

# Multiple Functions of Generic Drugs: Future Perspectives of Aureolic Acid Group of Anti-Cancer Antibiotics and Non-Steroidal Anti-Inflammatory Drugs

Hirak Chakraborty<sup>1,§</sup>, Pukhrambam Grihanjali Devi<sup>2,§</sup>, Munna Sarkar<sup>1,\*</sup> and Dipak Dasgupta<sup>2,\*</sup>

<sup>1</sup>Chemical Sciences Division and <sup>2</sup>Biophysics Division, Saha Institute of Nuclear Physics, Sector-I, Block-AF, Bidhannagar, Kolkata-700 064, India

**Abstract:** Non-steroidal anti-inflammatory drugs and aureolic acid group of anti-cancer drugs belong to the class of generic drugs. Research with some members of these two groups of drugs in different laboratories has unveiled functions other than those for which they were primarily developed as drugs. Here we have reviewed the molecular mechanism behind the multiple functions of these drugs that might lead to employ them for treatment of diseases in addition to those they are presently employed.

## 1. INTRODUCTION

'Old drugs' that have been in the market for long constitutes a large pool of compounds available for further research. Many of these drugs have outlived their patents, allowing them to be legally produced as generic drugs. Generic drugs have the same chemical ingredients as their brand name counterpart and show the same benefits and risks. Since they are 'off patented' the cost of production is low keeping their pricing much cheaper than their brand name counterparts. Many of these drugs in the market show unconventional functions, which are quite distinct from the function other than the intended one. Understanding the molecular mechanism behind these unconventional functions would allow the utilization of these 'old drugs' for new disease targets. Recently, phenotype and molecular target based screening of generic drugs against multiple targets have become an important strategy in accelerating rational drug design [1]. On the other hand, repositioning approach typically uses an interesting side effect of an approved medication to develop it for its new function [2]. An example is that of 'Propecia', a drug now used in the treatment of hair loss was developed to act against benign enlarged prostate gland. The advantage of using old medication for novel application is that their doses, *in vivo* pharmacokinetics and ADMET (Adsorption, Distribution, Metabolism, Excretion, Toxicity) profiles are well studied since they have passed the necessary clinical trials for their conventional use. Another conceptually interesting approach, aimed at reducing side effects of a drug, is based on targeted drug delivery using tumor specific peptides capable of translocating drugs across cell membranes [3]. This allows better internalization of the drugs allowing delivery of the dosage required for tumor elimination. Drug resistance in cancer therapy is a common problem. Recently, drug resistance against common cancer drug

cisplatin, has been overcome using gene therapy. Infection with a recombinant adenovirus expressing the human retinoblastoma tumor suppressor gene is sufficient to impart lethality in tumor cells in absence of cisplatin by triggering cell cycle arrest in the G1 phase [4]. Even though rapid screening against multiple targets allows identification of novel hits, understanding the molecular mechanism behind the multiple functions of generic drugs might lead to a better usage of these drugs. The approach helps to develop a new class of drugs based on the chemical platform of old drugs but aimed at a specific function. In this review we will discuss the multiple functions of two classes of generic drugs, viz. synthetically produced non-steroidal anti-inflammatory drugs (NSAIDs) and aureolic acid group of anti-cancer antibiotic obtained from bacterial sources.

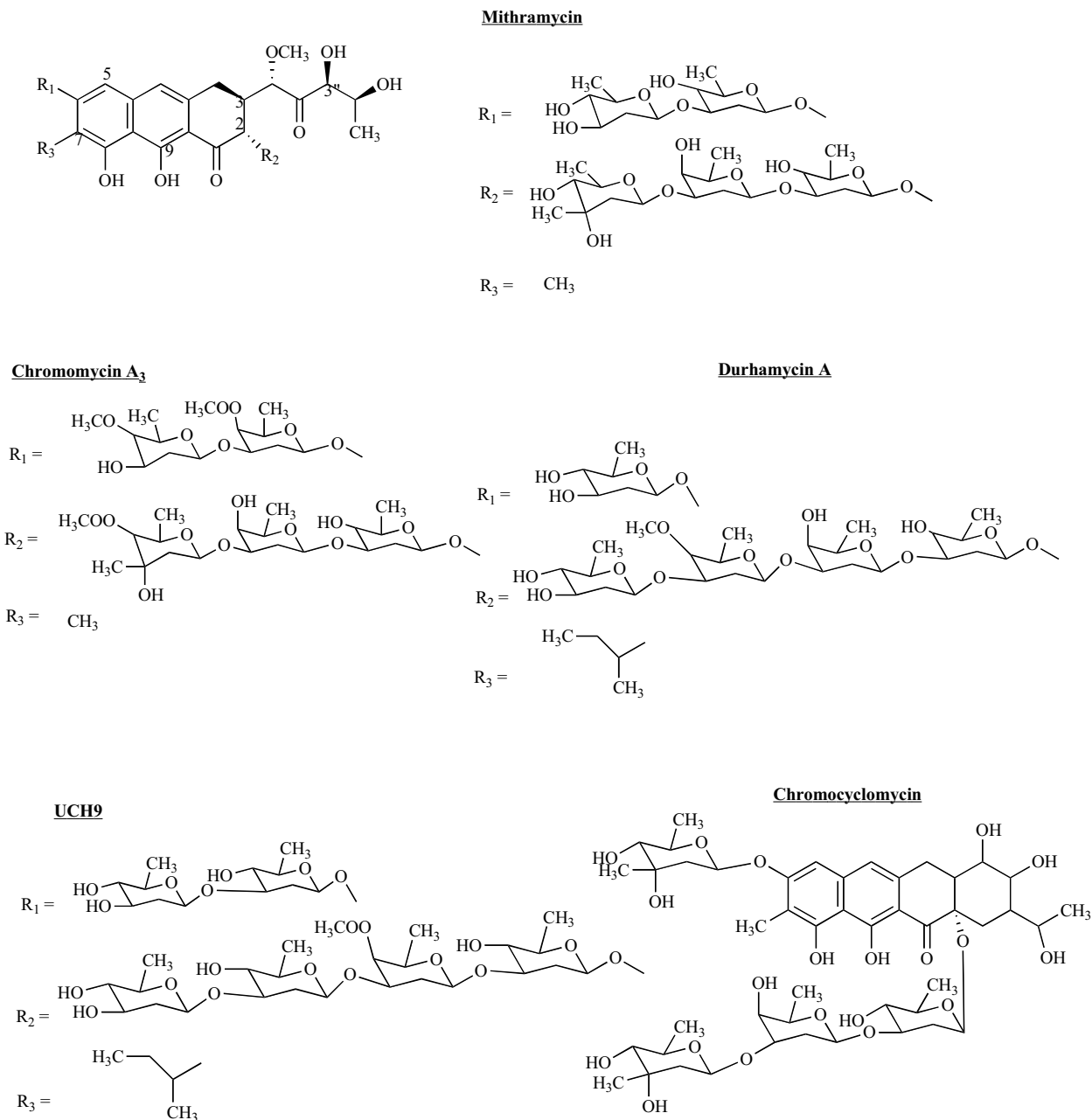
The conventional use of NSAIDs is to control pain and inflammation. This class of drugs has been in the market for a very long time with aspirin being the oldest drug that was marketed more than hundred years back. The principal targets for their conventional function are cyclooxygenases (COX) enzymes, which are membrane-associated proteins. There are two isoforms of COX, viz. COX-1 and COX-2 [5]. Over the past one decade research have shown that these NSAIDs can have several other functions which include chemoprevention [6,7] and chemosuppression [8,9] against several types of cancers, protection against neurodegenerative diseases like Alzheimer disease (AD) [10-12], UV-sensitizer [13-15], UV-protector [16,17] etc. Till date, there seems to be no general consensus as to the exact mechanism behind these novel functions. Several targets have been implicated in almost all the different functions. Not all NSAIDs show same kind of behavior towards a specific function and a great variation exist in the extent of their efficacies. Since NSAIDs encompasses several chemical motifs, a closer look at the chemical basis of their novel function could be a good approach for future drug discovery/ designing.

The aureolic acid anticancer antibiotics, chromomycin A<sub>3</sub> (CHR) and mithramycin (MTR), (Fig. (1)) are the two naturally occurring antibiotics first reported from *Streptomyces gresius*, and *Streptomyces plicatus*, respectively, in the 1950s

\*Address correspondence to these authors at the Chemical Sciences Division and Biophysics Division, Saha Institute of Nuclear Physics, Sector-I, Block-AF, Bidhannagar, Kolkata-700 064, India;

E-mail: dipak.dasgupta@saha.ac.in / munna.sarkar@saha.ac.in

§ Both authors contributed equally in writing the review.



**Fig. (1).** Structures of aureolic acid antibiotics.

and 1960s [18]. Since then a number of structurally related antibiotics have been reported from different bacterial sources. They are glycosylated aromatic polyketides with an intense yellow color and fluoresce under UV light, which is the genesis for the name of the family. With the exception of chromocyclomycin, which is a tetracyclic compound, the aglycons of this family consist of a tricyclic ring system fused to a unique dihydroxy-methoxy-oxo-pentyl aliphatic side chain attached at C-3. In some compounds, a small alkyl residue (methyl, isobutyl) is attached at position C-7. Each consists of the chromomycinone moiety, the aglycon ring either side of which is linked to six-membered sugar residues

via O-glycoside linkages [19]. Aureolic acids, MTR and CHR, were initially meant to be used for their antibiotic activity against gram-positive bacteria but now have clinical applications because of its anti-cancer property. Earlier studies with the antibiotics have proposed that they act by inhibiting DNA-dependent RNA synthesis both *in vivo* and *in vitro* via reversible interaction with (G.C)-rich DNA [18-21] in the presence of bivalent metal ion, like  $Mg^{2+}$ . They inhibit the expression of genes with (G.C) rich promoter. Even though their role as anticancer drugs has been studied well, there has been resurgence in the study of these antibiotics to examine their therapeutic potential for the treatment of

human disorders other than cancer. Extensive work of isolation and characterization has been done on the biosynthesis gene clusters of the two antibiotics [22,23]. The knowledge about the biosynthetic pathway of the antibiotics along with the identification of the associated gene cluster have opened the prospect of employing genetically modified structural analogues for therapeutic purpose.

