *et al*., 2004). In a model of experimental colon carcinogenesis in rats, long-term, low-dose administration of aspirin significantly reduced cytokine and matrix metalloproteinase release (Bousserouel *et al*., 2010). Based on the above findings, it would appear to be reasonable to suppose that research into the epigenetic modifying effects of NSAIDs, and inflammation itself (Maekawa & Watanabe, 2007), particularly with respect to alterations in DNA repair protein expression and MSI, should be a focus in the future.

Oxidative stress occurs as a consequence of the activation of the immune system, where oxidative bursts have a role in protecting the host against microbial invaders. Reactive oxide and nitrogen oxide species (ROS and RNOS) thus produced can ultimately cause DNA damage such as abasic sites, oxidised bases, DNA-intrastrand adducts, strand breaks, as well as RNA alkylation, and protein damage (Hussain *et al*., 2003). Reaction with lipids produces extremely reactive peroxidation products, including malondialdehyde (MDA) and *trans*-4-hydroxynonenal (4-HNE). 4-HNE can form etheno adducts in DNA and has been found to preferentially form adducts in codon 249 of the human p53 gene (Federico *et al*., 2007; Hu *et al*., 2002). Mutations in the tumour suppressor p53 are found in inflamed tissue in ulcerative colitis (UC) patients (Hussain *et al*., 2000). Accelerated telomere shortening, and DNA damage - as assessed by analysis of phosphorylated histone H2AX (γH2AX; a measure of DSBs) occurs in the bowel of UC patients (Risques *et al*., 2008). ROS and RNOS - whilst generally perceived as having a negative impact on cell function - do have important roles as secondary messengers (Valko *et al*., 2006). However, if the cellular defence mechanisms - anti-oxidant enzymes such as manganese superoxide dismutase and glutathione peroxidise, detoxification, and DNA repair systems - are overwhelmed, these reactive species are potentially mutagenic (Ferguson, 2010). The bystander effects from released cytokines and reactive signalling species may also occur some distance from the initial trauma. Redon *et al*. have shown that increased levels of DNA damage (measuring γH2AX levels) can occur systemically in mice implanted with a non-metastatic tumour (Redon *et al*., 2010). Animal model studies confirm the role of RNOS and the inflammatory process in contributing to cancer development (Hussain *et al*., 2003; Itzkowitz & Yio, 2004).

The base excision repair (BER) system is critically important for dealing with oxidative damage to DNA, such as removal of 8-oxo-G lesions (David *et al*., 2007; Lindahl & Wood, 1999; McCullough *et al*., 1999; Wood *et al*., 2001). Increased expression of the BER proteins AAG (a 3-methyladenine DNA glycosylase) and APE1 (apurinic endonuclease1; Ref-1) is seen in inflamed tissue from UC patients. Paradoxically a positive correlation of MSI was noted with overexpression of AAG, and for MSI-high tissues with increased APE1 expression. In model systems this adaptive response was confirmed to positively correlate with an in increase in MSI in human cells and frameshift mutations in *S. cerevisiae* (Hofseth *et al*., 2003). Dysregulated expression of BER proteins can generate a mutator phenotype (Glassner *et al*., 1998) (as cited in Hofseth, 2003). In a mouse model of UC, expression of the Mutyh BER protein, which can recognise 8-oxoG:A mispairs and oxidised adenines was actually found to influence the inflammatory response to dextran sulphate sodium induced oxidative stress (Casorelli *et al*. 2010). The biomediator signalling molecule nitric oxide (NO·) formed during inflammation has the capacity to inhibit *in vitro* and *in vivo* the formamidopyrimidine-DNA glycosylase, which can recognise abasic sites and 8-oxoguanine lesions (Wink & Laval, 1994). Thus, oxidative stress can compromise genetic stability.

RNOS, and inflammatory cytokines and chemokines can activate the transcription factors activator protein-1 (AP-1), HIF-1 and NF-κB, the latter resulting in transcription of target genes including COX-2 and TNF-α (for example, see Olson & van der Vliet, 2011; Valko *et al.*, 2006). DNA double strand breaks can also activate NF-κB (Rakoff-Nahoum, 2006), thus potentially creating a feedback loop where the inflammatory response is perpetuated. Persistent DNA damage can also trigger secretion of cytokines such as IL-6 (Rodier *et al.*, 2009). There is evidence that NF-κB and cyclooxygenase activation and HIF-1 signalling are interlinked (Jung *et al.*, 2003; Qiao *et al.*, 2010). Hypoxia can induce MSI (Kondo *et al.*, 2001): in human sporadic colon cancers HIF-1α over-expression is associated with loss of hMSH2 expression, and *in vitro* experiments confirm that in a p53 dependent manner HIF-1α can repress hMSH2 and hMSH6 (Koshiji *et al.*, 2005; To *et al.*, 2005). Recently HIF-1α has been suggested to regulate expression of an inhibitor of apoptosis protein, Survivin (Wu *et al.*, 2010), which is notably present in CRC (Chen *et al.*, 2004). There is increasing evidence that microRNAs (short, non coding RNAs that regulate translation) are mediators of the inflammatory process (Schetter *et al.*, 2010). Two micro-RNAs, miR-210 and miR-373 are up regulated in an HIF-1α dependent manner in hypoxic cells: forced expression of mir-210 reduced expression of homologous recombination factor RAD52, whilst reduced mir-373 expression was found to suppress RAD52 and RAD23B (Crosby *et al.*, 2009). Over-expression of another micro-RNA, miR-155 has been reported in colorectal cancer, and can regulate DNA MMR protein expression (Valeri *et al.*, 2010). Inflammation can up-regulate miR-155 expression and increase mutation frequency two to threefold in spontaneous hypoxanthine phosphoribosyltransferase gene mutation assays (Tili *et al.*, 2011). MiR-155 expression is associated with a poor prognosis in lung cancer (Yanaihara *et al.*, 2006). The cyclooxygenase family of enzymes (COX-1: constitutively expressed; COX-2: induced in inflammation; COX-3: splice variant of COX-1) catalyse the conversion of arachidonic acid into prostanoids (prostaglandins and thromboxanes). Cyclooxygenase expression is increased in CRC (Kutchera *et al.*, 1996). Prostaglandins promote epidermal growth factor receptor (EGFR) transactivation, increased cell proliferation, motility, invasion and angiogenesis (eg by altering vascular endothelial growth factor expression) and inhibit apoptosis, for example, by increasing Bcl-2 expression (Ghosh *et al.*, 2010; Pai *et al.*, 2003; Sheng *et al.*, 1998; Wang & DuBois, 2008). To summarise: inflammation and hypoxia can result in genetic instability and suppression of apoptosis.

