AMERICAN UNIVERSITY OF BEIRUT

CADMIUM INDUCES MIGRATION OF COLON CANCER CELLS: THE ROLES OF REACTIVE OXYGEN SPECIES, P38 AND CYCLOOXYGENASE-2

by
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AN ABSTRACT OF THE THESIS OF

<u>Sara Chafik Naji</u>   for   <u>Master of Science</u>
                                        <u>Major</u>: Pharmacology and Toxicology

Title: <u>Cadmium Induces Migration of Colon Cancer Cells: The Roles of Reactive Oxygen Species, p38 and Cyclooxygenase-2</u>

Background: Increasing evidence shows that exposure to environmental pollutants such as heavy metals is positively correlated with colorectal malignancy. Cadmium (Cd) is a heavy metal contaminant whose toxicity has been strongly associated with different types of cancer including colorectal cancer (CRC). Despite this, the underlying molecular mechanisms of Cd-induced CRC remain obscure.

Methods/Aims: HT-29 human adenocarcinoma cells were employed to the study the effects of acute low-level Cd exposure (100 nM) on their migratory (scratch assay) and proliferative (MTT) capacity. To this end, Luciferase reporter assay was used to assess COX-2 transcriptional activity, and Western blotting was used to detect p38 Mitogen Activated Protein Kinase (MAPK) and Akt phosphorylation as well as Cyclooxygenase-2 (COX-2) expression in the presence or absence of specific inhibitors. Prostaglandin E_2 (PGE_2) levels were measured using Enzyme Linked Immunosorbent Assay (ELISA). Finally, reactive oxygen species (ROS) formation was assessed using Dihydroethidium (DHE) stain.

Results: Our results show that the migratory capacity of cells treated with Cd were significantly higher than vehicle-treated cells. Cd caused a time-dependent increase in COX-2 transcription and protein expression. Treatment with celecoxib (10 µM), a COX-2 selective inhibitor, significantly reduced Cd-induced migration. Because ROS and p38 are implicated in COX-2 expression and migration, we determined if Cd modulates their levels. Indeed, Cd increased levels of ROS and phosphorylation of p38. Importantly, Cd-induced COX-2 expression and migration were significantly abolished when cells were pre-treated with N-Acetyl-Cysteine NAC (10 mM), a ROS scavenger, or SB202190 (10 µM), a specific p38 inhibitor. Furthermore, p38 phosphorylation was inhibited by NAC, suggesting that ROS acts through p38 to upregulate COX-2. In line with this, Cd (100 nM) caused a significant time dependent increase in PGE_2 levels. This Cd-increased PGE_2 was abrogated when cells were pre-treated with NAC, SB202190, or celecoxib. Remarkably, exogenous PGE_2 (1 µM) potentiated cell migration, indicating that it is sufficient for basal migratory ability. Since the PI3K/Akt pathway is well known to play a crucial role in CRC migration, we explored

whether Cd activates it. Cd caused a significant increase in Akt phosphorylation in a ROS mediated pathway. Moreover, Cd-induced migration was significantly attenuated by the phosphatidylinositol-3-kinase (PI3K) inhibitor, LY294 002 (10 µM), clearly implicating PI3K/Akt pathway as mediator of Cd-increased migration. Finally, Cd caused a paradoxical decrease in cell viability. This Cd-induced anti-proliferative effect was reversed by NAC, indicating that oxidative stress contributes to Cd cytotoxicity.

Conclusion: Taken together, our results suggest that exposure to low levels of Cd promotes a more migratory cancer phenotype in a ROS-p38-COX-2-PGE$_2$ pathway as well as ROS-Akt pathway. Therefore, COX-2, PGE$_2$ receptors or Akt represent potential targets in the treatment of CRC, particularly in patients exposed to Cd.

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CHAPTER I

INTRODUCTION

A. Epidemiology:

Colorectal cancer (CRC) is the third leading cause of cancer related mortalities globally being responsible for 774000 deaths in 2015 alone [1]. By 2030, the worldwide CRC burden is estimated to increase by 60 % with 2.2 million cases and 1.1 million deaths [2] .The incidence as well as the mortality rates of CRC are not uniform throughout the world [2].In fact, CRC is mostly prevalent in developed countries such as Australia, United States, New Zealand, and Western Europe [3, 4]. In Lebanon, a study conducted between 2003 and 2008 showed that CRC ranked as the fourth most prevalent type of cancer among males and second among females [5]. This study further showed that by 2018, CRC is expected to remain among the most common five types of cancer in both genders [5].

B. Risk factors:

1. Age and Hereditary factors:

There are multiple risk factors associated with CRC (Figure 1). The likelihood of developing CRC increases with age, especially after the age of 40 [6]. In fact, 90% of CRC cases are diagnosed in individuals aged 50 years or more [6, 7]. Additionally, CRC risk is known to be higher in individuals with a personal history of either colorectal polyps or inflammatory bowel diseases (IBD) whether ulcerative colitis or Crohn's disease [8]. Interestingly, it was reported that IBD patients with a disease duration of more than 25 years had 7 to 14% higher CRC risk [9]. Indeed, a positive family history of either adenomatous

polyps or CRC especially in first degree relatives is tightly linked with increased CRC risk [8].

Furthermore, certain inherited genetic conditions such as familial adenomatous polyposis

(FAP) and hereditary nonpolyposis colorectal cancer (HPNCC; also referred to as Lynch

syndrome) were shown to be positively correlated with increased CRC risk [6, 7].
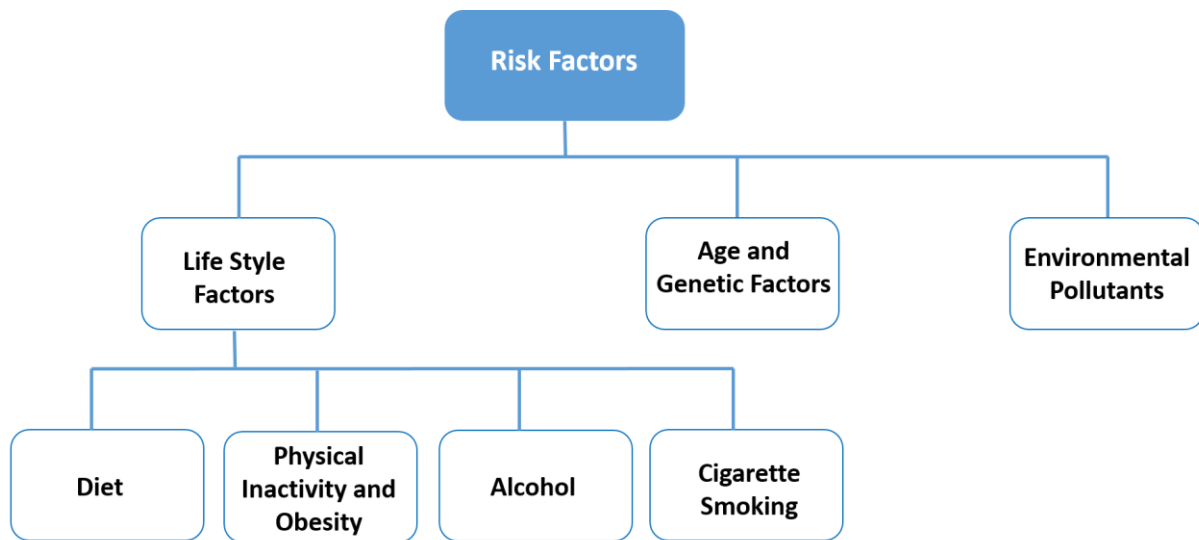
Figure 1: CRC Risk Factors

2. *Lifestyle factors:*

Diet, sedentary lifestyle, obesity, alcohol consumption and cigarette smoking are well

known to play a critical role in elevating the risk of CRC [6]. Indeed, a diet low in fibers, fruits

and vegetables but rich in fats, processed and red meat (referred to as Western diet) is

strongly associated with increased CRC risk [6, 10]. It has been postulated that fibers play a

protective role in CRC by modifying colonic pH, increasing feces bulk and thereby reducing

transit time and mucosal contact with carcinogens [6]. Epidemiological evidence on the other

hand has shown that high dietary cholesterol intake is positively correlated with a higher CRC

risk [11]. By increasing insulin resistance and reducing metabolic rates, obesity and lack of physical activity can contribute to increased likelihood of CRC [3, 6, 10].