In this review we shall briefly highlight the alternate functions of NSAIDs as well as that of aureolic acid anticancer antibiotics. An approach to understand the chemical basis of unconventional use of these drugs will also be discussed. It should be mentioned that NSAIDs encompasses several chemical motifs, whereas the principal chemical motif of the aureolic acid anti-cancer antibiotics are same with small changes in the sugar moieties. It is therefore expected that for the NSAIDs, the chemical structure should play a more determining role in the manifestation of their new functions. However, as will be shown later, even small changes in the sugar moieties of the aureolic acid antibiotics can affect the extent of their various functions.

## 2. PRINCIPAL FUNCTIONS OF NSAIDS AND AUREOLIC ACID ANTICANCER ANTIBIOTICS AND THEIR MECHANISM OF ACTION

Non-steroidal anti-inflammatory drugs (NSAIDs) have been commonly used to reduce pain and inflammation in different arthritic and post-operative conditions [5,24]. NSAIDs are also used as anti-pyretic, analgesic and uricosuric agents [25]. Their anti-inflammatory effect is mainly due to their ability to inhibit the activities of cyclooxygenases (COX) enzymes that mediate the production of prostaglandins from arachidonic acid, which is a dietary fatty acid (Fig. (2a)). There are two isoforms of cyclooxygenases viz., cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [5,26]. Prostaglandins are powerful signaling agents in the human body. Some prostaglandins, mainly synthesized by the COX-2 isoform are substantially involved in bringing about and maintaining inflammatory processes by increasing vascular permeabilities and amplifying the effects of other inflammatory mediators such as kinins, serotonin and histamin [27]. Hence reducing and controlling the formation of

these prostaglandins can reduce the swelling, heat and pain of inflammation. However, not all prostaglandins are harmful for the human body. Some of them, synthesized by COX-1, are important in protecting the stomach lining, promoting clotting of blood, regulating salt and fluid balance maintaining blood flow to the kidneys etc. [28]. Hence inhibition of COX-1 will lead to loss of many prostaglandins important for the homeostatic functions of the human body and is not desirable. Designing COX-2 specific inhibitors is an important strategy in controlling inflammatory processes.

X-ray crystallography of the 3-D structures of COX-1 and COX-2 has done much to illuminate how COX-2 specific NSAIDs can be designed. The two isoforms, COX-1 and COX-2 have very similar 3-D structures consisting of a long narrow channel with a hairpin bend at the end [5]. The isoforms are membrane-associated so arachidonic acid released from damaged membranes adjacent to the opening of the enzyme channel, which is largely hydrophobic, is sucked in, twisted around the hairpin bend, two oxygens are inserted and a free radical extracted, resulting in the five-carbon ring that characterizes prostaglandins [5]. From fluorescence quenching study of arachidonic acid it is found that older NSAIDs block both COX-1 and COX-2 about halfway down the channel. X-ray crystallography suggested that this blocking occurs by hydrogen bonding to the polar arginine at position 120 (Fig. (2b)) leading to non-specific inhibition of the two isoforms. A single amino acid difference is critical for the selectivity of many drugs. At position 523 there is an isoleucine molecule in COX-1 and a valine (smaller by a single methyl group) in COX-2. The smaller valine molecule in COX-2 leaves a gap in the wall of the channel (Fig. (2b)), giving access to a side pocket, which is thought to be the site of binding of many COX-2 selective drugs. The bulkier isoleucine at 523 in COX-1 is large enough to block access to the side pocket. So targeted single amino acid substitution of valine for isoleucine is sufficient to turn COX-1 into a protein that can be inhibited by COX-2 selective inhibitors [29, 30]. Various NSAIDs have been designed with differential specificity towards COX-1 and COX-2 using different chemical templates. NSAIDs can be classified according to their chemical structure.

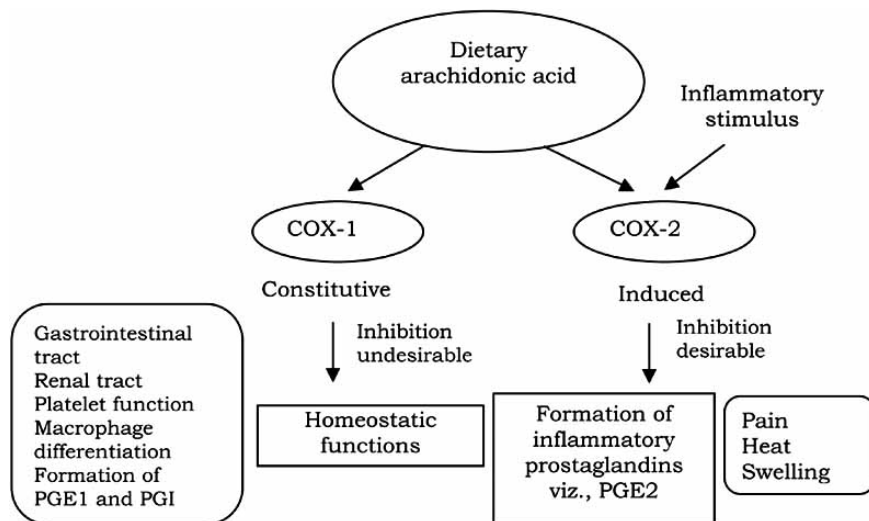


Fig. (2a)). Mechanism of action of NSAIDs: The Cyclooxygenase Pathway.

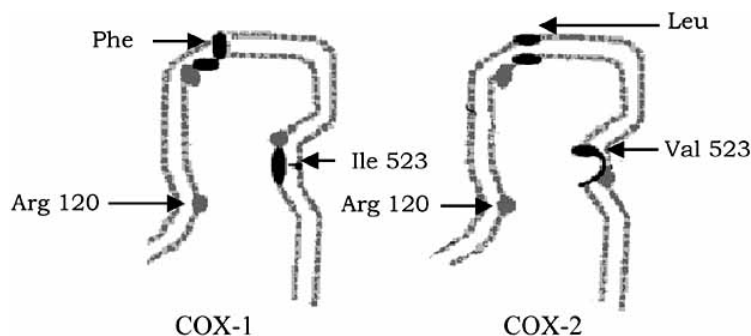


Fig. (2(b)). Schematic Diagram of COX-1 and COX-2.

Different chemical templates that are being used as NSAIDs have differential efficiency against COX-1 and COX-2. For a particular group of NSAIDs, different drugs are synthesized by the method of isosteric substitution considering a particular drug as the mother template [29]. For example, in the oxicam group of NSAIDs, piroxicam is the mother compound and meloxicam, tenoxicam, lornoxicam etc. are synthesized by small changes in the piroxicam chemical template. Generally NSAIDs are broadly divided into six major classes as shown in Table 1. Of these, aspirin, belonging to the salicylic acid group is the oldest NSAID in the market. NSAIDs, designed after the discovery of the structural differences between COX-1 and COX-2 isoforms, show better selectivity towards COX-2. Coxibs (rofecoxib, celecoxib) and NS-398 are highly selective towards COX-2 whereas flurbiprofen, ketoprofen etc show high level of selectivity towards COX-1. Dichlofenac, etodolac, meloxicam, nimesulide etc are relatively COX-2 selective whereas aspirin, ibuprofen, indomethacin have equal affinity towards COX-1 and COX-2. Though it was assumed that COX-2 inhibition is the most effective pathway in controlling pain and inflammation but there are several other functions of COX-2, which are important for homeostasis in health. COX-2 is expressed constitutively in kidney particularly in macula densa, cyclical induction of COX-2 has an important role in ovulation, uterine COX-2 induced at the end of pregnancy, where it is important for the onset of labor and COX-2 inhibitors cause fluid retention [5]. It is because of these homeostatic functions of COX-2, COX-2 specific inhibitors viz., rofecoxib and celecoxib show side effects leading to myocardial infarction and hence rofecoxib has been withdrawn from the market. Therefore use of COX-2 specific NSAIDs may land up into more complex situation of side effects than gastrointestinal ulceration caused by older non-specific COX-inhibitors. So in controlling pain and inflammation, the preferential COX-2 inhibitors or older NSAIDs may be much more effective because their side effects are well studied and can be managed by using combination drug treatment.

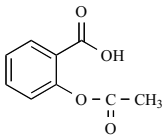
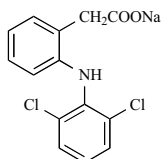
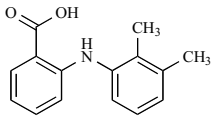
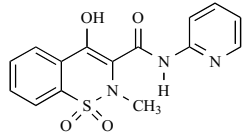
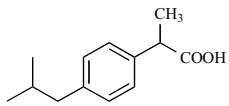
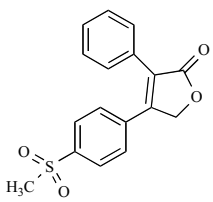
Despite the above problems it may not be wise to discard the COX-2 selective NSAIDs because they have immense potential against various diseases other than to control pain and inflammation. In the past few years it has been shown that COX-2 is a key player in many biochemical processes like apoptosis, angiogenesis, amyloidoses, etc., which will be discussed in the subsequent sections of this review. So modification may be made using the present day COX-2 inhibi-

tors as the main chemical templates to develop drugs against various diseases other than their traditional use, which will be more economic and time saving.

As has been mentioned before, the aureolic acid anticancer antibiotics chromomycin A<sub>3</sub> (CHR) and mithramycin (MTR) (Fig. (1)) act *via* inhibition of DNA-dependent RNA synthesis both *in vivo* and *in vitro*. The presence of bivalent metal ion, like Mg<sup>2+</sup>, is an obligatory factor for the transcription inhibitory property at physiological pH. The anionic antibiotic binds to the metal ion [31,32] and the resulting complex(es) is(are) the DNA binding ligand(s) at and above physiological pH. They bind to DNA *via* minor groove [33-35]. It was established in our laboratory from spectroscopic and thermodynamic studies that the modes of binding of the two ligands with natural DNA, polynucleotides and oligomeric duplexes are different [31-35]. We also illustrated the role of DNA minor groove size and the accessibility of the 2-amino group in the minor groove of guanosine in drug-DNA interaction using designed nucleotide sequences [35-37]. Detailed NMR studies from other laboratories have helped to understand how the bulky complex of the type [(drug)<sub>2</sub>Mg<sup>2+</sup>] is accommodated at the cost of a considerable widening of the minor groove in B-DNA type structure [38, 39]. In our laboratory we have shown from a detailed thermodynamic analysis of the association of the dimer complex with different DNAs, natural and synthetic, with defined sequences, that B to A type transition in the groove leads to a positive change in enthalpy. This is compensated by a positive change in entropy arising from the release of bound water in the minor groove. Sugars present in the antibiotics play a significant role during the association with nucleic acids [33-37]. Absence of substituents like acetoxy group in the sugar moieties of mithramycin imparts conformational flexibility to a greater degree than chromomycin. Therefore, the drug dimer of mithramycin has been found to have a better conformational plasticity than chromomycin when it binds to the minor groove of DNA. Its strong antitumor activity against a number of cancer cell lines has been ascribed to the DNA-binding property drug-metal complex(es).