2.2 Mechanistic aspects of NSAID cytotoxicity

The mechanism by which NSAIDs protect the host from colorectal cancer development has been under investigation for decades, and as a consequence a plethora of hypotheses (some of which may be competing) have been proposed to explain this phenomenon. In addition to the evidence alluded to above of epidemiological studies and animal models examining the protective effect of NSAIDs, there also exists a substantive literature reporting that NSAIDs exhibit a degree of specific toxicity *in vitro* to colorectal cancer cell lines. Because of the intrinsic anti-inflammatory activity of NSAIDs a significant number of researchers have focused on this aspect as a protective mechanism. For example, aspirin can acetylate the cyclo-oxygenases (COX) significantly reducing arachidonic acid metabolism and prostaglandin production, thereby reducing inflammation (Elwood *et al.*, 2009). Expression of the inducible COX, cyclooxygenase-2 is notably elevated in colorectal malignancies and in other cancers (Ferrandez *et al.*, 2003; Kutchera *et al.*, 1996; Soslow *et al.*, 2000), and this over-

expression has been actively implicated in the metastasic potential of tumours (Jang *et al.*, 2009; Tsujii *et al.*, 1997). These effects, however, may not be restricted only to colorectal cancer: a case control study has found that use of selective and non-selective COX-2 inhibitors (celecoxib, refecoxib and aspirin and ibuprofen) has utility in the chemoprevention of lung cancer (Harris *et al.*, 2007).

NSAIDs do not only affect cyclooxygenase activity: exposure to these drug can significantly alter gene expression and thus NSAIDs can be reasonably described as having pleiotropic effects, some of which may be relatively compound dependent. Chronic NSAID use can suppress CpG island hypermethylation of tumour suppressor genes [p14(Arf), p16(INK4a), E-cadherin] in the human gastric mucosa (Tahara *et al.*, 2009). Furthermore, NSAID toxicity may not be absolutely dependent on inhibition of COX activity: the proliferation of COX-2 negative cell lines can also be inhibited by NSAIDs (Lai *et al.*, 2008; Richter *et al.*, 2001) and the chemical precursor of aspirin - salicylate - which has weak anti-COX activity, can itself be anti-inflammatory (Amann & Peskar, 2002) and pro-apoptotic to CRC cells (Elder *et al.*, 1996). Additionally, whilst the NSAID sulindac sulfide and its sulfone derivative can both inhibit the HT-29 CRC cell line growth, sulindac sulfone is "devoid of prostaglandin inhibitory activity" (Piazza *et al.*, 1995). Additionally, aberrant crypt foci formation in a chemically induced rat model of CRC were suppressed by treatment with sulindac sulfone (Charalambous & O'Brien, 1996). Supporting these findings, a mechanism for NSAID toxicity (anti-proliferative and inducing apoptosis) – testing sulindac sulfide and piroxicam - toward a CRC line lacking cyclooxygenase activity (HCT-15) and thus independent of prostaglandin inhibition, has also been reported (Hanif *et al.*, 1996). Sulindac metabolites (sulphide and sulfone; see Fig.1) can inhibit the activation and expression of the EGF receptor (Pangburn *et al.*, 2005), with the down-regulated activity mediated by lysosomal and proteasomal degradation(Pangburn *et al.*, 2010). Rigas and others have cogently proposed that the anti-neoplastic activities of NSAIDs can be categorised as either being COX-dependent or COX-independent (Keller & Giardiello, 2003; Shiff & Rigas, 1999). This concept has been reviewed in some detail in (Ferrandez *et al.*, 2003). Smith *et al* examined the effects of NS-398 (a selective COX inhibitor), indomethacin (a non-selective COX inhibitor) and aspirin on the HT29Fu, HCA-7, SW480 and HCT116 CRC cell lines with respect to effects on cell proliferation, cell cycle arrest, apoptosis induction, β-catenin and COX-1 and COX-2 protein production. They concluded that NSAIDs act via COX-dependent and COX-independent mechanisms (Smith *et al.*, 2000). Indeed, acetylsalicylic acid regulates MMP-2 activity and inhibits colorectal invasion of B16F0 melanoma cells (Tsai *et al.*, 2009). Intriguingly, although medium conditioned by cultured colorectal cancer cell lines is capable of inducing endothelial cell (EC) tube formation (as a model for angiogenesis); aspirin and salicylate (both at 1mM) can reduce the ability of the conditioned medium from treated DLD-1, HT-29 and HCT116 CRC cell lines to promote EC tube formation (Shtivelband *et al.*, 2003).

Aspirin is principally metabolised to salicylate *in vivo* (Law *et al.*, 2000; Paterson & Lawrence, 2001), with plasma salicylate concentrations of 0.95-1.9 mM achievable in patients receiving aspirin as an anti-inflammatory agent (Amann & Peskar, 2002; Urios *et al.*, 2007; Yin *et al.*, 1998). The effect of significantly higher concentrations than that achievable physiologically has been used by a number of investigators in a number of *in vitro* studies and one must interpret cautiously data produced from these analyses. To facilitate interpretation, we have incorporated the concentrations utilised in the relevant publications. We should also point out that whilst vegetables and fruits have been considered to be a

natural source of salicylate, where it functions as a plant signalling molecule (Paterson & Lawrence, 2001; Schenk *et al.*, 2000, and refs therein), it has been reported that SA may be an endogenous compound, with SA found in the blood of carnivorous animals, for example in the burrowing owl (Paterson *et al.*, 1998; Paterson *et al* 2008).