It is well established that tobacco and alcohol consumption are associated with elevated CRC risk [3, 6]. Evidence demonstrates that long term smoking is correlated with a higher CRC mortality [12]. In fact, it has been suggested that 12% of CRC deaths are attributable to cigarette smoking [13]. Fedirko *et al.* showed that alcohol consumption (>1/day) is strongly linked with CRC risk [14]. Indeed, it has been hypothesized that the carcinogens as well as the carcinogenic metabolites found in cigarettes [15] and alcohol (namely acetaldehyde) [16, 17] are probably responsible for contributing to increasing CRC chance.

3. *Environmental pollutants:*

Increasing evidence indicates that pollution is positively associated with incidence of CRC [18]. Workers exposed to pollutants as asbestos or wood dust are reported to have higher CRC risk [19]. Moreover, increased exposure to certain types of pesticides has been correlated with elevated CRC risk [20]. Remarkably, higher plasma levels of different types of pesticides were reported to be associated with CRC [21].

Among the many other factors contributing to environmental pollution are the heavy metals, which are generally characterized by having a specific density of more than 5 g/cm^3 [22].These metal contaminants are widely spread in the environment and originate from either natural or anthropogenic sources [23]. Examples of heavy metals include: Arsenic, lead, mercury, chromium and cadmium [22, 24].

A huge body of evidence indicates that exposure to heavy metals is strongly correlated with variety of diseases such as pulmonary [25], renal [26], neurodegenerative [27], cardiovascular diseases [28, 29] and cancer [30] including CRC. Higher plasma levels of cadmium and lead were shown to be positively linked to metastatic colon cancer [31]. Other epidemiological evidence demonstrated that soil arsenic exposure was significantly associated with colon cancer mortality [32]. Yang *et.al* has shown that reducing arsenic exposure by improving the supply system of drinking water is associated with reduced colon cancer mortality in males [33]. A cross sectional study further demonstrated that lead levels were significantly higher in colorectal cancer tissues when compared to healthy tissues, probably implying that lead exposure may play a role in CRC development [34]. Indeed, Cadmium is among these metal toxicants whose exposure has been suggested to be correlated with CRC (to be discussed later).

C. Cadmium:

1. *Environmental occurrence and Human Exposure:*

Cadmium (Cd) is a naturally occurring heavy metal of considerable toxicity. It occurs in the earth's crust, atmosphere, soil and water [35]. These natural sources along with anthropogenic ones are the major sources for its environmental levels. Indeed, industrial activities largely account for its release to the environment [36]. In fact, Cd is used as a color pigment, plastic stabilizer, anticorrosive agent and in the production of nickel-cadmium batteries [36].

Since Cd is widely spread in the environment, humans are highly exposed to this contaminant. Different exposure routes have been suggested. These include the

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gastrointestinal, pulmonary as well as dermal routes [36]. Cd contaminated food is the major

source of the gastrointestinal exposure [36]. As a matter of fact, the average daily intake of Cd

in most countries ranges between 0.1-0.4 µg/kg body weight [35, 37]. Since tobacco leaves

contain considerable amounts of Cd [38], cigarette smoking is considered to be the major

source of Cd exposure via inhalation [36]. Indeed, blood Cd levels of smokers are generally 4-

5 times higher than non-smokers [39]. Very little is known about Cd dermal absorption.

However, it has been postulated that Cd is able to penetrate the skin reaching systemic

circulation by 2 mechanisms: associating with sulfhydryl groups of cysteine in epidermal

keratins, or inducing and forming complexes with metallothionein (MTN) [36, 40].

Following exposure, and upon reaching the systemic circulation, a major fraction of

Cd gets bound to albumin and is ultimately taken up by the liver stimulating the synthesis of

MTN as a body protective mechanism [41]. Owing to its low rate of renal elimination, Cd

tends to bioaccumulate in tissues. It has been postulated that slow Cd excretion might be due

the fact Cd-MTN complex has a low molecular weight and is therefore easily reabsorbed in

the renal tubules [41]. Cd has an unusual long biologic half-life in humans which is estimated

to be between 6 to 38 years in the kidney and 4 and 19 years in the liver [42].

Cd levels could be measured in blood or urine .While urinary Cd levels primarily

reflect cumulative exposure, blood levels are indicative of both recent and cumulative

exposures [35]. Despite the fact that much higher plasma concentrations of Cd have been

described for environmental exposure (more than 10 µg/L) and occupational exposure (up to

50 µg/L), the normal blood levels in the general population range from 0.4-1 µg/L for non-

smokers and 1.4-4 µg/L for smokers [35, 37]. It is worth mentioning that a whole blood level

of 5 µg/l or more is considered to be hazardous, according to the Occupational Safety and Health Administration (OSHA).

2. *Cellular uptake and Signaling Pathways:*

To exert its toxic effects, Cd should first enter target cells. It could be taken up by cells via active transport and facilitated diffusion [43]. Several Cd uptake mechanisms have been suggested. These include the ionic mimicry, molecular mimicry as well as receptor mediated endocytosis [44]. Because Cd mimics the essential divalent cations (zinc, calcium and iron), it is thought that Cd could act similar to these metals at their binding sites within the channels/transporters responsible for their cellular uptake [44]. Furthermore, Cd possesses the ability to associate with certain sulfhydryl-containing molecules such as Glutathione (GSH) and Cysteine, forming complexes acting as molecular mimics of endogenous molecules at the site of their transport [44, 45]. Finally, by biding to albumin or MTN, Cd could be taken up by some epithelial cells via receptor mediated endocytosis [44, 46].

The pathology of Cd toxicity is probably attributed to its ability to disrupt important cellular signaling players such as calcium (Ca), cyclic adenosine monophosphate (cAMP), nitric oxide (NO), Mitogen activated protein kinases (MAPKs), nuclear factor kappa B (NF-kB) signaling and others [47]. In addition to its non-genomic effects, Cd is also able to influence the expression of genes [48]. It is important to note that oxidative stress largely contributes to Cd toxicity [49]. In fact, Cd is able to alter the cellular redox balance by several mechanisms [49]. Being a non-fenton metal, Cd is not able to directly generate ROS [47]. Rather, it induces oxidative stress indirectly by (1) liberating redox-active metals such as iron [50], (2) depleting antioxidant biomolecules such as GSH [51], (3) depressing the activity of

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anti-oxidant enzymes such as superoxide dismutase (SOD) [51], (4) impairing [52] the

mitochondrial electron transport chain [47, 49, 53]and (5) inducing the activity/expression of

ROS generating enzymes such as NOX family of nicotinamide adenine dinucleotide

phosphate (NADPH) oxidases [53-55].