As these antibiotics specifically bind to GC-rich regions, they are employed as strong inhibitors of specific promoter regions like *c-myc* [40] and *c-src* [41] thus preventing the association of regulatory proteins and transcription factors like Sp1 and the resulting formation of the transcription initiation complex. It has an adverse effect upon the general processes such as transcription elongation. DNase I foot

Table 1. Different Chemical Groups of NSAIDs

Class	Example	Template
Acetyl salicylic acid	Aspirin	 Aspirin
Acetic acid	Diclofenac, Indomethacin Ketorolac, Nabumetone, Sulindac, Tolmetin	 Diclofenac sodium salt
Fenamates	Meclofenamate, Mefenamic acid	 Mefenamic acid
Oxicam	Piroxicam, Meloxicam Tenoxicam, Lornoxicam	 Piroxicam
Propionic acid	Ibuprofen, Ketoprofen, Naproxane, Oxaprozin	 Ibuprofen
Coxib	Celecoxib, Rofecoxib Valdecoxib	 Rofecoxib

printing has identified that MTR binds to the P1 and P2 promoter regions of the *c-myc* gene [42]. This also explains their antiviral property of deactivating the HIV-I provirus [43]. Treatment of different cancer cell lines with MTR was found to facilitate different apoptotic pathways such as TNF [44], tumor necrosis factor- $\alpha$ -related apoptosis-inducing ligand (TRAIL) [45] and Fas [46]. These properties give MTR (trade name Plicamycin) its clinical application as an FDA approved drug. MTR is clinically employed for the treatment of neoplastic diseases like chronic myelogenous leukemia, testicular carcinoma and Paget's disease [18].

Since the above promoters are part of the chromatin, we have studied the effects of these drugs upon the chromatin structure [47-51]. Spectroscopic studies such as absorbance, fluorescence and CD have demonstrated directly the associa-

tion of the above complexes with chromatin and its components under different conditions [47,48]. The reduced binding affinity of the antibiotic:  $Mg^{2+}$  complexes to nucleosome or chromatin might be a consequence of bending of double helix or, additionally, of unusual DNA conformations induced by the histone binding [49,50]. Presence of histones might also reduce the accessibility of the minor groove to this class of groove binders. Alternatively, one can say that histone-DNA contacts and N-terminal tail domains of individual core proteins in nucleosome core particle reduce the accessibility of nucleosomal DNA to antibiotic:  $Mg^{2+}$  complexes [51]. In the chromatin, presence of linker H1 further reduces the binding potential of the ligand. These drugs also induce instability in nucleosome leading to DNA release.

These antibiotics also show other functions quite diverse in nature. They are briefly described below.

### 3. OTHER FUNCTIONS OF THESE DRUGS AND MECHANISM OF ACTIONS

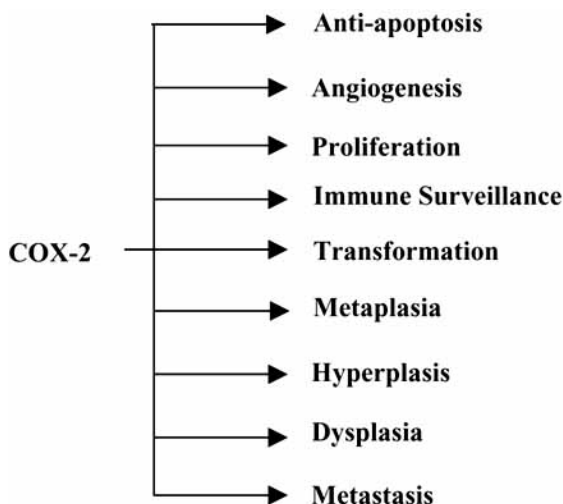
#### 3.1. Other Functions of NSAIDs

##### 3.1.1. Chemoprevention and Chemosuppression

Numerous experimental, epidemiological and clinical studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) have a great potential as anticancer agents [7,9, 52-57]. Again nonrandomized epidemiological studies have found that long-term users of aspirin or other NSAIDs have a lower risk of colorectal adenomatous polyposis and colorectal cancer than non-users [58]. There exists a wealth of data which show that NSAIDs have both chemoprevention and chemosuppression ability. Two schools of thoughts exist regarding the molecular mechanism of these drugs as chemopreventive and chemosuppressive agent. There is enough literature, which supports the COX-dependent mechanism whereas many data exist in literature, which opposes the COX-dependent pathway. We will present some key studies that demonstrate both schools of thoughts.

##### 3.1.1.1. COX-Dependent Pathway

Apart from producing different prostaglandins from arachidonic acid multiple lines of compelling evidences support that COX-2 plays a crucial role in carcinogenesis (Scheme-1). Molecular studies on the relationship between polyunsaturated fatty acid metabolism and carcinogenesis have revealed novel molecular targets for cancer prevention and treatment [59,60]. Literature data exists that show overexpression of COX-2 in tumor cells of colon carcinoma [61], colorectal carcinoma [62], esophageal carcinoma [63], pancreatic carcinoma [64], malignancies of breast, skin, cervix, ovary, bladder, head, neck, etc [65]. In addition to the finding that COX-2 is commonly over-expressed in premalignant and malignant tissues, there exists considerable evidence that links COX-2 to the development of cancer. The most specific data that support a cause-and-effect connection between COX-2 and tumorigenesis come from genetic studies. Multiparous female transgenic mice are engineered to overex-



Scheme 1.

press human COX-2 in mammary glands, develop focal mammary gland hyperplasia, dysplasia and metastatic tumors [66]. These findings are consistent with the idea that under some conditions, increased expression of COX-2 induces tumor formation. In a related study, transgenic mice that overexpress COX-2 in skin develop epidermal hyperplasia and dysplasia. Consistent with these studies, knocking out COX-2 markedly reduces the development of intestinal tumors and skin papillomas.

Colon cancers are thought to arise as a result of a series of histopathologic and molecular changes that transform normal colonic epithelial cells into a colorectal carcinoma, with an adenomatous polyp as an intermediate step in the process.

Analysis of COX-2 expression shows that it is elevated in up to 90% of sporadic colon carcinomas and 40% of colonic adenomas but is not elevated in the normal colonic epithelial cells [67]. Increased level of COX-2, prostaglandins or both are found in adenomas of the patients with familial adenomatous polyposis (FAP) and in experimentally induced colon tumors in rodent models [68]. COX-2 overexpression in normal alveolar type II cells may be directly involved in increasing the sensitivity of these cells to the effects of carcinogens and enhancing tumor development after initiation [69]. The Ras/ERK signaling pathway appears to play a role in the regulation of COX-2 expression. Human non-small cell lung cancer cell lines with mutations in Ki-Ras have high expression levels of COX-2, and inhibition of Ras activity in these cell lines decreases COX-2 expression [70]. It has also been found that gall bladder cancer cell growth can be stimulated by mitogens and that mitogens can decrease apoptosis. A specific COX-2 inhibitor can decrease the mitogenic stimulus, and the COX-2 inhibition can increase apoptosis. The decreased mitogenesis and increased apoptosis produced by the COX-2 inhibitors were associated with decreased PGE<sub>2</sub> formation [7]. Eberhart *et al.* [71] and others demonstrated that COX-2 enzyme is overexpressed in human colorectal tumors compared with adjacent normal colonic mucosa [71,72]. Employing a specific COX-2 inhibitor, NS-398, Tsuji *et al.* [73] demonstrated that proliferation of a gastric cancer cell lines and a colon cancer cell lines could be inhibited by the COX-2 enzyme inhibitor [73,74]. Others have subsequently demonstrated that in a variety of epithelial cell lines, specific COX-2 inhibitors decrease mitogenesis [75,76]. The studies in animal models also confirm that the polyposis is directly related to the COX-2 expression. Mice containing mutations in both the adenomatous polyposis coli (APC) gene and the COX-2 gene developed fewer intestinal polyps than mice with a functional COX-2 gene [77]. A new selective COX-2 inhibitor, SC58125, suppressed tumor growth *in vivo* and induced apoptosis *in vitro* in cell lines that express high levels of COX-2, but the COX-2 inhibitor was ineffective in HCT-116 cells, which have undetectable level of COX-2 expression [76]. These studies place COX-2 in a central position of colon carcinogenesis and suggest that selective COX-2 inhibition may be a useful approach for chemoprevention or even treatment of the cancers, particularly those with high levels of COX-2 expression.

There are evidences that COX-2 inhibitors can also act as anti-angiogenic agents. The link between the COX-2 activity and vascular endothelial growth factor (VEGF) production

and action has been established. The disruption of COX-2 gene in mice dramatically suppressed VEGF production in fibroblasts [78] and tumor cells [79]. Again COX-2 inhibitors prevented VEGF-induced MAPK activation in endothelial cells. Ruegg *et al.* [80] have established a link between COX-2 and integrin  $\alpha$ V $\beta$ 3-mediated endothelial cell migration and angiogenesis [80-82]. Inhibition of COX-2 activity in endothelial cells by NSAIDs suppressed  $\alpha$ V $\beta$ 3-dependent endothelial cell spreading and migration *in vitro* and FGF-2 induced angiogenesis *in vivo* [83-85]. Exogenous PGE<sub>2</sub> rescued endothelial cell spreading and migration in the presence of COX-2 inhibitors [81,86]. The effect of NSAIDs was due to the inhibition of  $\alpha$ V $\beta$ 3-dependent activation of Cdc42 and Rac, two members of Rho family of GTPases that regulate cytoskeletal organization and cell migration. Besides promoting Rac activation and cell spreading, the COX-2 metabolite PGE<sub>2</sub> also accelerates  $\alpha$ V $\beta$ 3-mediated endothelial cell adhesion [86]. The important role of Rac in angiogenesis was also demonstrated by VEGF-required Rac activation [82,87] and the inhibition of the Rac effector p21-activated kinase (PAK)-1, suppressed endothelial cell tube formation *in vitro* and angiogenesis in the chick CAM assay *in vivo* [88,89].