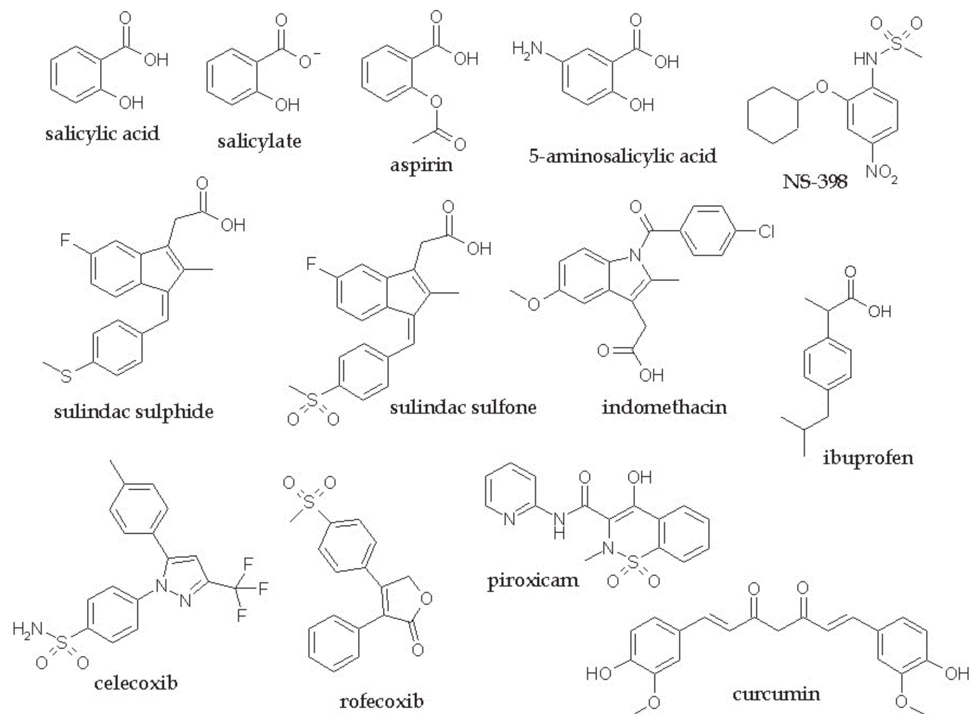

Fig. 1. The non-steroidal anti-inflammatory drugs (NSAIDS) referred to in this chapter can be sub-categorised into five groups. 1. Salicylates (salicylic acid/salicylate anion, aspirin, 5-aminosalicylic acid). 2 Acetic acid derivatives (indomethacin, sulindac sulphide, sulindac sulphone) 3. Propionic acid derivatives (ibuprofen). 4. Enolic acid derivatives or oxicams (piroxicam). 5. Selective COX-2 inhibitors (celecoxib, rofecoxib, NS-398). Curcumin has a dimeric structure with two identical phenolic moieties suggestive of salicylate-like character.

2.2.1 COX independent effects of NSAIDs

A substantial number of hypotheses regarding NSAID cytotoxicity not directly involving inhibition of COX activity exist (reviewed in: Goke, 2002; Watson, 2006). Much of the data is obtained from examining the effect of NSAIDs on cultured cell lines. For example, salicylate can cause cell cycle arrest: in a range of adenoma and carcinoma derived cell lines, incubation with 5 mM resulted in a decrease of cells in the S-phase with cells accumulating in the G_o-G_1 phase (Elder *et al.*, 1996). Activating mutations stabilizing the Wnt pathway and β-catenin are common in CRC (Behrens, 2005; Bienz & Clevers, 2000). In four CRC cell lines (SW948, SW480, HCT116, LoVo), aspirin (5 mM) and indomethacin (400 μM) downregulated cyclin D1 expression, and the authors proposed this was a consequence of reduced

transcriptional activity of the β-catenin/TCF complex (Dihlmann *et al.*, 2001). Bos *et al* using reporter assays also showed that aspirin (up to 5 mM) can down-regulate APC-β-catenin-TCF4 signalling in the SW480 CRC cell line and increase the phosphorylation of protein phosphatase 2A (PP2A), resulting in decreased PP2A enzymatic activity. This alteration is stated to be essential for the observed effect on the Wnt/β-catenin pathway activity (Bos *et al.*, 2006). Mutations in the APC or β-catenin genes may also result in increased peroxisome proliferator-activated receptor δ (PPAR δ) activity; indeed, sulindac sulfide and indomethacin have been reported to suppress (by direct inhibition) in a dose-dependent fashion, PPARδ activity (He *et al.*, 1999). However, it should be noted that regulation of PPARδ by the APC/ β -catenin/TCF4 pathway and the effect of NSAIDs on PPAR function is a field not without some controversy; for example, Foreman *et al* recently reported not finding decreased PPARβ/δ expression following NSAID treatment in a number of human CRC cell lines (Foreman *et al.*, 2009).

2.2.2 NSAIDS, signalling pathways and apoptosis

Other transcriptional pathways affected by NSAID use include NF-κB signalling, which is critically involved in regulating immunity, inflammation and apoptosis and in cancer development (Nakano, 2004; Olivier *et al.*, 2006; Staudt, 2010). Aspirin and sodium salicylate can antagonise the NF-κB pathway (Kopp & Ghosh, 1994), via inhibition – by direct, but not covalent binding- of IκB kinase-B (IKK β kinase) (Yin *et al.*, 1998). Yamamoto *et al* extended this hypothesis by reporting that sulindac, sulindac sulfide and sulindac sulfone (but not indomethacin) also inhibits the NF-κB pathway by decreasing IKK β kinase activity (Yamamoto *et al.*, 1999). In HT-29 CRC cells, nitric oxide (NO)-donating aspirin inhibited NF-κB transcription more potently than aspirin as assessed by electrophoretic mobility shift assays (Williams *et al.*, 2003).