3. *Cadmium and Health Effects:*

Accumulating evidence suggests that Cd is associated with a wide range of adverse

health effects (Figure 2). Positive relationship between blood Cd levels and blood pressure has

been reported [56]. Furthermore, Cd exposure in rats was found to induce endothelial damage

[54] and to cause an elevation in their blood pressure [57, 58].
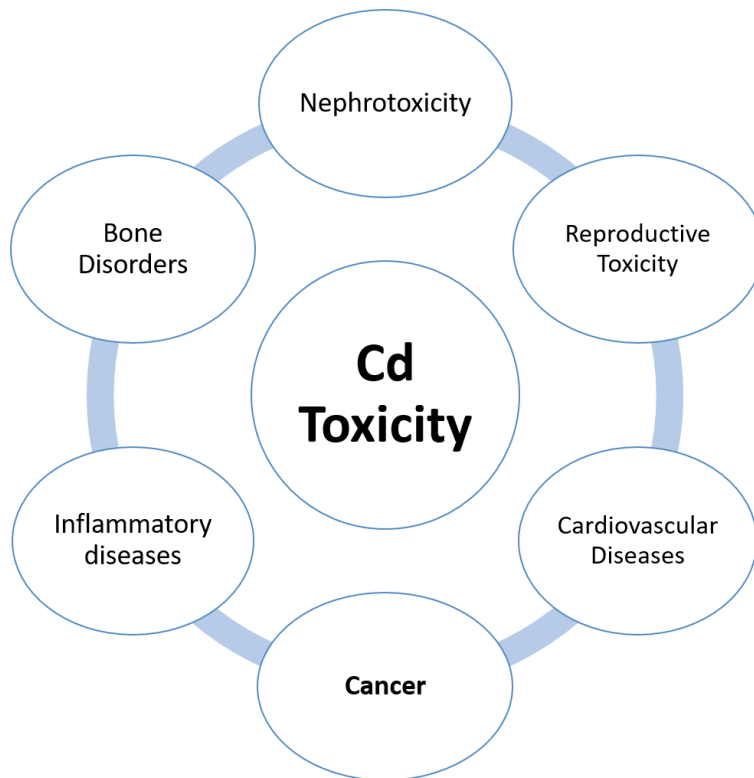
Figure 2: Cadmium Associated Health Effects

Besides cardiovascular effects, Cd exposure has been implicated in bone related disorders. Increased skeletal fragility and decreased mineral density was reported in group of rats that were exposed to long term dietary Cd at levels similar to environmental exposures in humans [59]. Epidemiological evidence further demonstrates that long term Cd exposure is associated with increased fracture risk and abnormalities in serum calcium, phosphorous, parathyroid hormone and vitamin D_3 levels [60].

Cd toxicity appears to target the reproductive system as well. By mimicking estrogen, Cd contributed to enhanced mammary glands growth and increased uterine weight [61]. Moreover, Cd induced sperm dysfunction by significantly reducing sperms motility and viability [62].

Inflammation has been also linked to Cd exposure [63]. In fact, inflammatory bowel diseases are among those that have been positively correlated with Cd. Oral Cd exposure in rats was reported to increase the severity of colonic inflammation and to delay the healing of acetic acid-induced colitis [64]. Experimental evidence further demonstrates that Cd initiates a pro-inflammatory response by inducing COX-2 upregulation in mouse neuronal cells [65].

It is well established that Cd is associated with increased risk of cancer. In fact, Cd is classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC). Different cancer cases have been linked to Cd [35] such as lung, kidney, prostate, breast and endometrial cancer in females and CRC. The exact molecular mechanisms underlying Cd-induced malignancy are poorly understood [66]. Nevertheless, it has been postulated that reactive oxygen species (ROS) play a central role in Cd mediated carcinogenicity [66, 67]. For instance, Cd was reported to act as mutagen by inducing ROS

mediated DNA damage [68]. Additionally, by inducing the overexpression of proto-oncogenes in a ROS dependent pathway, Cd was shown to mediate BALB/c-3T3 cell transformation and tumorigenesis [69]. MAPKs (extracellular signal-regulated protein kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK) are among the signaling players known to be activated by ROS [70] and have been implicated in many diseases including cancer [71]. It is not surprising then that Cd, by inducing ROS, could activate MAPKs and hence provoke carcinogenesis [49].

CRC is among the different types of cancers associated with Cd. A significantly higher Cd plasma level was reported in metastatic CRC patients when compared to healthy group [31]. CRC patients have elevated erythrocyte catalase concentration, an antioxidant enzyme that serves as a body protective mechanism in cancer [72]. It was reported that Cd acts as carcinogen by generating ROS and thereby inhibiting catalase activity [72]. One of the etiologic factors for colon cancer is a defective DNA mismatch repair mechanism [73]. In fact, experimental evidence demonstrates that Cd acts a mutagen by suppressing DNA mismatch repair mechanism [74]. Accordingly, it has been postulated that Cd could play a role in CRC development. Interestingly, Cd induced transformative and carcinogenic effects in human colorectal cells CRL-1807 [75].The injection of nude mice with Cd transformed cells resulted in the development of tumor that grew significantly over a period of 1 month compared to those injected with untreated cells [75]. This study further showed that Cd transformed cells differentially expressed multiple proteins that are likely to play a crucial role in transformation and carcinogenicity [75].

D. Inflammation and CRC

Increasing evidence indicates that inflammation is strongly correlated with the initiation and development of cancer [76, 77]. In fact, persistent inflammation as a result of infection or injury could predispose to cancer development. For instance, chronic infections as in hepatitis B or C are associated with elevated hepatocellular carcinoma risk [76]. Moreover, chronic inflammation of intestine as in patients with IBD is strongly linked to increased CRC risk [8]. Immune cells as macrophages and neutrophils as well as pro-inflammatory mediators such as cyclooxygenases have been suggested to play a key role in the early steps of CRC development [78]. Indeed, inflammation activates multiple cellular signaling pathways associated with tumor initiation, metastasis [79, 80], cell growth, proliferation and invasion [80].

1. Cyclooxygenase pathway

The arachidonic acid (AA) pathway is a central modulator of inflammatory responses [81]. AA is a 20-carbon polyunsaturated fatty acid released from plasma membrane under the action of cytoplasmic phospholipase A2 ($cPLA_2$), serving as a substrate for 3 different enzymatic pathways: the cyclooxygenase (COX) pathway ,the lipoxygenase (LOX) pathway and the cytochrome450 (CYP 450) pathway [82] (Figure 3).