So the multiple lines of evidences indicate that COX-2 is an important pharmacological target for anti-cancer therapy. Epidemiological studies show that use of NSAIDs, prototypic inhibitors of COX-2, is associated with a reduced risk of several malignancies, including colorectal cancers. Consistent with this, tumor formation and growth are reduced in animals that are engineered to be COX-2 deficient or treated with a selective COX-2 inhibitor. In the clinical trial, it has been found that treatment with celecoxib, a selective COX-2 inhibitor, reduced the number of colorectal polyps in patients with familial adenomas polyposis (FAP) [90,91]. Based on these findings many clinical trials are under way to assess the potential efficacy of selective COX-2 inhibitors in preventing and treating human cancers.

### **3.1.1.2. COX-Independent Pathway**

In the previous section we have presented evidences of COX-2 as being an important target for the chemopreventive and chemosuppressive functions of NSAIDs. However, the precise mechanisms by which various NSAIDs exert their antiproliferative effects on cancer cells are still controversial. Emerging evidences suggest that these effects can, at least in some cases, be exerted through COX-2 independent pathways. In this section of the review, we will discuss the recent progress in understanding the different COX independent pathways that lead to chemoprevention and chemosuppression by the NSAIDs.

Several independent studies have shown that various NSAIDs can show apoptotic effect in cell lines irrespective of their level of expression of COX-1 and COX-2. For example, indomethacin, a non selective COX-inhibitor, induced apoptosis in both Seg-1 (COX-1/2 positive) and Flo-1 (COX-1/2 negative) esophageal adenocarcinoma cells [92]. Sulindac sulfide and sulindac sulfone induced apoptosis in malignant melanoma cell lines independent of COX-2 expression [93]. Using cell lines with controlled COX-2 expression, they were unable to detect any differences between

COX-2 expressing and COX-2 deficient Caco-2 cell clones in the ability of celecoxib to inhibit the cell cycle [94]. Indomethacin and NS398 had antiproliferative activity on both COX-2 positive cell line (HT29 and HCA7) and COX-2 negative cell line (SW480 and HCT116) [95]. Sulindac sulfide and piroxicam induced apoptosis in both COX-2 expressing HT29 human colon cancer cell lines and COX-2 deficient HCT15 cells [96]. Furthermore, though the COX-2 inhibiting ability of rofecoxib and celecoxib is similar, but celecoxib has a much higher antiproliferative activity in COX-2 positive A549 epithelial cells and COX-2 negative BALL1 hematopoietic cells than rofecoxib [97]. NS398, a COX-2 selective inhibitor, induced apoptosis in HT29 (COX-2 positive) and S/KS (COX negative) human colorectal carcinoma cell lines with comparable IC<sub>50</sub> [98]. In addition to these studies with a spectrum of cancer cell lines it has also been demonstrated that cells genetically engineered to lack expression of COX-1 and COX-2 or both can remain sensitive to the antiproliferative effects of NSAIDs indicating that NSAIDs can bypass COX to exert their anti-cancer effect.

The heterozygote Min/+ mouse model, like patients with FAP, carries a nonsense mutation in the APC gene that results in the spontaneous development of intestinal adenomas (100% incidence). Administration of sulindac to Min/+ mice reduced the tumor number but did not alter the level of PGE<sub>2</sub> and leukotriene B<sub>4</sub> in intestinal tissues [99]. Furthermore, increasing PGE<sub>2</sub> and interleukine B<sub>4</sub> levels with dietary arachidonic acid supplementation had no effect on tumor number or size [99]. Similarly, when PGE<sub>2</sub> is given to rats concomitantly with indomethacin does not reverse the tumor reducing effect of indomethacin in these animals [100]. In support, it has been shown that celecoxib has an antitumorigenic effect in COX-2 deficient tumors in the nude mice model and also induces apoptosis in the cells, which do not express COX-2 [101]. Again some NSAID derivatives that do not inhibit COX activity retain their chemopreventive activity in the Min/+ mouse model of intestinal polyposis [102]. R-flurbiprofen induces cell cycle blocking and apoptosis in human colon carcinoma cell lines HCT116 by activating C-Jun-N-terminal Kinase (JNK) and down-regulating cyclin D1 expression [103]. Sulindac sulfone, the oxidative metabolite of sulindac, is completely devoid of COX-inhibitory activity but inhibits growth and induces apoptosis in variety of human cancer derived cell lines [104].

Additional studies indicate that sulindac sulfone and its derivatives CP248 and CP461, which activate PKG, lead to rapid and sustained activation of JNK1, a kinase known to play a role in the induction of apoptosis by other cellular stress related events. Mechanistic studies indicate the existence of a novel PKG-MEKK1-SEK1-JNK1 pathway for the induction of apoptosis by sulindac sulfone [105]. Once activated JNK1 plays a role in apoptotic signaling pathways, JNK1 can phosphorylate and inactivate the anti-apoptotic proteins Bcl-2 and Bcl-XL [106] and it can include the expression of pro-apoptotic proteins (Bad and Bim) through activation of the transcription factor AP-1. MEK/ERK signaling may regulate mitochondrial events that lead to activation of caspases.

Akt plays a key role in tumorigenesis and cancer progression by stimulating cell proliferation and inhibiting apoptosis [107]. The Akt is composed of a -NH<sub>2</sub> terminal pleckstrin homology domain and a -COOH terminal kinase catalytic domain. It is activated by a dual regulatory mechanism that requires both translocation to the plasma membrane and phosphorylation. Recently, Wu et. al. have demonstrated that celecoxib regulates the phosphorylation of Akt and inhibited PDK1 and PTEN phosphorylation in cholangiocarcinoma cells [108]. The anti-sense depletion of COX-2 failed to alter the level of phospho-Akt, which indicates the existence of COX-2 independent effect. This result is supported by the studies from other investigations showing that celecoxib induces apoptosis *via* COX-2 independent mechanism in other human cancer cell lines. In a separate study Lai et. al. [109] showed that in suppression of rat cholangiocarcinoma (cultured C611B cells) and neu-transformed WB344 rat liver epithelial stem-like cells (WBneu cells), concentration of celecoxib needed to suppress growth and induce apoptosis was markedly higher than that needed for effective inhibition of PG production by these malignant cell types. Studies also show that celecoxib reduces neointimal hyperplasia after angioplasty through inhibition of Akt signaling in a COX-independent manner. Results suggested that celecoxib affects the Akt/GSK signaling axis, leading to vascular smooth muscle cells (VSMC) proliferation and an increase in VSMC apoptosis [110]. Several reviews have been devoted to highlight the detail effect of different NSAID and/ or COX-2 inhibitors on different cancers. Different COX-independent targets have been indicated that might play a crucial role for the NSAID to exert their anticancer effect. There are many established COX-independent pathways, which includes cell surface death receptor-mediated pathway [111,112]. This

pathway is initiated by extracellular hormones or agonists that belong to the tumor necrosis factor (TNF) super family including TNF $\alpha$ , Fas/CD95 ligand and Apo2 ligand. These agonists recognize and activate their corresponding receptors, members of TNF/NGF receptor family, such as TNFR1, Fas/CD95 and Apo2. Another important target in the COX-independent pathway is nuclear factor kappa B (NF- $\kappa$ B) [104]. In vertebrates Rel/NF- $\kappa$ B homodimers and heterodimers bind to DNA target sites, collectively called  $\kappa$ B sites and directly regulate gene transcription. Many NSAIDs inhibit the NF- $\kappa$ B followed by induction of apoptosis [113]. NSAIDs like salicylate was shown to inhibit the activation of p70S6 kinase, which results the down regulation of c-myc, cyclin D1, cyclin A and might contribute to salicylate induced growth arrest [114]. NS-398 and piroxicam block JNK phosphorylation and inhibit AP-1 activity, resulting in induction of apoptosis [115]. Peroxisome proliferation-activated receptors  $\alpha$ ,  $\gamma$  and  $\delta$  (PPAR  $\alpha$ ,  $\gamma$  and  $\delta$ ) are members of a class of nuclear hormone receptors involved in controlling the transcription of various genes that regulate energy metabolism, cell differentiation, apoptosis and inflammation [116]. Some of the NSAIDs activate the PPAR  $\alpha$ ,  $\gamma$  and  $\delta$ , which enhances the apoptosis. Fig. (3) shows COX-independent targets of NSAID. Up regulation or down regulation by NSAIDs is indicated by color code. Possible NSAIDs affecting a particular target are also indicated.

All these studies make it clear that NSAIDs can have multiple targets to exert their anticancer effects. What is important from literature is that not all NSAIDs act equally well, rather a selective group of NSAIDs work on a specific target. To understand this preference one needs to look for common chemical templates involved in the interaction with

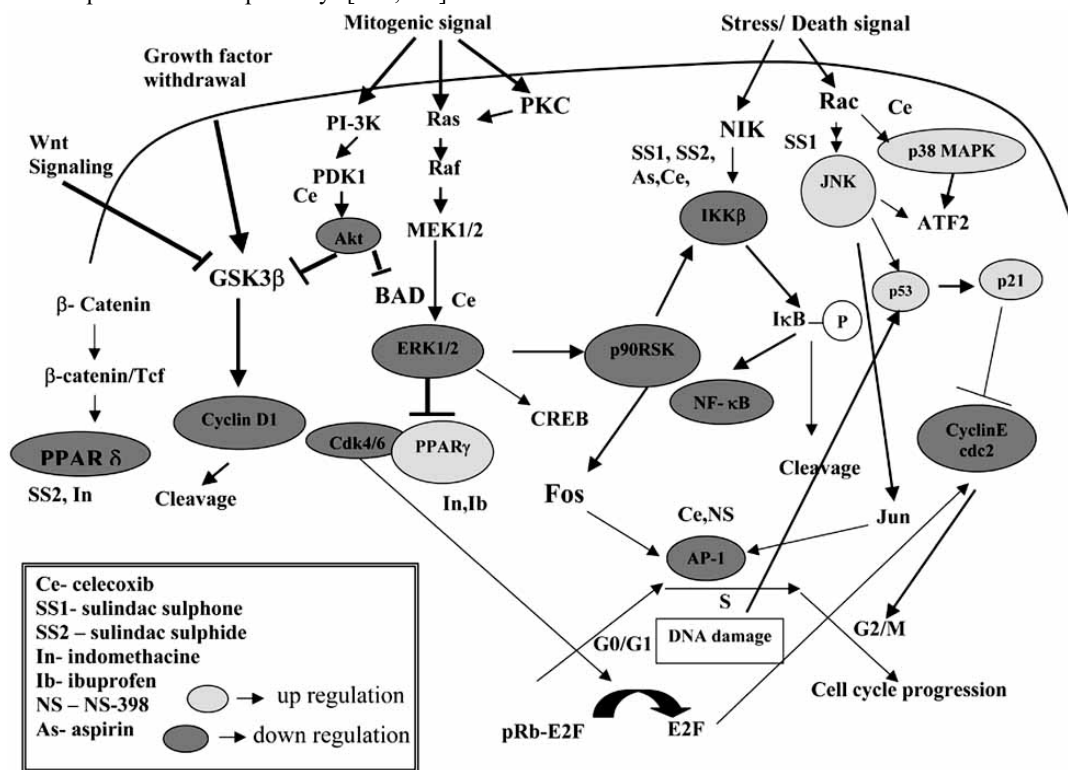


Fig. (3). Different COX-2 independent targets of NSAIDs.



a specific target. However there exists very few literature where this approach has been made and some of them will be discussed later.