Zerbini *et al* reported that NF-κB can mediate the repression (assessed by RT-PCR analysis) of the growth arrest and DNA damage inducible (*GADD*) *45*α and *GADD45γ* genes and that this repression is both necessary and sufficient for cell survival, and that GADD45α and γ activity can contribute towards apoptosis of prostate cancer cells (assessed by transfection studies in DU145 and PC-3) (Zerbini *et al.*, 2004). The melanoma differentiation associated gene 8 (mda-7/IL-24) was induced by NSAIDs and affected growth arrest and apoptosis *in vitro*, in a manner dependent on GADD45α and γ expression affecting the p38/JNK pathway (Sarkar *et al.*, 2002; Zerbini *et al.*, 2006). However, it has been suggested that recombinant IL-24 lacks apoptosis inducing properties to melanoma cells (Kreis *et al.*, 2007). In a recent review, Siafakas and Richardson have suggested that the GADD family of proteins can be considered to be molecular targets for anti-tumour agents; indeed, commonly used NSAIDs can up-regulate GADD45α expression (Rosemary Siafakas & Richardson, 2009). GADD45α overexpression in NIH 3T3 cells has been shown to promote global DNA demethylation and loss of expression has been shown to induce DNA hypermethylation (including in *hMLH1*). Such findings led Barreto *et al* to conclude that GADD45α can relieve epigenetic silencing by promoting DNA repair (Barreto *et al.*, 2007). It is noteworthy that GADD45γ can be inactivated by epigenetic mechanisms in multiple tumours (Ying *et al.*, 2005).

In a review of the role of the NF-κB pathway in inflammation and cancer, the authors suggested that, '...constitutive NF-κB activation is likely involved in the enhanced growth properties seen in a variety of cancers' (Yamamoto & Gaynor, 2001). Specifically targeting

the NF-κB pathway may thus have utility in cancer treatment (Olivier *et al.*, 2006): for example, attempts are ongoing to identify clinically useful and novel IKK-β inhibtors (for example see (Lauria *et al.*, 2010)), and inhibition of NF-κB (by siRNA) can enhance the chemosensitivity of HCT116 CRC cells to the DNA topoisomerase inhibitor, irinotecan (Guo *et al.*, 2004). In marked contrast however, Stark *et al*, have suggested that aspirin can activate NF-κB signalling *in vitro* (SW480, HRT-18, HCT116, CT26 cell lines) and has the capacity to induce apoptosis in *in vivo* (xenografts and APC $^{Min+/-}$) models of CRC (Stark *et al.*, 2001; Stark *et al.*, 2007). Moreover, these effects are cell type specific to aspirin (for CRC cells): the induction of apoptosis was independent of COX-2 expression, APC and β-catenin mutation status, and DNA mismatch repair proficiency (Din *et al.*, 2004).

A number of other hypotheses have been invoked for the observed cytotoxicity of NSAIDs to CRC cell lines. Cytosolic phospholipase A_2 (cPLA2) expression is decreased in NIH 3T3 cells treated with either aspirin or sulindac (Yuan *et al.*, 2000) and in CRC cell lines (SW480, Colo320 and HT-29) when treated with low mM (2.5-10) concentrations of aspirin (Yu *et al.*, 2003). This observation is intriguing given that $cPLA_2$ expression has been proposed to participate in intestinal tumorigenesis (eg (Lim *et al.*, 2010) and refs therein). Law *et al* have indicated that (high mM concentrations of) salicylate (in Balb/MK cells) can inhibit the activity and phosphorylation of the mitogen activated protein kinase, $p70^{s6k}$ independent of p38 MAPK, with a concomitant reduction in DNA synthesis, cell proliferation and in expression of proliferation associated proteins such as c-MYC, cyclin D1, and PCNA and led the authors to conclude that salicylate may act via the mTOR pathway (Law *et al.*, 2000). In contrast, it has been suggested that salicylate-induced apoptosis in HCT116 CRC cells occurs through activation of p38 MAPK and p53; however, the concentration tested was - in our opinion - high (10mM), and thus casts doubt on the utility of the findings (Lee *et al.*, 2003). NO-donating aspirin has also been reported to activate p38 and JNK MAP kinase pathways in HT-29 colorectal cancer cells (Hundley & Rigas, 2006). Using subtractive hybridization, Baek *et al* identified an increase in the expression of the NSAID activated gene (NAG-1) – a member of the TGF-β superfamily, with pro-apoptotic properties - in indomethacin treated HCT116 cells. A range of NSAIDs including sulindac sulfide and aspirin also increased NAG-1 expression (Baek *et al.*, 2001). NCX-4040 (*para*-NO-aspirin), an NO donating aspirin derivative can also induce NAG-1 expression and it was confirmed that NAG-1 has a pro-apoptotic role (Tesei *et al.*, 2008, and refs therein). Another nitro-derivative of aspirin, NCX-4016 inhibited EGFR and STAT3 signalling in cisplatin-resistant human ovarian cancer cells (Selvendiran *et al.*, 2008). NSAIDs can also up-regulate 15-lipoxygenase-1 (15-LOX-1) in CRC cells: NS-398 and sulindac sulfone both induced expression in the DLD-1 (COX-1 and COX-2 negative) cell line; significantly, inhibiting 15-LOX-1 blocked the induction of apoptosis (Shureiqi *et al.*, 2000).

With respect to characterisation of the pathway by which NSAID toxicity occurs, reports have been rather contradictory: *in vitro* studies have suggested that whilst aspirin and other NSAIDs can cause cell cycle arrest and inhibit CRC proliferation, this may, (Din *et al.*, 2004; Elder *et al.*, 1996; Piazza *et al.*, 1995; Yu *et al.*, 2002; Yu *et al.*, 2003) or may not (Shiff *et al.*, 1996; Smith *et al.*, 2000) occur with the induction of apoptosis, or may occur as consequence a combination of activation of both apoptotic and necrotic 'pathways' (Lai *et al.*, 2008). Notwithstanding the apparently contradictory reports, an absence of Bax expression in CRC cells has been found to abolish the apoptotic response to NSAIDs (Zhang *et al.*, 2000). These authors reported finding Bax mutations in indomethacin resistant cells. Bax and BCl-2