Membrane phospholipids

Hydroxyeicosatetraenoic acids (HETEs)

Epoxyeicosatrienoic acids (EETs)

CYP 450

cPLA$_2$

Arachidonic Acid

LOX

Leukotrienes

'housekeeping'

COX-1

NSAIDs

Inducible

COX-2

PGs,TXA2

PGE2

Physiological functions

EP1-4

Inflammation Malignancy
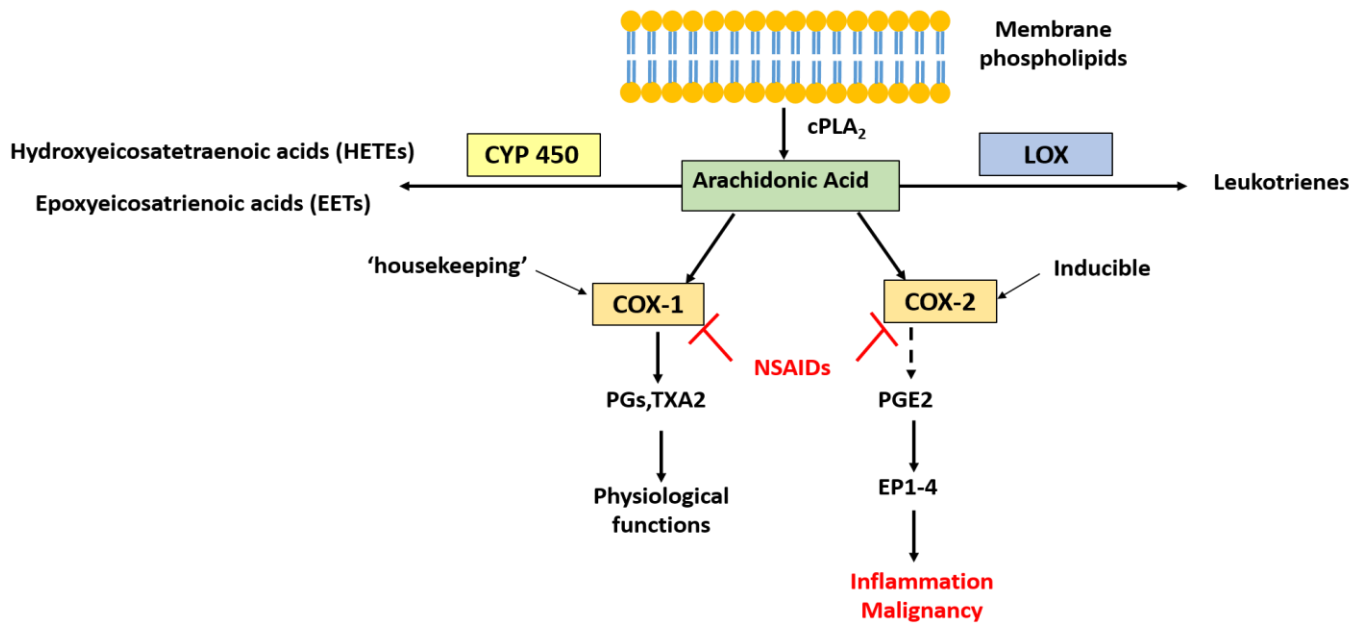
Figure 3: Arachidonic Acid Pathway

The COX enzyme, also known as prostaglandin endoperoxide synthase, exists in 2 major isoforms: COX-1 and COX-2. Both mediate the conversion of AA to prostaglandin H$_2$ (PGH$_2$), which under the action of tissue specific synthases is converted to prostaglandins (PGs) and thromboxanes (TX) (collectively known as prostanoids) [83]. The function as well as the pattern of expression of these 2 isoforms differ dramatically. COX-1 is constitutively expressed in most tissues and serves as a housekeeping gene via production of prostanoids associated with homeostatic function such as platelet aggregation and protection of gastric mucosa [84]. COX-2, on the other hand, is mostly an inducible enzyme stimulated by mitogenic and inflammatory stimuli. Prostaglandin E$_2$ (PGE$_2$), is the major COX-2 product released under tumorigenic and inflammatory conditions [84]. Notably, COX-2 is only constitutively expressed in the brain, testis and renal parenchyma [85]. It is worth mentioning

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that both COX-1 and COX-2 serve as targets for the Non-steroidal Anti-inflammatory drugs (NSAIDs), a class of medications used in the management of pain, fever and inflammation.

2. COX-2 in CRC

Overwhelming evidence shows that COX-2 is positively correlated with autoimmune and inflammatory diseases as well as carcinogenesis [86]. In fact, COX-2 has been implicated in number of tumors such as lung [87], breast [88], pancreatic [89] and colon cancer [90].

The potential association between COX-2 and CRC has been extensively studied. Patients with Gardner's syndrome (which is characterized by multiple intestinal polyposis) treated with NSAIDs displayed a reduction in adenoma number [91]. This was the first indication that COX might be involved in colorectal malignancy and the first clinical evidence that NSAIDs could be used as effective chemopreventive agents in cancer. Several lines of evidence now clearly indicate that COX-2 is involved in colorectal inflammation and carcinogenesis. COX-2, but not COX-1, gene expression was reported to be highly elevated in human colorectal cancer mucosa when compared to the normal one [92]. Moreover, COX-2 overexpression was found to be associated with worse survival among CRC patients [93]. Interestingly, a prospective cohort study has demonstrated that the regular use of aspirin (an NSAID) after diagnosis with CRC is associated with a lower CRC specific and overall mortality [94]. A significant reduction in the occurrence of sporadic colorectal adenomas was reported in patients receiving the selective COX-2 inhibitor celecoxib (400 mg once daily) [95]. Similarly, patients with familial adenomatous polyposis (FAP) receiving celecoxib (400 mg twice daily) displayed a significant reduction in the number of colorectal polyps [96].

Animal models provided additional evidence on the role of COX-2 in colorectal tumorigenesis. Pharmacological inhibition or genetic deletion of COX-2 in Apc $^{\Delta716}$ knockout mice (model of FAP) caused a significant decrease in number and size of intestinal polyps [97]. Furthermore, chemical induction of colon cancer in rats using azoxymethane (AOM) resulted in marked elevation in COX-2 but not COX-1 gene expression [98]. Importantly, the selective COX-2 inhibitor NS-398 significantly reduced the number of colon pre-neoplastic lesions in AOM treated rats [99].

Increased cell invasiveness and survival are among the cancer hallmarks that have been linked to COX-2 expression. Elevated expression and activity of matrix metalloproteinase (MMP) is needed to degrade the extracellular matrix to allow cell invasion and migration [100]. The permanent transfection of human colon cancer Caco-2 cells with COX-2 resulted in the activation of metalloproteinase-2 and increased RNA levels for the membrane-type metalloproteinase and thereby enhanced invasiveness [101]. COX-2 forced over expression on the other hand lead to the inhibition of apoptosis and increased the levels of the pro-survival protein Bcl-2 [102].

Pharmacological studies *in vitro* using NSAIDs provide a strong evidence on the tumorigenic role of COX-2 in CRC. For instance, COX-2 inhibition using NS-398 resulted in decreased levels of MMP-2 and 9 accompanied by a significant reduction in the migration and invasion of CRC mouse cell line MC-26 [103]. Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their polarity and become more invasive, migratory and resistant to apoptosis by transforming into mesenchymal cells [104]. Remarkably, celecoxib inhibited invasiveness and EMT induced by hypoxia and/or epidermal growth factor in the

COX-2 positive HT-29 cells. Importantly, celecoxib was not effective in reducing

invasiveness and EMT of SW-480, a COX-2 negative colon cancer cell line, clearly indicating

that celecoxib inhibition of EMT is mediated by COX-2 [105]. In line with this, the selective

inhibition of COX-2 using rofecoxib attenuated the CRC metastatic potential in mice [106]

and humans [107]. Anti-proliferative effects of NSAIDs have been also described. Treatment

of HT-29 cells with NSAIDs (aspirin, indomethacin, naproxen, and piroxicam) reduced

proliferation and induced apoptosis (all except aspirin) [108]. These studies along with many

others contributed to the recognition of COX-2 as a potential target to treat and/or prevent

CRC.