### 3.1.2. Beneficiary Effects on Alzheimer Disease (AD)

Alzheimer disease (AD) is a neurodegenerative disorder characterized by impairment in memory and cognition. The pathogenesis of AD is characterized by cerebral deposits of amyloid  $\beta$ -peptides ( $A\beta$ ) as amyloid plaques and neurofibrillary tangles (NFTs), which are surrounded by inflammatory cells. Plaque material mainly consists of extra-cellular aggregates of  $A\beta$  peptides. Misfolding of the soluble native peptide leads to self association to form oligomers, protofibrils or other intermediates in the fibril formation pathway. Oligomers of  $A\beta$  can be detected *in vitro* [117] in cell culture transgenic mouse model of AD [118-120] and also in post-mortem of AD patients' brain specimens [121]. NFTs mostly consist of intra-cellular aggregation of phosphorylated tau protein. Severe inflammatory response develops around the  $A\beta$  deposition [122,123], which is initiated by the activation of microglia and the recruitment of astrocytes. These cells secrete many inflammatory cytokines and chemokines that may contribute to neural degeneration and cell death by various mechanisms [124,125]. It is still controversial as to which event is the key player in AD pathogenesis, whether it is the formation of amyloid peptides ( $A\beta$ ) by the neurons that mediate neurodegeneration [126] or the inflammatory response associated with the presence of neuritic plaques that cause the neurotoxicity [125,127]. Even though there exist a wealth of experimental data, there is still neither a direct correlation nor do we understand the mechanism by which amyloidosis or neuroinflammation mediate neurodegeneration. Epidemiological studies have found strong correlation between long term use of NSAIDs with reduced risk for developing AD and delay in the onset of the disease [128]. Since NSAID group of drugs are primarily used to control pain and inflammation it is an obvious expectation that they would target neuro inflammation to exert their beneficiary effects on AD. However, the picture is not so clear and the mechanism behind the role of NSAID in AD pathogenesis is controversial and riddled with several hypotheses.

Selective NSAIDs viz., Sulindac sulfide, ibuprofen, indomethacin and flurbiprofen reduces  $A\beta$  levels in cultured cells from peripheral, glial and neuronal origins [129-133]. Other NSAIDs like naproxen, acetaminophen, aspirin and celecoxib showed no effect on amyloid pathway. This effect is proposed to be by a mechanism independent of COX-pathway by directly affecting amyloid pathology in the brain that reduces  $A\beta_{42}$  peptide levels. This is achieved by subtly modulating  $\gamma$ -secretase activity [133] without perturbing Amyloid Precursor Protein (APP) or Notch processing. Among the  $A\beta$ -effective NSAIDs, flurbiprofen is particularly important [129]. The R-enantiomer of flurbiprofen does not inhibit COX but does reduce  $A\beta_{42}$  levels *in vitro* and *in vivo* supporting the fact that this NSAID does indeed reduce  $A\beta_{42}$  levels by COX independent mechanism [130]. NSAIDs like nimesulide, ibuprofen and indomethacin have been shown to favor nonamyloidogenic APP processing by enhancing  $\alpha$ -secretase activity thereby reducing the formation of amyloidogenic derivatives [134]. NSAIDs have also been implicated to target different components of neuroin-

flammation. Neuroinflammation is secondary to neuritic plaques. Activated microglia and reactive astrocytes surrounding extracellular deposits of  $A\beta$  protein initiate an inflammatory response [124,128,135]. Microglial COX expression is considered to be important in the pathogenesis of AD [135]. However, in adult human microglia *in vitro*, COX-1 is constitutively expressed but not COX-2, on exposure to  $A\beta$  or plaque associated cytokines. So COX-1 is said to be a better target than COX-2 [128]. This could explain the failure of COX-2 specific inhibitors like celecoxib to produce any beneficiary effect in AD pathogenesis. Pilot trial with therapeutic dose (dose of COX inhibition) of traditional NSAIDs showed promise but higher dose may be required.

Another target of neuroinflammation i.e., peroxisome proliferator activated receptor- $\gamma$  (PPAR- $\gamma$ ) has been implicated in the mechanism of action of NSAIDs [124]. PPAR- $\gamma$  belongs to a family of nuclear receptors that is able to regulate the transcription of proinflammatory molecules. NSAIDs have been hypothesized to activate PPAR- $\gamma$  thereby reducing the inflammatory response. In Fig. (4), we show the various stages of APP processing that leads to formation of  $A\beta$ -peptides, which then misfolds, oligomerizes to form fibrils. The possible steps that can be affected by NSAIDs in the entire pathway are also indicated. It is obvious that the NSAIDs can exert their effects on AD pathogenesis by various mechanisms, which is still controversial. However, it is also now an established fact that only selected NSAIDs show beneficiary effects against AD. Till date, there has been no study devoted to understand the chemical basis for this preference for a few selective NSAIDs.

This could be an important approach, which could lead to specific target identification in the AD pathogenesis. A recent report on the inhibitory effects of NSAIDs on  $A\beta$  fibril formation spans NSAIDs having different chemical templates which contradict the importance of chemical motifs in determining the mechanism. However, this study shows that drugs like naproxen, aspirin etc that show no effect on AD pathogenesis can inhibit fibril formation. It is therefore important to look for the chemical basis of action of NSAID on AD pathogenesis.

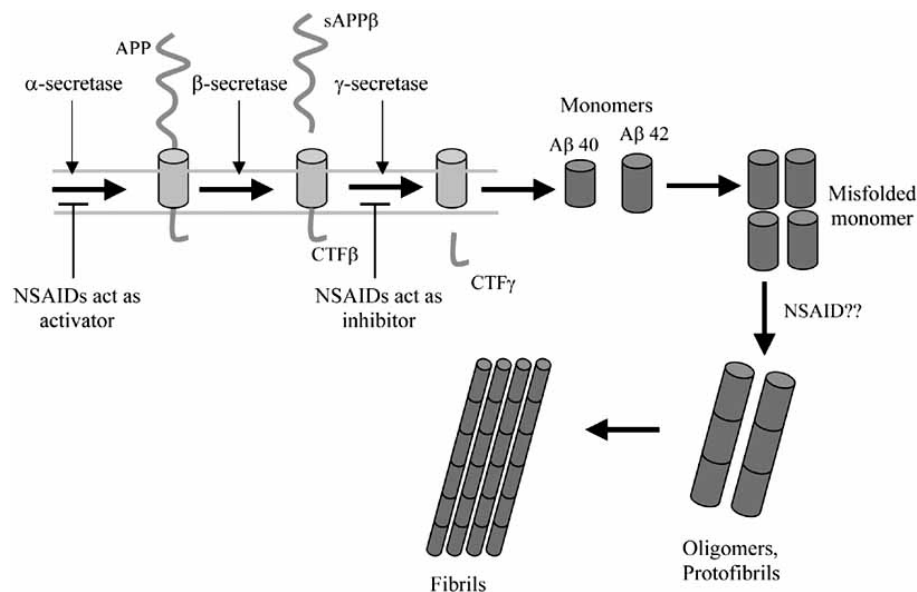
## 3.2. Other Functions of Aureolic Acid Group of Antibiotics

### 3.2.1. Inducer of Erythroid Differentiation and Fetal Hemoglobin Production

Mithramycin is a potent inducer of  $\gamma$ -globin mRNA accumulation and fetal hemoglobin (HbF) production in erythroid cells from healthy human subjects and  $\beta$ -thalassemia patients [136,137]. Results from the study suggest potential clinical application of MTR for induction of HbF in patients affected by  $\beta$ -thalassemia or sickle cell disease. The authors proposed that the mechanism of action involves alteration in the pattern of protein binding to the  $\gamma$ -globin promoter, leading to transcriptional activation. They did not rule out the possibility of a direct effect of the antibiotic on other genes involved in the activation of erythroid differentiation.

### 3.2.2. Prolongation of Survival in Mouse Model of Huntington's Disease (HD)

Pharmacological treatment of a transgenic mouse model of HD (R6/2) with mithramycin extends survival by 29.1%,



**Fig. (4).** Different APP processing pathways leading to formation of Aβ-42 peptide, which leads to oligomerization and fibril formation.

greater than any single agent reported to date. Improved motor performance and markedly delayed neuropathological sequelae are the important recoveries. The functional mechanism for the healthy effects of mithramycin was linked to the prevention of the increase in H3 methylation observed in R6/2 mice. The enhanced survival and neuroprotection might be ascribed to the alleviation of repressed gene expression important to neuronal function and survival. These findings are also significant because they demonstrate the penetrability of mithramycin across the blood– brain barrier to exert some of its beneficial effects. These protective effects appear to require GC-DNA specificity, because an AT-DNA binding antibiotic, distamycin, has no effect on either R6/2 transgenic mouse survival or rotorod performance. However, the pathway of action is not clear. While displacement of the Sp1 family of transcriptional activators from their canonical GC DNA binding sites is a plausible pathway, the transcriptional activation of a hitherto unknown gene/s involved in promoting neuronal death could be another possibility. Sp1 could be acting directly or indirectly to repress transcription of a prosurvival gene(s). Mithramycin treatment might also work by decreasing htt transgene expression in R6/2 mice. Indeed, the demonstration that mithramycin can reduce striatal lesions induced by the mitochondrial toxin 3-NP also suggests that the protective action of mithramycin is downstream of mutant htt [138-140].

### 3.2.3. Potential Therapy for Neurological Diseases Associated with Aberrant Activation of Apoptosis

Mithramycin A and its structural analog chromomycin A<sub>3</sub> are reported to be effective inhibitors of neuronal apoptosis induced by glutathione depletion-induced oxidative stress or the DNA-damaging agent camptothecin. It indicates the potential of mithramycin A and its structural analogs as effective agents for the treatment of neurological diseases associated with aberrant activation of apoptosis which can be induced by many pathological stimuli. The displacement of the transcription factors Sp1 and Sp3 from binding at their cog-

nate GC box was attributed the reason behind the protective effects of mithramycin A [141].