expression can be up and down-regulated respectively, in a dose-dependent fashion, (up to 10 mM) in SW480 cells incubated with aspirin (Lai *et al.*, 2008). BCL-2 expression can be reduced by aspirin in SW480 cells (Yu *et al.*, 2002) and can also suppress apoptosis in CRC cells (Jiang & Milner, 2003). Pretreating LNCap (human prostate) and CX-01 (colorectal carcinoma) cell lines with aspirin was found to enhance the capacity of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to intiate apoptosis, an effect – according to the authors - related to down regulation of *BCL-2* gene expression resulting from the inhibition of NF-κB (of which BCL-2 is a known target) (K.M.Kim *et al.*, 2005). HeLa cells incubated with aspirin undergo apoptosis as assessed by cleavage of procaspase 3, PARP and PKC-δ, and annexin V staining. Moreover, the caspase inhibitor zVAD-fmk suppressed cell death, Bax was noted to translocate from the cytosol to mitochondria, and cytochrome c release from mitochondria was seen using time-lapse confocal microscopy (Zimmermann *et al.*, 2000). Sulindac sulfide (up to 500 μM tested) can inhibit growth of NIH3T3 and SAOS cells: an inhibtion of ras mediated proliferation and transformation was noted (Herrmann *et al.*, 1998). Hughes *et al.,* (2003) showed that NSAID treatment of colorectal cancer cell lines caused a decrease in intracellular polyamine content and was cytotoxic. Polyamines are growth factors, and involved in protein synthesis. An increase in intracellular polyamine concentration is observed in the early stages of carcinogenesis. When polyamines were re-introduced to NSAID treated cells, apoptosis was inhibited suggesting that the polyamine pathway is affected by NSAID treatment in CRC cell, and that modulation of this pathway may explain the chemoprotective effects of NSAIDs (Hughes *et al.,* 2003). Dikshit *et al.*, reported that aspirin disrupted proteasome and mitochondrial function in mouse Neuro 2a cells (Dikshit *et al.*, 2006). Hardwick *et al.,* (2004) analysed changes to gene expression employing DNA microarray in the colorectal cancer cell line HT-29 upon aspirin treatment, and found a significant increase in *Rac1* gene and protein expression in a time and concentration dependant manner. Rac1 is involved in intestinal epithelial cell differentiation (Stappenbeck & Gordon, 2000) proliferation, motility and resistance to apoptosis (Parri & Chiarugi, 2010). Spitz *et al* report that aspirin and salicylate can inhibit purified phosphofructokinase (PFK) in a dose-dependent manner: as cancer cells utilise glycolysis for energy production in which PFK is the rate limiting step, the authors suggest that these NSAIDs may be pro-apoptotic through disturbing the glycolytic pathway (Spitz *et al.*, 2009). We have recently identified novel derivatives of di-aspirin (bis-carboxyphenol succinate) to have potential as anti-colorectal cancer agents (Deb *et al.*, 2011).

2.3 NSAIDS and DNA repair

A relatively unexplored area of research is that the anti-tumour mechanism of action of aspirin may be via 'interaction' with the DNA repair systems. This is of key interest particularly in the context of hereditary and sporadic colorectal cancer where DNA repair defects can be a causative factor. It has been shown that the MSI mutator phenotype is suppressed by aspirin. For example, treatment of the colorectal cancer cell line HCT116 - which is DNA MMR deficient with a defect in *hMLH1* - reduced the MSI phenotype and induced apoptosis (Ruschoff *et al.*, 1998). It is speculated that aspirin exposure resulted in genetic selection against cells with MSI and that MSI unstable cells were weeded out by apoptosis (Ruschoff *et al.,* 1998). This observation is important as MSI resulting from a genetic mutation in *hMLH*1, accounts for 5 - 10% of colonic tumours. Nitric oxide releasing NSAIDS (NO-NSAIDS) suppressed MSI in DNA mismatch repair deficient colorectal cancer cells (McIlhatton *et al.*, 2007), and the authors suggested that since these NO containing

derivatives of aspirin are more effective than aspirin itself, they should be considered for use in chemopreventitive trials in patients at high risk of developing CRC. Recently, in a mouse model of HNPCC, McIlhatton *et al.*, reported that aspirin and low dose NO-aspirin increased life span. However, though the intestinal tumours in aspirin treated animals showed less microsatellite instability, low dose NO-aspirin had 'minimal effect on MSI status' and high dose NO-aspirin decreased life span and increased MSI (McIlhatton *et al.*, 2011). In addition to the phenomenon of aspirin selecting for microsatellite stability in colorectal cancer cells (Ruschoff *et al.*, 1998), aspirin *in vitro* can inhibit CRC growth and increase the level of the DNA MMR proteins hMLH1, hMSH2, hMSH6 and hPMS2 in DNA MMR proficient cells (Goel *et al.*, 2003). Increased DNA MMR levels could conceivably facilitate programmed cell death. This is a highly significant observation as defects in DNA MMR proteins are ultimately responsible for HNPCC and this study speculates in particular on the involvement of MLH1 function in the chemoprotective effect of aspirin. In addition to this, recent evidence has shown that although MLH1 expression is decreased in cases of sporadic colorectal adenoma, MLH1 expression was found to be increased in cases of sporadic colorectal adenomas with regular aspirin use (Sidelnikov *et al.*, 2009). Aspirin is not alone in having the capacity to select for microsatellite stability: the NSAID mesalazine (5-aminosalicylic acid), used to treat patients with inflammatory bowel disease (IBD), experimentally reduced frameshift mutations in cultured CRC cells (HCT116 and HCT116+chr 3) independent of DNA mismatch repair proficiency (Gasche *et al.*, 2005). *In vitro*, mesalazine inhibits the growth of HCT116 and HT-29 cells, a result reported a consequence of the compound inhibiting CDC25A, a cell cycle protein (Stolfi *et al.*, 2008). Studies have also implicated cell-cycle arrest as a cellular response to aspirin exposure (Lai *et al.*, 2008; Ricchi *et al.*, 1997). Decreased transcription of CCNB1 (Cyclin B1) which regulates cell-cycle progression at the G_2/M phase by sulindac treatment has been observed (Iizaka *et al.*, 2002). To summarise: NSAIDs can select for microsatellite stability and cause cell-cycle arrest.