3. PGE_2 and EP receptors in CRC

As noted earlier, the tumorigenic effects of COX-2 are thought to be largely

attributed to its major metabolite, PGE_2. In fact elevated PGE_2 levels was reported among

patients with colonic adenomas as well as carcinoma [109]. Furthermore, AOM treated rats

injected with PGE_2 (intraperitoneal injections, 7.7 μg) once a week for 25 weeks displayed a

significant increase in the colon tumor incidence, strongly suggesting that PGE_2 enhances

colon carcinogenesis [110].

The cellular levels of PGE_2 are regulated by PGE_2 synthases as well as PGE_2

metabolizing enzymes. Microsomal PGE_2 synthase 1 (mPGES-1) is an inducible enzyme

responsible for the conversion of COX-2 derived PGH_2 to PGE_2. Remarkably, mPGES was

reported to be overexpressed in 83% of colon cancers [111]. In line with this, genetic ablation

of mPGES-1 in mouse models of intestinal cancer resulted in a marked suppression of

intestinal tumorigenesis [112]. Metabolizing enzymes on the other hand ,such as 15-

hydroxyprostaglandin dehydrogenase (15-PGDH), greatly reduce the biological activity of PGE$_2$ [113].Interestingly, while 15-PGDH is highly expressed in normal colon mucosa, it is significantly down regulated in human colorectal cancers [114].

The biological functions of PGE$_2$ are meditated by binding to its cell surface receptors which are G-protein-coupled receptors known as the E type prostanoid (EP) receptors [115]. Four different EP receptors have been characterized: EP1, EP2, EP3 and EP4, each showing distinct downstream signaling pathways (Figure 4). Being coupled to Gq, EP1 receptor acts through the phospholipase C (PLC)/inositol triphosphate signaling, leading to intracellular mobilization of calcium. While EP2 and EP4 are coupled to Gs, which upon stimulation activate adenylyl cyclase (AC) causing increase in intracellular cAMP, EP3 is coupled to Gi which inhibits AC and results in a consequent decrease in intracellular cAMP [115].
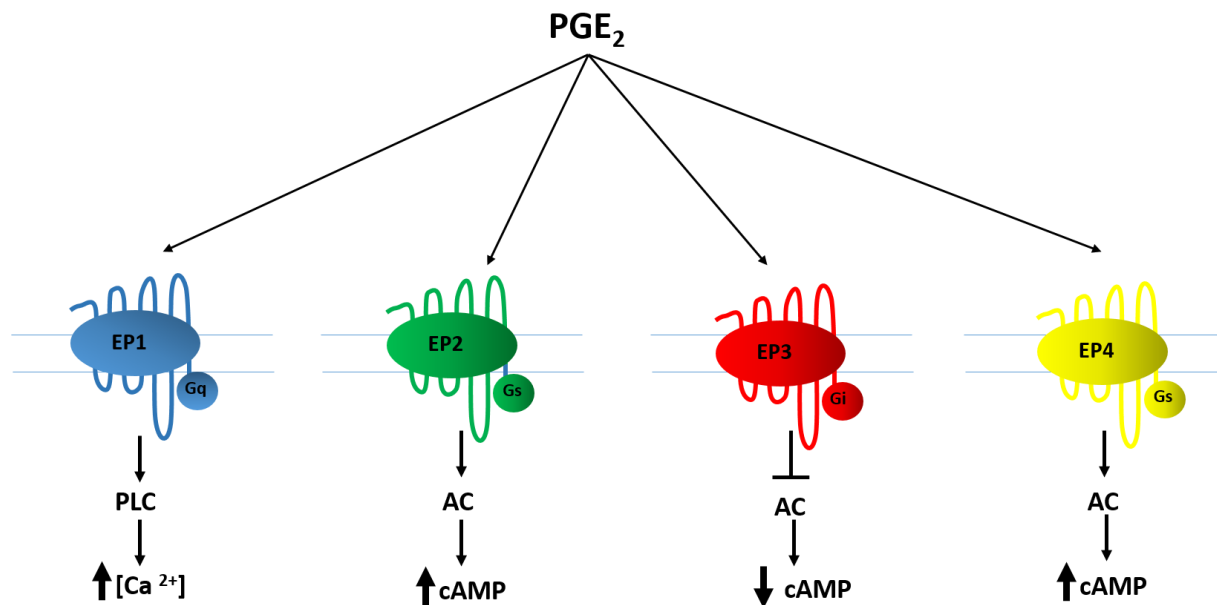

Figure 4: Signal transduction pathways of the four PGE$_2$ receptors subtypes. EP1 is coupled to Gq and therefore causes a rise in intracellular Ca^{2+}. EP2 and EP4 are coupled to

Gs and thereby increase intracellular cAMP. EP3 is coupled to Gi and hence causes a

decrease in intracellular cAMP.

Recent evidence suggests that EP receptors, mainly EP2 and EP4, play a central role in colorectal neoplasia. Increased EP4 receptor expression was reported in colorectal cancers (100%) as well as adenomas (36%) when compared with normal colon epithelium [116].The same study showed that EP4 receptor over-expression favored an anchorage-independent phenotype, suggesting that EP4 plays a role in the transition from colorectal adenoma to carcinoma [116]. Enhanced tumorigenic behavior and suppression of apoptosis was observed in HT-29 human adenocarcinoma cells overexpressing EP4 receptors [117]. Moreover, the genetic deletion or the pharmacological inhibition of EP4 receptor (using ONO-AE2-227) [118] and EP1 receptor (using ONO-8711) [119] in AOM tumor induced mice model caused a significant inhibition of tumor growth, suggesting that EP1 and EP4 receptors play a role in colon tumorigenesis. Interestingly, in the same model, genetic ablation of EP3 receptor was associated with enhanced colorectal neoplasia, reflecting a tumor suppressive role for EP3 in the intestine [120]. To further confirm the roles of EP receptors in colorectal inflammation, other animal models were used. $Apc^{\Delta716}$ mice lacking EP2 receptor displayed a significant reduction in the number and size of intestinal polyps, clearing indicating that EP2 is positively associated with colorectal inflammation [121].

PGE$_2$ exerts a plethora of effects in colorectal cancer promoting proliferation, survival, migration, invasion and angiogenesis. Multiple signaling pathways were shown to be implicated in COX-2/PGE$_2$ mediated carcinogenicity. Phosphatidylinositol 3-kinase (PI3K)/Akt, also known as the survival pathway, is among those known to play a critical role

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in the regulation of cell motility, survival and growth [122]. PGE$_2$, by activating EP4 receptor, caused a significant increase in growth, migration and invasion of LS-174T colorectal carcinoma cells in a PI3K/Akt mediated pathway [123]. These investigators further demonstrated that PGE$_2$ induced activation of PI3K/Akt pathway and subsequent increase in migration and invasion occur via rapid transactivation of epidermal growth factor receptor (EGFR) [124]. Consistently, PGE$_2$ (1, 5 or 10 μM) enhanced the migratory capacity of the human LoVo colon cancer cell line. Of interest, in these cells, PGE$_2$ induced COX-2 upregulation via initiating an EP2/EP4 mediated positive feedback loop in a PI3K/Akt dependent pathway [125].