### 3.2.4. Downregulation of Proinflammatory Cytokine Induced MMP Gene Expression

Osteoarthritis (OA) and rheumatoid arthritis patients show articular cartilage degeneration as a major pathological manifestation with increased levels of several matrix metalloproteinases (MMPs) in cartilage, synovial membrane and synovial fluid. MMP-3 and MMP-13 cleave collagens and aggrecan of cartilage extracellular matrix. Proinflammatory cytokines, interleukin-1 (IL-1), IL-17 and tumor necrosis factor (TNF)-α are also increased in arthritic joints thereby inducing catabolic pathways leading to an enhanced expression of MMPs. Reduction in the damage in arthritic tissues can be attained by an inhibition of these proteases. Mithramycin downregulates MMP-3 and MMP-13 gene expression induced by IL-1β, TNF-α and IL-17 in human chondrosarcoma SW1353 cells and in primary human and bovine femoral head chondrocytes. Treatment with the drug also leads to a suppression of constitutive and IL-1-stimulated MMP-13 levels in bovine and human cartilage explants. Interference of Sp1 binding by MTR is proposed as one of the possible mechanisms behind the above function of the drug and its potential to reduce cartilage degeneration. However, the authors did not rule out the possibility of unidentified mechanisms of action [142].

### 3.2.5. Activation of Fas Death Pathway in Leukemic Cell Lines

MTR induces apoptosis in Fas sensitive Jurkat cells and Fas resistant KGa1 cell lines. The cell lines showed morphological changes with Fas aggregation, DISC (Death Inducing Signalling Complex) formation and caspase processing when treated with MTR ending in chromatin condensation and cell apoptosis. The authors speculate that cytotoxicity and severe side effects of MTR such as sepsis and toxic hepatitis could

be a result of activation of Fas apoptotic pathway in these cells. On the other hand, sublethal doses of MTR sensitize the Fas-resistant cells to other chemotherapeutic agents such as Aracytin and Mitoxantrone thus advocating the use of MTR in combination therapy [46].

### 3.2.6. Other Functions of Aureolic Acids

The above reports aim at exploration of the additional therapeutic potential of the aureolic acid antibiotic, mithramycin. However, these studies propose that promoter binding ability culminating in the alteration of the gene expression is the predominant pathway for the therapeutic property of the drug. We want to summarize those results from our laboratory which emphasize an alternate site and pathway of its action. We have earlier reported the association of the antibiotics with the cytoskeletal protein, spectrin both in the absence and presence of  $Mg^{2+}$ . The association is as strong as its spectrin-ankyrin interaction [143]. The observation suggests the cytoskeletal proteins as another class of potential target inside the cell. During our in-depth analysis of the energetic of binding of the antibiotics with different levels of chromatin structure, it has not escaped our notice that some of the results indicate interaction of the antibiotics with histones. Therefore, it might be conjectured that these antibiotics might either interact with histone(s) or modulate the histone modification. Effect upon the histone modification would culminate into alteration of gene expression [144]. One report has suggested that MTR might interfere the acetylation of a core histone like H3. ESET/SETDB1 gene expression and histone H3 (K9) trimethylation in Huntington's disease is inhibited by a combination of drugs, mithramycin and cystamine [140]. A recent report has shown that mithramycin exerts multiple points of control in the regulation of Hdm2 protein synthesis. The oncoprotein plays a key role in the regulation and activation of p53 protein. Firstly, the constitutive hdm2-P1 promoter is inhibited by MTR. Secondly, MTR induces p53-dependent transcription from the hdm2-P2 promoter. Thirdly, and critically, MTR also inhibits Hdm2 synthesis at the post-transcriptional level, with negative effects on hdm2 mRNA nuclear export and translation [145].

### 3.2.7. Metal Chelation Property of the Aureolic Acids as an Alternate Mode of Action

An interesting property of these antibiotics that has been overlooked for a potential therapeutic value is its metal chelating property. The importance of the metal chelating property of the aureolic acid antibiotics and their intracellular action on bivalent metal-dependent enzymes and proteins, e.g. Zn-dependent proteins like transcription factors and matrix metalloproteases (MMPs) cannot be overlooked. This property and its consequence have not been adequately pursued to evaluate its therapeutic potential in the treatment of diseases relating to metal dyshomeostasis. In the absence of DNA, MTR and CHR bind to many bivalent metal ions like  $Mg^{2+}$  [31],  $Zn^{2+}$  [146] and  $Cu^{2+}$  forming antibiotic-metal complexes. The metal binding potential of these antibiotics have also been demonstrated for  $Fe^{2+}$ ,  $Mn^{2+}$ ,  $Co^{2+}$ ,  $Cd^{2+}$  and  $Ni^{2+}$  [147-149]. It has also been shown that MTR forms complexes with monovalent and trivalent metal ions like  $Li^+$ ,  $Na^+$ ,  $K^+$ ,  $Tb^{3+}$  and  $Gd^{3+}$  but these cations are not able to promote MTR-DNA interaction [150]. Fluorescence microscopic studies also showed the presence of metal-drug complex in the cellular nucleus [149]. Studies from our laboratory have shown that MTR and CHR forms two different types of complexes with  $Mg^{2+}$ . The stoichiometries of these complexes are 1:1 (complex I) and 2:1 (complex II) in terms of antibiotic: $Mg^{2+}$  [31-34]. In contrast, it forms only one type of complex of type II with other metal ions. Recently, we have made a detailed report based on the biophysical characterization of the complex formed upon the association of the antibiotics with  $Zn^{2+}$  and  $Mg^{2+}$  ions [146]. In another study from our laboratory, we have also characterized the formation of the complex between  $Cu^{2+}$  and the drugs. Like the zinc ion, they form only 2:1 type complex with  $Cu^{2+}$  (unpublished data). Table 2 summarizes the affinity parameters for the association of these antibiotics with the metal ions.

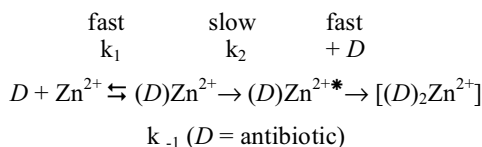
$Mg^{2+}$  and  $Zn^{2+}$  ions show similarities in many physical properties [151], however, there are major differences also. The 100-fold slower exchange of water from the  $[Mg(H_2O)_6]^{2+}$  complex as compared to that of  $[Zn(H_2O)_6]^{2+}$  might be a plausible reason for the formation of two types of complexes

**Table 2. Binding Constants and Stoichiometry for the Formation of Antibiotic-Metal Complexes**

Complex	Antibiotic	Stoichiometry (antibiotic:metal)	Dissociation Constant ( $M^2$ )
$Mg^{2+}$	MTR	1:1 (Complex I)	$5.5 \times 10^{-5}$ (M)
		2:1 (Complex II)	$3.5 \times 10^{-8}$
	CHR	1:1 (Complex I)	$5.3 \times 10^{-5}$ (M)
		2:1 (Complex II)	$9.1 \times 10^{-8}$
$Zn^{2+}$	MTR	2:1	$10.55 \times 10^{-10}$
	CHR	2:1	$3.20 \times 10^{-10}$
$Cu^{2+}$	MTR	2:1	$0.67 \times 10^{-10}$
	CHR	2:1	$0.15 \times 10^{-10}$

with  $Mg^{2+}$  during the association with the antibiotics. There are similarities in the spectral changes of the antibiotics upon 2:1 complex formation with the two metal ions [34,35,146]. The structure of the  $[(D)_2Mg^{2+}]$  ('D' is the antibiotic) complex has been proposed to be octahedral [150-155] with  $Mg^{2+}$  having a co-ordination number of six of which four are satisfied by two each from the carbonyl oxygen (C1-O) and the negatively charged oxygen of hydroxyl group (C9-OH) of the chromomycinone ring (see Fig. (1)). The other two coordination sites are satisfied by water as ligands. However, as  $Zn^{2+}$  ion has zero crystal field stabilization energy as a result of its filled d-orbital, it shows no preference for a particular geometry. Thus, tetra coordination is a plausible option. This is supported from the results of  $^1H$  NMR spectroscopy that favors the possibility of the complex,  $[(D)_2Zn^{2+}]$ , containing tetra-coordinated  $Zn^{2+}$  without the participation of water as ligand [146].

Hou and co-workers showed the stabilization of the  $[(CHR)_2Mg^{2+}]$  complex, derived from X-ray crystal structure where the metal ion has octahedral coordination, is due to mutual stacking of the aromatic part of the chromophore of one CHR molecule with the C-D glycosidic linkage of the other molecule. They also stated from the NMR structure of the same complex that the metal ion has a tetrahedral geometry [156,157]. The formation of the antibiotic-metal complex is always entropy driven with the following kinetics [146]:



The ability of the bivalent metal ions to bind with the drug(s) at physiological pH coupled with their intracellular concentrations and the stability parameters of the drug(s)-metal complex imply that the mode of action of these drugs inside the cell need not be limited to the DNA binding property of the drug-dimer complex only. In addition, their clinical use may be extended for the purpose where diseases occur due to misbalance of metal homeostasis inside the cell.

Recently, we have also shown the negative effect for the preincubation of Zn(II)-containing alcohol dehydrogenase (ADH) with MTR (& CHR) upon their enzymatic activity which is a consequence of induced structural alteration as a sequel to its complex formation with the  $Zn^{2+}$  ions present in the enzyme [158]. Alcohol dehydrogenase (E.C. 1.1.1.1) is a tetrameric enzyme containing two zinc ions per subunit, one each at the catalytic and structural sites, was studied. A negative effect for the pre-incubation of ADH with MTR/CHR upon its enzymatic activity was observed which was characterized by a decrease in the rate and the extent of NADH formed. In general, the rate of the reaction and the product formation decreased with the increase in pre-incubation concentration of the antibiotic. The enzymatic inhibition of ADH by the antibiotic(s) follows non-competitive inhibition.

Our studies have opened up the possibility to exploit the metal binding potential of the aureolic acid antibiotics for therapeutic purpose as discussed above.