2.3.1 NSAIDs and DNA repair protein expression

Microarray analysis has been extensively utilised to examine alterations in gene expression in response to NSAID exposure in a number of colon cancer cell lines (Germann *et al.*, 2003; Hardwick *et al.*, 2004; Huang *et al.*, 2006; Iizaka *et al.*, 2002; Yin *et al.*, 2006). Hardwick *et al.*, (2004) reported that cell-cycle related genes and NF-κB were repressed upon aspirin treatment. Iizaka *et al.*, (2002) reported an increase in *GADD45*α upon treatment with the NSAID sulindac. However, although, microarray analysis has been previously carried out to determine the effects of aspirin on colorectal cancer (Hardwick *et al.*, 2004; Iizaka *et al.*, 2002) these studies have not looked specifically at DNA damage signalling pathways. In addition, Hardwick *et al.*, (2004) and also Yin *et al.*, (2006) have shown that notably different gene expression patterns are seen when different concentrations of NSAID are used. Both studies showed that at low concentrations (1 mM) aspirin elicited activation of a different set of genes in the colorectal cancer cell line HT-29, compared to cells incubated with 5 mM aspirin. There is a lack of consistency in the literature regarding dosages utilised for both clinical and experimental studies. Intriguingly, clinical trials have shown that low dose aspirin (81mg) is more protective against colorectal cancer than 'high' dose aspirin (325mg) (Baron *et al.*, 2003). The variation in experimental design, especially with regards to what dose is clinically relevant makes it problematic when deciding on appropriate drug concentrations for experimental work.

In addition to the report by Iizaka *et al.*, (2002), a number of other studies have also shown NSAID regulation of growth arrest and DNA damage (GADD) gene expression. Germann *et al* (2003) analysed gene expression of CC531 colorectal cancer cells treated with 4.5mM Butyrate or 3mM aspirin and showed that *GADD153* is up-regulated upon aspirin treatment. *GADD153* expression has also been shown to be induced by celecoxib treatment in cervical cancer cells (Kim *et al.*, 2007). Microarray analysis of the colorectal cancer cell line HT-29 treated with 1mM and 5mM aspirin showed an up-regulation of *GADD45α* (Yin *et al.*, 2006). Further to this, microarray analysis also shows *GADD45* gene expression to be up-regulated by NS-398 (Huang *et al.*, 2006) and celecoxib (Fatima *et al.*, 2008) in colorectal cancer cells and by ibuprofen in human gastric adenocarcinoma cell line (Bonelli *et al.*, 2010). Suppression of *GADD45*α expression confers resistance to sulindac and indomethacin induced gastric mucosal injury and apoptosis (Chiou *et al.*, 2010) .
Other genes involved in DNA repair have also been shown to be regulated by NSAIDs. High concentrations of NS-398, ibuprofen, and RNAi mediated inhibition of COX-2 in human prostate carcinoma cells affected genes involved in DNA replication, recombination and repair (John-Aryankalayil *et al.*, 2009). *PCNA* gene expression has been shown to be up-regulated by ibuprofen treatment of a human gastric adenocarcinoma cell line (Bonelli *et al.*, 2010), and also in the human hepatocellular carcinoma cell line HepG2 treated with 25mM vanillin (Cheng *et al.*, 2007). In contrast, down-regulation of *PCNA* gene expression has been reported in colorectal cancer cell lines treated with celecoxib suggesting that these compounds may modulate cell cycle regulation in these model (Fatima *et al.*, 2008). Interestingly, *FADD* gene expression is down-regulated by NS-398 but up-regulated by indomethacin in the colorectal cancer cell line Caco-2 (Huang *et al.*, 2006). Sulindac has been reported to down-regulate *XRCC5*, *ERCC5* and *UNG* gene expression in colorectal cancer cell lines (Iizaka *et al.*, 2002). Celecoxib up-regulated *ATM*, *MAP3K2*, *CDKN1A* and *Bax* gene expression in the colorectal cancer cell line HCA-7; in contrast, *Bax* gene expression was down-regulated in the HCT116 cell line upon celecoxib treatment (Fatima *et al.*, 2008) suggesting that there may be variation in response from cell line to cell line to NSAID exposure.
We have recently reported finding that XRCC3 protein expression in SW480 cells was increased upon 1 mM aspirin treatment for 48 hours (Dibra *et al.*, 2010). The altered expression of *XRCC3* upon aspirin exposure may have implications to the sensitivity of cells to chemotherapeutic agents. Indeed, previous research in the breast cancer cell line MCF7 demonstrated that over-expression of XRCC3 induced cisplatin resistance (Xu *et al.*, 2005). Studies have shown that in contrast to XRCC3 over-expression and cisplatin resistance, *XRCC3* deficient HCT116 cells have increased sensitivity to cisplatin and also mitomycin C (Yoshihara *et al.*, 2004). Depletion of XRCC3 by siRNA in MCF7 cells inhibited cell proliferation, leading to accumulation of DNA breaks and triggering activation of p53-dependant cell death (Loignon *et al.*, 2007). Although some studies have shown no association between polymorphisms in *XRCC3* and colorectal cancer risk (Mort *et al.*, 2003; Yeh *et al.*, 2005; Tranah *et al.*, 2004; Skjelbred *et al.*, 2006) some studies have (Improta *et al.*, 2008), and in *XRCC3* polymorphisms have also been associated with breast and lung cancer susceptibility (Smith *et al.*, 2003; Jacobsen *et al.*, 2004). One essential point to note is that there is a lack of information in current literature about the effects of NSAIDs on normal human colonocytes. The information that we have relates to cell lines/*in vitro* studies and murine models. There is a need to understand the effects of these compounds on normal, healthy colon cells and how these effects prevent carcinogenesis in these cells. With the majority of the

models used in studies already established as cancerous, it is difficult to separate the effects of these compounds on normal cells rather than cells which are already cancerous and subject to pathway dysregulation. In addition to NSAIDs, flavonoids have also been associated with anti—carcinogenic properties. It is interesting to note that a recent study has shown that these compounds, which occur naturally in fruits and vegetables, not only have a protective effect against oxidative DNA damage but also increase repair activity *in vitro* (Ramos *et al*., 2010). To summarise: NSAIDs can alter the expression of DNA repair, and pro- and anti-apoptotic proteins.