Since PGE$_2$ and peroxisome proliferator activated receptor δ (PPAR δ) are known to promote cell survival, a group of investigators hypothesized that PGE$_2$ induced anti-apoptotic effects could be mediated by PPAR δ [126]. PGE$_2$ promoted cell survival by indirectly transactivating PPAR δ via a PI3K/AKT dependent pathway [126]. In line with this, PGE$_2$ suppressed apoptosis and induced Bcl-2 protein expression via the activation of MAPK/ERK pathway in HCA7 human colonic carcinoma cells [127].

Hepatocyte growth factor (HGF)/c-Met signaling is ideally associated with invasion and metastasis of cancer cells [128]. PGE$_2$ was shown to transactivate HGF receptor in a pathway involving EGFR leading to enhanced colon cancer cells invasion [129].

COX-2/PGE$_2$ pathway has been also shown to be implicated in angiogenesis. For instance, Wang *et al.* demonstrated that PGE$_2$ induced the expression of CXCL1, an angiogenic chemokine [130]. Moreover, PGE$_2$ was reported to stimulate the expression of

vascular epidermal growth factor (VEGF), a key regular of tumor neovascularization, in HCT116 human colon cancer cells [131].

Regarding the growth promoting effect of PGE_2 on colorectal cancer cell lines, studies yielded conflicting results (Figure.5). This might be due to different experimental settings such as PGE_2 concentration, presence or absence of serum, proliferation time frame and others [132]. For instance, Qiao *et al.* reported that PGE_2 at a maximal concentration of 10^{-6} M for 72 hours stimulated proliferation of HT-29 human colorectal adenocarcinoma [133]. On the other hand, other investigators failed to observe any proliferative effect of PGE_2 (100 nM) in HT-29 cells [134]. An anti-proliferative effect of PGE_2 was even reported. Parker *et al.* showed that PGE_2 ($> 2x10^{-5}$ M) inhibited proliferation and had no proliferative effect at concentrations ranging from 10^{-9} M up to 10^{-5} M in HT-29 cells [135]. Interestingly, the mitogenecity of PGE_2 was correlated with its concentrations and consequently intracellular cAMP levels. Low PGE_2 (nanoM) levels activate EP3 receptor causing a subsequent decrease in cAMP and thereby increased proliferation of CRC cells. Higher concentrations (microM) on the other hand, activate EP2 and EP4 receptors resulting in elevated cAMP levels and consequent antiproliferative effects [132].

Effect of PGE$_2$ on CRC	Methods	Concentration	Time (Hours)	Serum
Stimulates proliferation	Cell counting	$10^{-10}<10^{-8}<10^{-6}$M	72	10% FBS
Inhibits proliferation	Cell counting MTT	$> 2\times10^{-5}$M $\rightarrow$inhibits 10^{-9} M up to 10^{-5} M $\rightarrow$no effect	72	10% FBS
Has no effect	Cell counting Bradford Assay	10^{-7}M	48 72	1% FBS, serum starved for 24 hours
Both stimulates and inhibits	Thymidine incorporation Automated cell counting	10nM$\rightarrow$ EP3$\rightarrow$decrease cAMP$\rightarrow$ increase in proliferation 1 µM$\rightarrow$ EP2/EP4$\rightarrow$increase cAMP$\rightarrow$ decrease in proliferation	48-72 Proliferation evident at 72	Absence of serum for 16-18 hours (overnight)

Figure 5: Conflicting Literature Data on PGE$_2$ Growth Promoting Effects

E. Hypothesis:

Cd is a heavy metal contaminant known to be associated with inflammation. It is well established that inflammation, namely the COX-2/PGE$_2$ pathway, plays a central role in CRC. Previous findings indicate that Cd activates the Akt pathway and induces COX-2 upregulation [65] in neuronal cells. Despite the fact that Cd is a known human carcinogen and its toxicity has been associated with CRC, the underlying molecular mechanisms of Cd-induced colorectal malignancy remain vague. **This prompted us to hypothesize that Cd increases the migration of CRC cells by increasing PGE$_2$ production via a ROS-p38-COX-2 dependent mechanism, concomitant with activation of the ROS-Akt pathway**.

CHAPTER II

MATERIALS AND METHODS

A. Reagents:

Cadmium Chloride dihydrate ($CdCl_2$) was a kind gift from the chemistry department at American University of Beirut (AUB). Rabbit monoclonal anti-COX-2 (ab15191), anti-β actin (ab119716), anti-p38 (phospho T180+Y182 ab38238) anti-total p38 (ab7952), anti-pan AKT (ab8805) and anti-pan AKT (phospho T308 ab38449) were obtained from abcam. Goat anti-rabbit secondary antibody IgG (H+L), HRP conjugate was obtained from Invitrogen.

N-Acetyl-Cysteine (#1761C311) was obtained from amresco. SB202190 (ab20638) and Celecoxib (ab141988) were purchased from abcam. LY294 002 was purchased from Sigma-Aldrich. PGE_2 was obtained from Cayman Chemical.

B. Cell culture:

HT-29 human colorectal adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown in 10 mm dishes and maintained in a humidified incubator at 37°C with 5% CO_2 atmosphere. Culture medium was changed every 48 hours and cells were split in a ratio of 1 to 4 once reaching 90% confluence. Culture media and FBS were purchased from Sigma-Aldrich. Penicillin/streptomycin was obtained from Lonza.

C. Migration Assay:

Cell migratory capacity was assessed using the scratch or wound healing assay .Cells were seeded in 12-wells plate ($6\text{x}10^5$ cells/well) in 1 ml complete DMEM. Once reaching 90-95% confluence, a scratch was created manually through the confluent monolayer using a white tip ($2–20\,\mu$l). The culture medium was then aspirated, and wells were washed with PBS to remove cellular debris. Complete fresh medium was then added along with the indicated treatments. Photomicrographs were taken at baseline (0 hour) and at the indicated time points. ZEN lite from Zeiss Microscope software was used to measure the width of the scratch which reflected the extent of cell migration. The distance migrated was measured in μm.

D. Viability Assay:

Cell viability was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. MTT was obtained from abcam (ab146345). Briefly, HT-29 cells were seeded in 96-well plates ($1\text{x}10^4$ cells/well) in 100 μl complete DMEM. 24 hours later, media were aspirated and fresh medium was added along with the indicated treatments for the specified time points. 10 μl MTT (5mg/ml) was then added to each well and cells were incubated at 37 ºC for 1 hour. All culture media were then removed and the formazan violet crystals were dissolved in 100 μl DMSO. Treatments were performed in duplicates. Cell viability was assessed colorimetrically at a wavelength of 550 nm using Elisa Multiscan EX Reader (Thermo), where the intensity of violet color is directly proportional to the number of viable cells.

E. Western blotting:

Cells were lysed using 10 mM Tris pH 6.8, 2% SDS. Proteins were quantified by DC protein assay kit (Bio-Rad, USA). Protein samples were then loaded (30-50µg) on 5-11% SDS-PAGE along with protein ladder (Abcam), electrophoresed at 70 V (Bio-Rad, USA) and then transferred to PVDF membranes (Bio-Rad, USA). After blocking with 5% non-fat dry milk in TBS-T (TBS and 0.05% Tween 20) for 1 hour at room temperature, the blots were probed with primary antibody at 4 ^{o}C overnight. Blots were then washed three times with TBS-T and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:2000) for 1 hour at room temperature. Immunoreactive bands were finally detected by ECL chemiluminescent substrate (Bio-Rad, USA). Blot images were taken by using Chemidoc MP Imaging system.