### 3.2.7.1. Zn(II)-Containing Proteins and Enzymes as Potential Targets

Zinc is the second most abundant trace metal in higher animals with the average human body containing 2-4 g of zinc. Zn(II)-containing proteins are known to have functions extending from catalysis of metabolic pathways and macromolecular synthesis to the regulation of gene expression. With the demonstrated ability of MTR and CHR to inhibit the function of the Zn(II)-containing enzyme, alcohol dehydrogenase [158] and the possibility of interaction of MTR with MMP during its inhibition of MMP gene expression [142], Zn(II)-containing enzymes and proteins might be explored as an alternative therapeutic target as these proteins are involved in most pathological conditions. It is known that proteases make up nearly 2% of the human genome and represent 5-10% of all known potential drug targets. In particular, there are nearly 200 distinct metalloproteases whose functions are linked to a number of clinically relevant conditions, most notably cancer [159]. All MMPs share a common active-site motif: in which three histidine residues bind to a zinc ion. Many of the inhibitors currently undergoing clinical testing are small peptide mimics that chelate the zinc ion and block the function of the enzymes. As new and valuable information to understanding the complex functional roles of metalloproteases in disease progression come up, clinical trials with MTR as matrix metalloprotease inhibitor could be considered for therapeutic gain.

### 3.2.7.2. Potential Agent in Chelation Therapy in Neurodegenerative Diseases Showing Metal Dyshomeostasis such as Alzheimer Disease (AD), Wilson's Disease (WD) and Prion Diseases

Alzheimer disease (AD) is a devastating neurodegenerative disease with progressive and irreversible damage to thought, memory, and language. The etiology of AD is not well understood but accumulating evidence supports oxidative stress generated by various mechanisms to be a major risk factor that initiates and promotes neurodegeneration. Many studies show the elevated concentrations of metal like iron, copper, zinc and aluminum in the brain of AD patients which indicate that the environmental conditions in AD, exacerbated by imbalances in several metals, has the potential for catalyzing and stimulating free radical formation and enhancing neuron degeneration thus opening the door for metal chelation therapy. A metal chelator with affinity for multiple metals such as aluminum, copper, and zinc turn out to be useful rather than damaging effects since various metals are implicated as oxidative instigators. This may be the reason behind the demonstrated therapeutic benefits of metal chelators like desferrioxamine (DFO) in patients with AD [160]. Wilson's Disease (WD) is a disorder in copper metabolism involving mutation in the ATP7B gene and is manifested by copper overload in the cells. Metal chelator like penicillamine has been used to treat patients with WD [161]. As the aureolic acids like MTR and CHR are metal chelators with affinity for multiple metals like iron, zinc and copper, they have potential as therapeutics in metal chelation therapy for the treatment of neurodegenerative diseases involving metal dyshomeostasis like AD and WD.

It was reported that prion proteins (PrP) might play a crucial role in zinc homeostasis within the brain, either through binding the metal ion at the synapse and being involved in a re-uptake mechanism, or as part of a protective sequestering system and/or a cellular sensor, thus preventing cellular toxicity of excess metal. However, in prion disease, when the protein undergoes a conformational change to the infectious form, this function of PrP in zinc homeostasis might be compromised thereby contributing to disease pathogenesis and changes in zinc binding. Studies with scrapie infected mice showed zinc content being reduced to 20% and the copper content reduced to 35–50% of the levels in uninfected mice while the blood content of zinc increased significantly. These data suggest that in prion disease, zinc is displaced from its normal localization and displaced in the blood [162]. As metal chelators have proved to be efficient therapeutics in other neurodegenerative diseases, it opens up the potential for the aureolic acids as potential therapeutic in prion diseases also.

### 3.2.8. Potential Therapeutic Functions of the Modified Antibiotics

It is well known that chromatin-interacting small molecules are of exceptional importance in medicine, accounting for a significant portion of all anticancer drugs. Majority of the clinical anticancer drugs introduced through 2002 are natural products or natural product derivatives, and exert their effects by acting on genomic DNA and /or histone(s). Although very few minor groove binders are clinically used, they are thought to hold the most promise for de novo design [163]. This holds great promise for the aureolic acid antibiotics MTR and CHR, which are minor groove binders. Therefore, we have included a section to give a brief overview of the potential leads for better and novel aureolic acid antibiotics with improved therapeutic index. The biosynthetic pathways of the parent antibiotics have been extensively studied and there have been reports of new structurally modified antibiotics. All leads are however based upon their potential as transcription inhibitors *via* reversible association with (G.C) rich DNA. Mithramycin SK, a novel antitumor drug with improved therapeutic index, Mithramycin SA, and demycarosyl-mithramycin SK are three new products generated in the mithramycin producer *Streptomyces argillaceus* through combinatorial biosynthesis [164]. Other new DNA-binding ligands were designed to mimic Chromomycin A3 (CHR), which contains a hydroxylated tetrahydroanthracene chromophore substituted with di and trisaccharides. In these new model compounds, a simple alkyl group attached to the chromophore part mimics the trisaccharide part of CHR. These have been successfully demonstrated for their  $Mg^{2+}$ -coordinated dimer complexes to exhibit DNA-binding affinity [165]. Recently, there was another report on a new analog, Mithramycin SDK, obtained by targeted gene inactivation of the ketoreductase MtrW catalyzing the last step in MTR biosynthesis. SDK exhibits greater activity as transcriptional inhibitor compared to MTR with a high degree of selectivity toward GC-rich DNA-binding transcription factors. SDK inhibited proliferation, inducing apoptosis in ovarian cancer cells with minimal effects on normal cells viability. The new MTR derivative SDK could be an effective agent for treatment of cancer and other diseases originating

from abnormal expression or activity of GC-rich DNA-binding transcription factors [166].

## 4. CHEMICAL DISSECTION OF THE DRUGS TO UNDERSTAND ALTERNATE FUNCTIONS

Efforts to understand the molecular mechanism behind multiple functions of NSAIDs have resulted in controversies and no general consensus exist as to the actual mechanism behind any one of the unconventional functions of NSAID. Multiple targets and molecular pathways have been implicated for various functions. However, it is now clear that only selective NSAIDs exhibit a specific function and that too not at an equal level. It is therefore important to look at the chemical basis behind the unconventional functions of NSAID to identify the structural features required for a particular function. This would help to develop new classes of drugs aimed at a specific function using the chemical templates of NSAIDs. Such an approach was taken by Chen and co-workers [107]. Celecoxib, a newer generation COX-2 inhibitor, was approved for FAP, an inherited predisposition to colorectal cancer in 1999. Its chemopreventive effect is achieved by sensitizing cancer cells to apoptotic signals. Equally powerful COX-2 inhibitor, rofecoxib, is at least two orders of magnitude less potent, which implied that the anti-tumor effect of celecoxib is independent of its COX-2 inhibition.

Chen looked at the structural difference between rofecoxib and celecoxib and used a systematic approach to modify the structures of both the drugs to produce 50 compounds. These compounds were then tested for their ability to induce apoptosis in human prostate cancer cells. The pathways through which apoptosis acts were monitored and molecular models were used to identify the key structural features involved in apoptosis. The structural requirements for the induction of apoptosis were found to be distinct from those that mediate COX-2 inhibition. Apoptosis required bulky terminal phenyl ring, a heterocyclic system with negative electrostatic potential and a benzene sulfonamide or benzene carboxamide moiety (Fig. (5)). To prove their observation, Chen et al. modified the structure of rofecoxib to create four compounds to mimic the surface electrostatic potential of celecoxib, one of which showed substantial increase in pro-apoptotic activity. They found that apoptosis was mediated by down regulating the serine/ threonine kinase Akt and extracellular signal regulated kinase-2 (ERK2). They therefore demonstrate how understanding the chemical basis behind the apoptotic function of a NSAID, viz. cele-

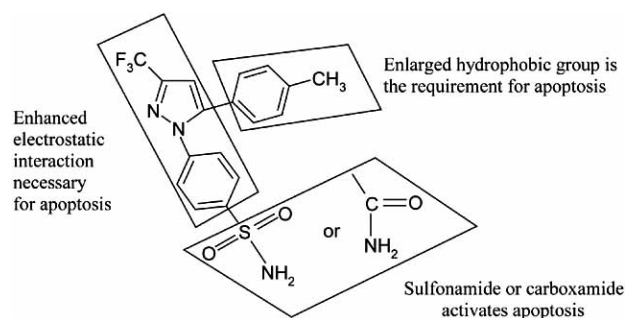
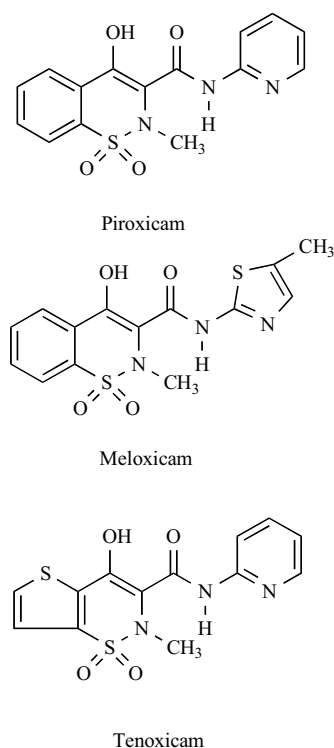


Fig. (5). Chemical basis of pro-apoptotic functions of celecoxib.

coxib can help in designing a new class of compounds that induce apoptosis by targeting the Akt ERK2 signaling pathways in human prostate cancer cells using the molecular template of the NSAID. Efforts were also given to understand the different functions of similar drug molecules. Our group is working on the oxamicam group of NSAIDs, which consist of piroxicam (Px), meloxicam (Mx), tenoxicam (Tx) etc. These molecules are structurally very similar to one another. Mx, Tx can be synthesized by the method of isosteric substitution considering Px as the mother template (Fig. (6)). The advantage of using these kinds of established drugs over the newly synthesized compounds is that the biochemical properties and the ADMET profile of the known drugs are well studied. From Fig. (6), it is evident that the structural difference between tenoxicam and that of meloxicam and piroxicam is small.