2.3.2 Curcumin and DNA repair

Curcumin is a naturally occurring turmeric derivative with anti-inflammatory properties and apoptotic, anti-proliferative, anti-oxidant and anti-angiogenic effects (Shehzad *et al*., 2010). *In vivo* studies have shown that curcumin decreased intestinal polyp formation in the $APC^{min/+}$ mouse model (Murphy *et al*., 2010). Curcumin is considered an attractive compound for chemopreventative use and there are at present clinical trials ongoing testing the effectiveness of the compound against different types of cancer (Shehzad *et al*., 2010). However, as with aspirin, a known mechanism of action of curcumin is yet to be elucidated with a range of molecular targets proposed (as reviewed in Shehzad *et al*., 2010 and Ravindran *et al*., 2009). As discussed in a recent review (Burgos-Moron *et al*., 2010), although curcumin is widely regarded as a potential chemopreventative drug its safety and efficiency is yet to be fully elucidated and results from studies, clinical or otherwise, should be interpreted with this in mind. Curcumin has been shown to affect DNA damage and repair. Curcumin has been found to induce DNA damage in mouse-rat hybrid retina ganglion cells (Lu *et al*., 2009). The study reported that curcumin decreased expression of the DNA repair genes *ATR*, *ATM*, *BRCA1*, *DNA-PK* and *MGMT*. Curcumin has also been seen to cause DNA damage, as tested in the Comet Assay, in gastric mucosa cells and human peripheral blood lymphocytes (Blasiak *et al*., 1999). Curcumin has been shown to induce DNA single strand breaks (Scott & Loo, 2004) and induce the expression of the pro-apoptotic gene *GADD153* in HCT116 colonocytes. It is suggested that the up-regulation of GADD153 is a direct response to DNA damage caused by curcumin, ultimately resulting in the induction of apoptosis (Scott & Loo, 2004). Microarray analysis of human lung cancer cells after curcumin treatment saw an upregulation of GADD45 and GADD153 gene expression (Saha *et al*., 2010) and microarray analysis has also shown an up-regulation of GADD45 by curcumin treatment in a human breast cancer cell line (Ramachandran *et al*., 2005). It has recently been proposed that DNA MMR may play a role in the cellular response to curcumin (Jiang *et al*., 2010): DNA MMR proficient cells showed a greater accumulation of double strand breaks (DSB) upon curcumin treatment compared to MMR deficient cells suggesting that DSB formation induced by curcumin is primarily a DNA MMR-dependent process; further to this, curcumin was reported to activate ATM/Chk1 and cause cell-cycle arrest and apoptosis in human pancreatic cancer cells. DNA MMR proficient cells showed activation of Chk1 and induction of the G(2)-M cell cycle checkpoint (Jiang *et al*., 2010) suggesting that the curcumin induced checkpoint response may be a DNA MMR dependent mechanism. Interestingly, microarray analysis of gene expression of invasive lung adenocarcinoma (CL1-5) cells exposed to curcumin found an induction in MLH1 gene expression and reduction in MMP expression (Chen *et al*., 2004). Curcumin is also reported to affect the Fanconi anemia (FA)/BRCA pathway. Curcumin sensitises ovarian and breast cancer cells with a functional FA/BRCA pathway to cisplatin

(Chirnomas *et al.*, 2006). This is a clinically significant finding as resistance to chemotherapeutic drugs such as cisplatin is common and monoketone analogs of curcumin are now being developed as a new class of FA pathway inhibitors (Landais *et al.*, 2009). Recently, curcumin inhibition of the FA/BRCA pathway has also been suggested to be a mechanism for the reversal of multiple resistance in a multiple myeloma cell line (Xiao *et al.*, 2010). In a recent phase IIa clinical trial report, curcumin reduced aberrant crypt foci formation (Carroll *et al.*, 2011).

2.3.3 Interaction of NSAIDs with anti-cancer treatments

The capacity for specific cyclooxygenase inhibitors and NSAIDs to enhance the cellular response to chemotherapeutic and radiotherapeutic agents has been examined. Additive, synergistic and antagonistic effects have been reported. For example, the effect of a selective COX-2 inhibitor SC-236 (4-[5-(4-chlorophenyl)-3-trifluoromethyl)-1H-pyrazol-1-yl) benzene sulphonamide) was tested on cells derived from a murine sarcoma in the absence and presence of γ-ray irradiation, and a clonogenic cell survival assay confirmed that the compound significantly enhanced cell radiosensitivity (Raju *et al.*, 2002). In the human hepatocellular carcinoma cell line HepG2, indomethacin and SC-236 enhanced doxorubicin toxicity reportedly via inhibiting P-glycoprotein and the multidrug resistance-associated protein 1 (MRP1) (Ye *et al.*, 2011). Exogenous prostaglandin E2 addition failed to reverse the cellular accumulation and retention of doxorubicin and the authors concluded that the action of the drugs was via a COX-independent mechanism. Synergistic cell death characterised as being apoptotic based on Bax expression, DNA fragmentation and TUNEL assay, was observed in HT-29 cells co-treated with aspirin and 5-fluorouracil (Ashktorab *et al.*, 2005). In marked contrast to the above findings, an antagonistic activity of celecoxib and SC-236 to cytotoxicity mediated by cisplatin to human esophageal squamous cell carcinoma cells has been observed; mechanistic analysis indicated that the compounds decrease cisplatin accumulation and DNA platination, in a COX-2 independent manner (Yu *et al.*, 2011). As previously intimated, there is evidence that NSAIDs can affect double strand break repair pathways. It is known that the homologous recominbation protein Rad51 is overexpressed in chemo-radioresistent carcinomas. A study by *Ko et al.*, (2009) showed that celecoxib enhanced gefitinib induced cytotoxicity in NSCLC cells: combined celecoxib/gefitinib treatment resulted in the reduction of Rad51 protein levels. Degradation of Rad51 occurred via a 26S proteasome-dependent pathway. Celecoxib has been shown to inhibit growth of head and neck carcinoma cells and enhance radiosensitivity in a dose-dependent manner: celecoxib downregulated Ku70 protein expression and inhibited DNA-PKcs kinase activity which is known to be involved in DSB repair (Raju *et al.*, 2005). Further to this, sodium salicylate has been shown to inhibit the kinase activity of ATM and DNA-PK which suppresses their DNA damage response (Fan *et al.*, 2010). A recent study in prostate cancer demonstrated that treatment of the prostate cancer cell line PC-3 with NO-sulindac increased the rate of single strand DNA breaks and that there was slower repair of these lesions (Stewart *et al.*, 2011). We thus suggest that caution is exercised in situations where patients are prescribed NSAIDs when undergoing chemotherapy or radiotherapy as unexpected additive or antagonistic reactions may arise, and thus potentially compromise treatment effectiveness. However, there is also the promise that NSAIDs (either known or novel) may significantly synergise and enhance responses to chemotherapeutic, biologic, or radiotherapeutic modalities.