F. Dihydroethidium staining (DHE):

ROS production was assessed using DHE stain. HT-29 cells were seeded on a cover slip in 12-well plates ($3x10^5$ cells/well) in 1 ml complete DMEM and incubated until reaching 50% confluency. The cells were treated as indicated. Medium was then removed, cells were washed twice with cold PBS. DHE stain (5 µM final concentration) was added to the wells (1ml/well) in the dark and cells were incubated for 1 hour. DHE stain was removed and cells were washed once with cold PBS. Stained cells on the cover slips were imaged using Microscope Zeiss Axio.

G. Transient Transfections:

COX-2 transcriptional activity was measured using the luciferase reporter assay. Cells were transiently transfected with COX-2 luciferase expression reporter plasmid using Lipofectamine 2000, to achieve an approximate 80% transfection efficiency. Cells were then allowed to recover overnight in complete growth medium. The next day, cells were treated as indicated. For the luciferase analysis, cells were washed, lysed in luciferase lysis buffer (Promega), snap-frozen, and then thawed at room temperature. This was followed by the centrifugation of the cell lysates at 9,300 g for 10 minutes. The luciferase activity was finally determined in the supernatant.

H. Enzyme linked immunosorbent assay (ELISA):

HT-29 cells were subjected to the specified treatment conditions for the indicated time points and PGE_2 production was evaluated using the PGE_2 Enzyme linked immunosorbent assay (ELISA) kit (R&D Systems) according to manufacturer's protocol.

I. Statistical Analysis

Statistical analysis was performed by a student's t-test for either paired or unpaired observations using GraphPad Prism version 5.0 and InStat3 Software (GraphPad software, Inc. San Diego, CA). Data was presented as mean $\pm$ SEM, where n is equal to the number of times an experiment is repeated (n will be = 3). When more than two means are used for comparison, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). $p < 0.05$ is considered significant.

CHAPTER III

RESULTS

A. Migration

1. Cd induces the migration of HT-29 cells

Cd at a concentration of 100 nM significantly ($p < 0.05$) promoted wound healing at 9 and 12 hours (Figure 6 A and B). On the other hand, 1000 nM Cd significantly ($p < 0.05$) increased the migration only at 9 hours (Figure 9B).

A.
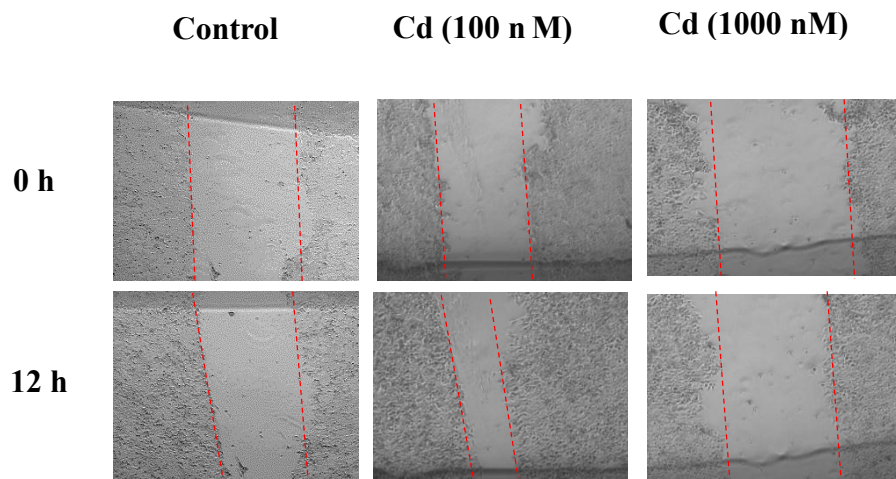
B
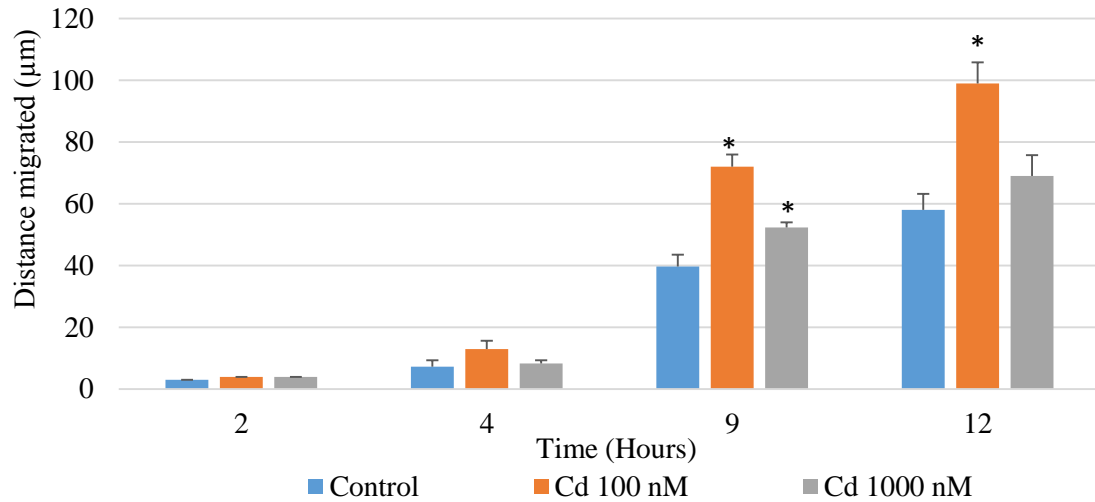
Figure 6: Cd increases the migration of HT-29 cells. *Cell migration was examined by the wound-healing assay. HT-29 cells were treated with Cd (100 and 1000 nM). Images were taken at the indicated time points. Values are represented as mean ± SEM of n=3. * denotes p < 0.05.*

2. ***Cd induces the formation reactive oxygen species:***

As shown in Figure 7, Cd treatment significantly increased ROS production as early as 5 minutes.

Control   5 minutes   10 minutes   30 minutes
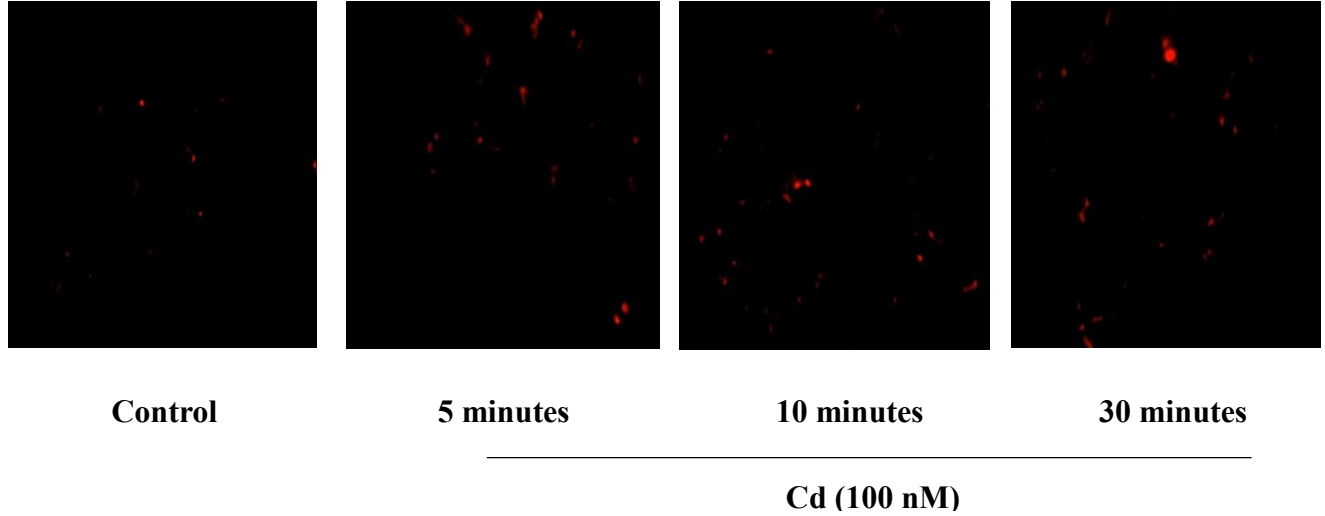
Cd (100 nM)

Figure 7: Cd increases the production of ROS. *HT-29 cells were treated with Cd (100 nM) for 5, 10, and 30 minutes and DHE staining assay was performed.*

3. Cd-induced migration is ROS mediated

Pre-treating cells with the ROS scavenger N-Acetyl-Cysteine; NAC (10 mM) for 30 minutes significantly ($p < 0.05$) attenuated Cd-induced migration (Figure 8 A and B).