**Fig. (6).** Chemical structure of piroxicam, meloxicam and tenoxicam.

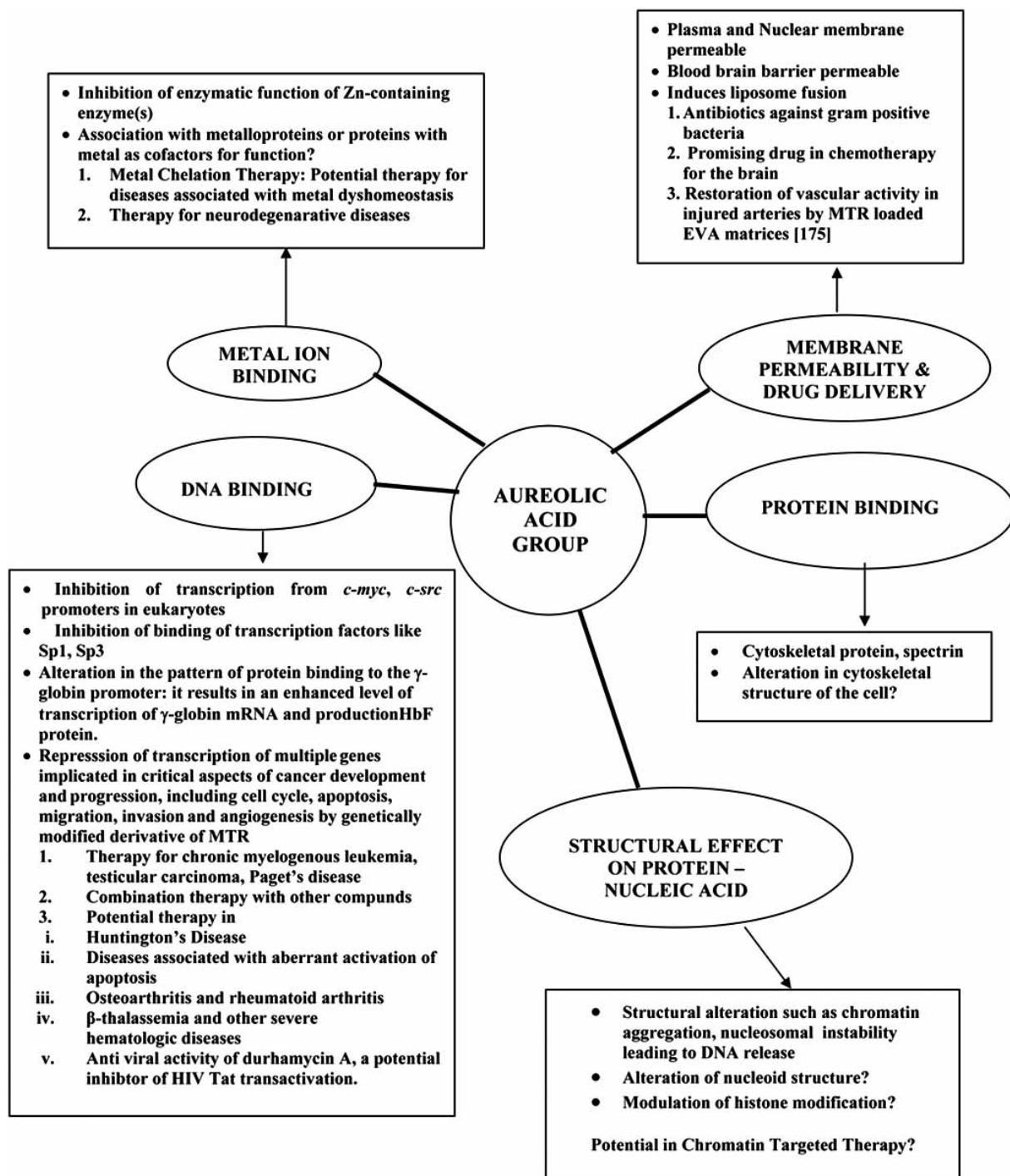
Even such small changes in chemical structure can lead to completely different chemical / biological property. Oxamicam group of NSAIDs are not equally efficient against different cancer though they are structurally very similar. For example even though Px and Mx show anticancer properties, Tx show no such effects. In the *in vitro* study it was observed that the Cu(II) complex of piroxicam and meloxicam bind with the calf thymus DNA resulting in alteration in the DNA backbone whereas tenoxicam does not interact with the DNA backbone at all [167]. Another important property of oxamicam NSAIDs is their structural dynamism. Structural dynamism is reflected in the ease with which the drugs can 'switchover' or convert from one prototropic form to the other guided by their environment [168]. In the interaction of these drugs with membranes/ membrane mimetic systems it has been

found that one prototropic form of the drug is converted to the other depending on the nature of the membrane mimetic systems/ membranes [169]. This switchover equilibrium is fine tuned by the electrostatics of the membrane surface, hydrophobicity of the membrane core, presence of counterion and steric effects guiding the water penetration within the membrane [169-174]. Thus the diverse nature of biomembrane *in vivo*, characterized by their different membrane parameters, should be the decisive factor in choosing which structural form of the drug will be finally presented to its target cyclooxygenases. This raises the intriguing possibility that the reason behind the different functions of these drugs could be due to their structural flexibility. However, further studies are required to establish this hypothesis. Px can alter membrane morphology leading to fusion and rupture of mitochondrial membrane [175]. This has been implicated as a possible cause for the release of cytochrome c from mitochondria into the cytosol *in vivo*, leading to the activation of pro-apoptotic events like caspase-3 activation. Px is also capable of reducing cytochrome c with small changes in protein structure. The clinical implications of these new functions of Px are yet to be established.

Aureolic acids, MTR and CHR, on the other hand, are structurally very similar compounds having the same pharmacore, the chromomycinone moiety, with little differences in the sugar residues. The presence of the acetoxy group on the B-ring in CHR is believed to be a plausible reason behind its greater toxicity than MTR. It is also noticed that the modes of action of the two antibiotics on metal chelation and DNA binding are the same but the extent of their activity differs. The absence of substituents like the acetoxy group in MTR imparts conformational flexibility to a greater extent than CHR when the antibiotic:metal complex(es) binds to the minor groove of DNA. It was also demonstrated from Biospecific Interaction Analysis (BIA) and Surface Plasma Resonance (SPR) analysis that the  $(MTR)_2 Mg^{2+}$ -DNA complexes are highly unstable as compared to that of the  $(CHR)_2 Mg^{2+}$ -DNA complexes which might explain its lower toxicity [176]. The rate of the association of CHR with bivalent metal ions like  $Zn^{2+}$  is also higher than that of MTR [146]. Both CHR and MTR have the tricyclic ring system fused to a unique dihydroxy-methoxy-oxo-pentyl aliphatic side chain attached at C-3. It has been shown that chemical modification of this 3-aliphatic side chain can generate novel aureolic acid compounds with greater therapeutic index such as MTR SDK, which has a diketo group in the C-2'' and C-3'' aliphatic side chain. The greater activity of SDK as transcriptional inhibitor in cells with tighter DNA binding, efficient protein binding and rapid accumulation in cells as compared to MTR might be a direct consequence of the structural modification [166].

## CONCLUSION

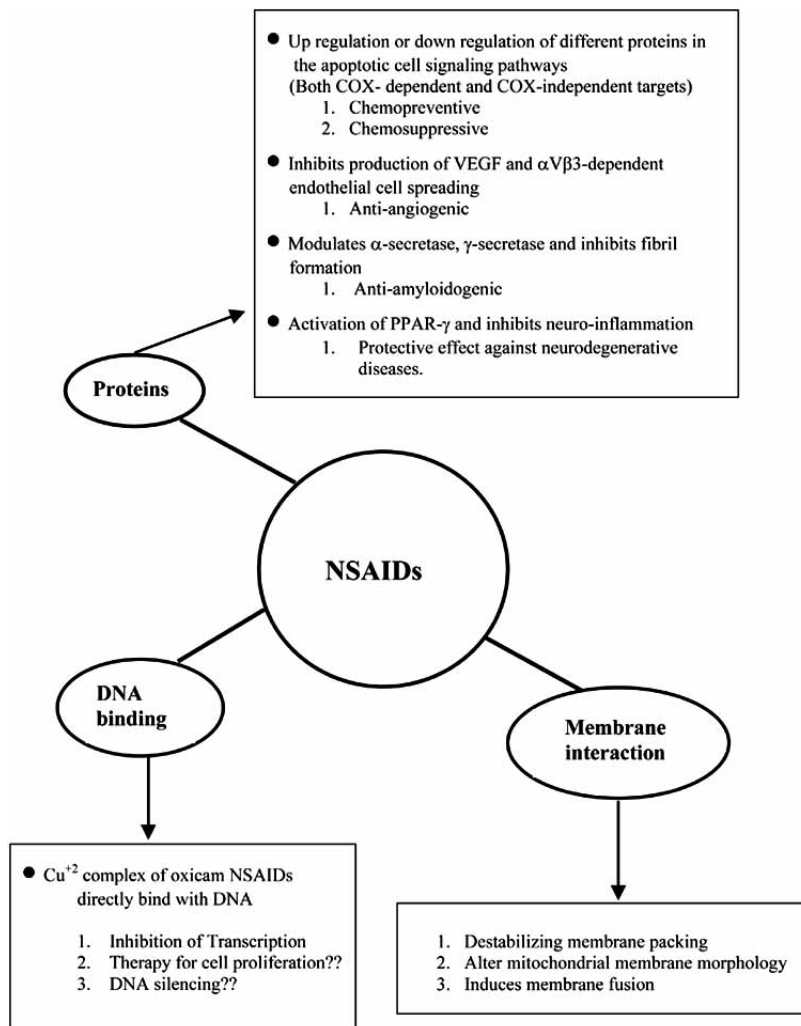
In this review we have given an overview of function based therapeutic potential of two classes of drugs. NSAIDs have larger market in comparison to aureolic acid group of drugs whose use is at present is confined to several types of cancer. In the two cartoons given below, we have catalogued the targets reported till date for the two classes of drugs. We have also indicated the pharmacological uses of the drugs that might employ these targets. An examination of the



**Scheme 2.** Sites of action and therapeutic potential of the aureolic acid group of antibiotics.

schemes (2 and 3) clearly suggests that the potential of the two classes of drugs, as therapeutics, has not been explored to the maximum. For the aureolic acid group of antibiotics, recently there has been a plethora of reports aiming to explore the alternate potential. The identification of the gene cluster for the biosynthesis of the aureolic acid group of antibiotics has further opened up the vista towards a series of modified antibiotics with less toxicity, and improved potential as drug.

The diverse milieu of the potential interaction sites in a cell suggest that it may be an uphill task to predict its potential as a drug for the diseases mentioned in the schemes. In a multicellular organism, there is another degree of complexity arising from the differences in the cellular function and the underlying mechanisms. Notwithstanding the above limitations, it may be worth looking for the other therapeutic uses of the drugs as described above. Trial and error experiments will be the tool of choice. Systems biology approach based



**Scheme 3.** Sites of action and therapeutic potential of NSAIDs.

on an integration of a high throughput experimental, mathematical and computational science in an iterative approach would definitely lend further help. In both cases an incisive knowledge about the sites of action leading to alternate therapeutic potential of the drugs is an important input parameter.

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