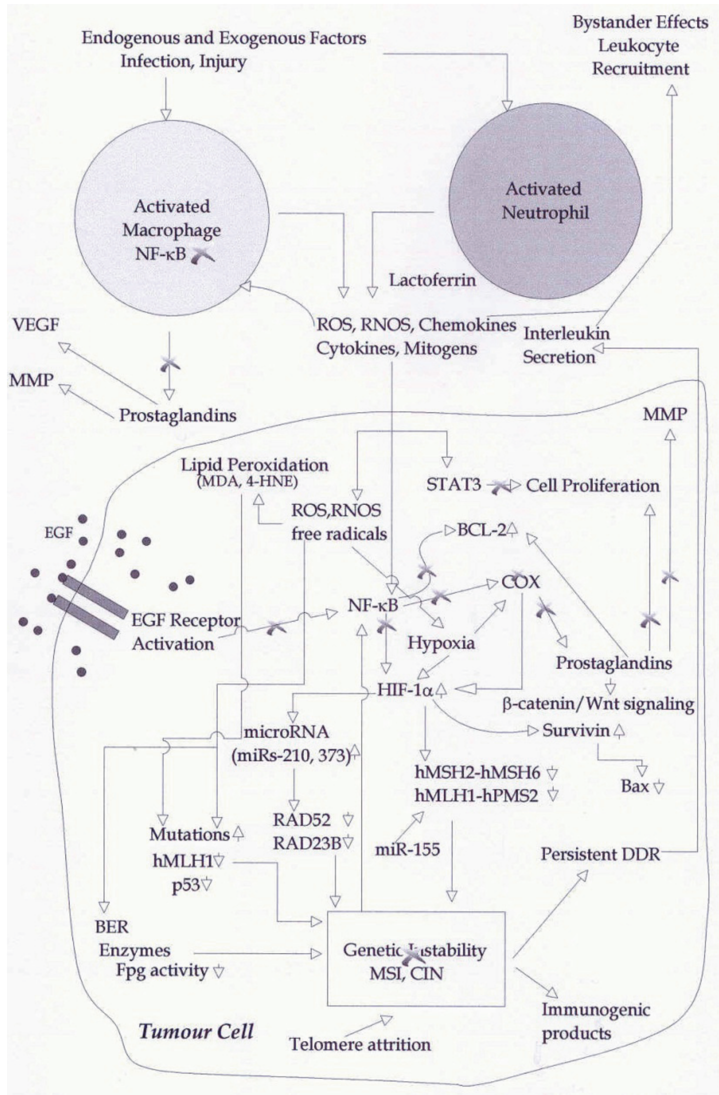

Fig. 2. A schematic illustration (composite) of the possible interconnections of the molecules and activities cited in the text. Where NSAIDs can potentially act is illustrated with **X.** Abbreviations: BER, base excision repair; CIN, chromosomal instability; COX, cyclooxygenase; DDR, DNA damage response; EGF, epidermal growth factor; Fpg, formamidopyrimidine-DNA glycosylase; 4-HNE, trans-4-hydroxynonenal; HIF-1α, hypoxia inducible factor-1α; MDA, malondialdehyde; MIN, microsatellite instability; MMP, matrix metalloproteinase; miR, micro RNA; NF-κB, nuclear factor kappa B; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor. For reasons of clarity, chemokine and cytokine receptor interactions are not shown.

3. Conclusion

The accumulated findings from a significant body of research indicates that NSAIDs – including aspirin- act via multiple mechanisms in reducing the morbidity and mortality of cancer, and that aspirin and other NSAIDs reduce inflammation via COX-dependent *and* COX-independent pathways. NSAIDs can select for DNA mismatch repair competency and microsatellite stable cells thus inhibiting at least one well recognised pathway of colorectal cancer progression; furthermore, *in vitro* data suggest that NSAIDs exhibit 'direct' and relatively specific and rapid toxicity to colorectal cancer cells, with evidence suggesting that this may (but almost certainly not exclusively) involve DNA repair pathways. There is a paucity of information regarding the effect of NSAIDS on gene expression in non-transformed cell lines, including colonocytes and stromal cells and stem cells. Epidemiological evidence strongly indicates that NSAIDs are particularly protective against adenocarcinoma formation. Given the potential as chemopreventative agents, significant effort should be directed into producing novel NSAID derivatives that do not produce the adverse gastrointestinal and cardiovascular effects but retain the multiple and potent protective actions that are involved in suppressing adenocarcinoma formation.
We feel that the following questions need to be addressed:

a. What is the mechanism by which aspirin selects for microsatellite stability?
b. What is the mechanism by which aspirin and other NSAIDs show their relatively specific toxicity to colorectal cancer cells?
c. Does long term usage of aspirin and other NSAIDs preferentially reprogram the colonic epithelium via epigenetic mechanisms *eg* altering histone deacetylase and DNA methyltransferase activities?
d. How do NSAIDS affect gene expression in non-transformed cells, including colonic stem cells? Do NSAIDs affect epithelial miRNA profiles?

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