A.
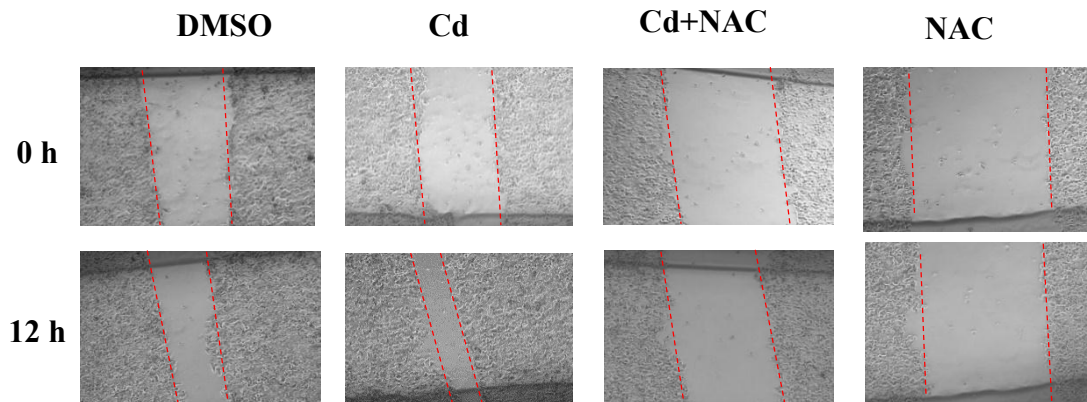
B.
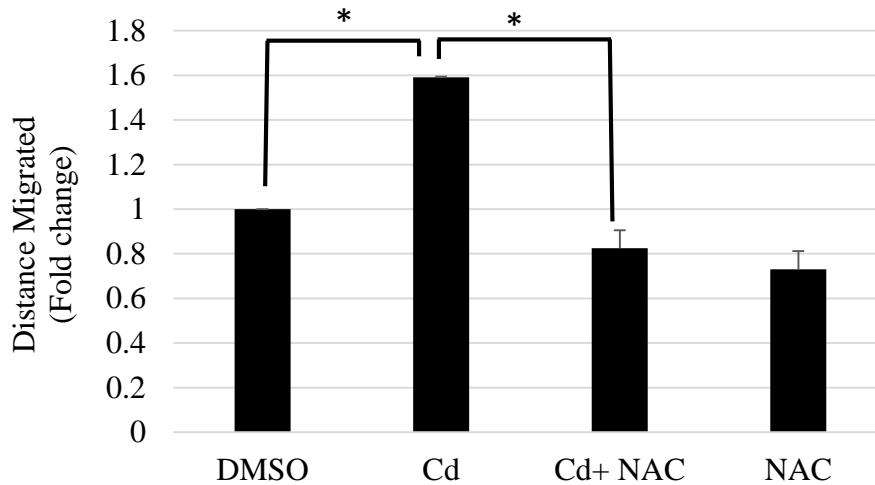
Figure 8: Cd-induced ROS production increases HT-29 migration. *HT-29 cells were treated with Cd (100 nM) in the presence or absence of the ROS scavenger NAC (10 mM) and migration was assessed by scratch assay .Values are represented as mean ± SEM of n=3. * denotes p < 0.05.*

4. p38 MAPK is activated in response to Cd and is crucial for migration

Cd caused more than 2 fold increase in p38 phosphorylation (p < 0.05) after 10 and 30 minutes of treatment (Figure 9A, 9B). Furthermore, pretreating the cells with SB202190 (10 μM), a specific p38 MAPK inhibitor, significantly (p < 0.05) abrogated Cd-induced migration (Figure 9C, 9D).

A.

Cd 100 nM

| Ctrl | 5 | 10 | 30 | 60 | mins |

Phospho-p38

Total p38

B.

Phospho-p38 / Total p38 (Fold increase)

ctrl   5   10   30   60   minutes

Cd 100 nM

28
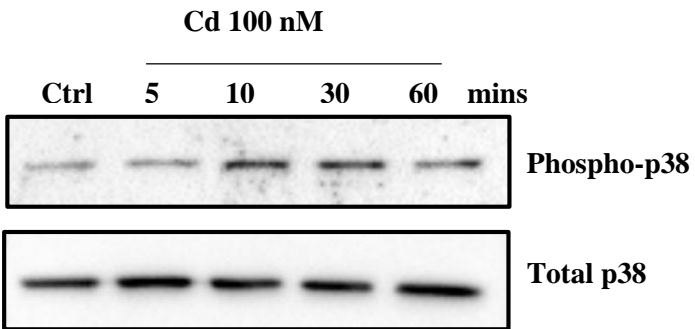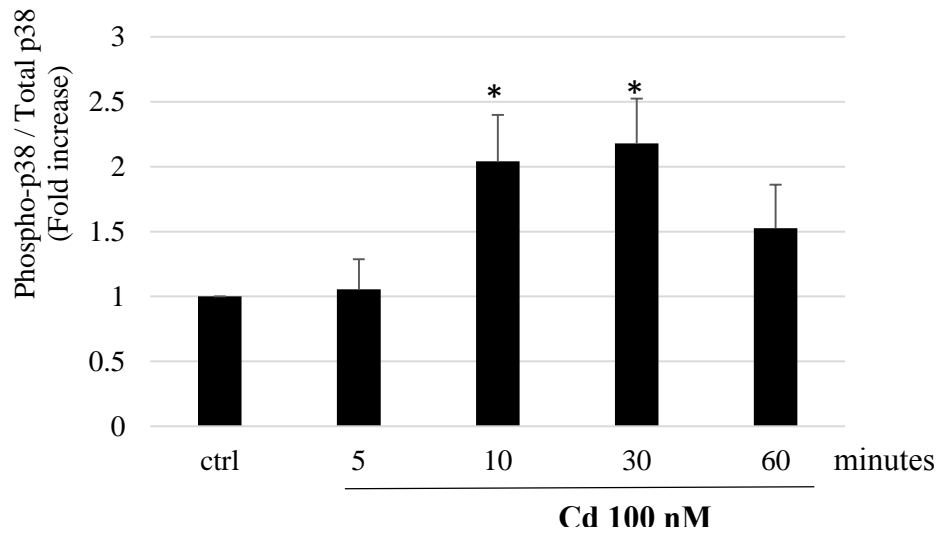
C.
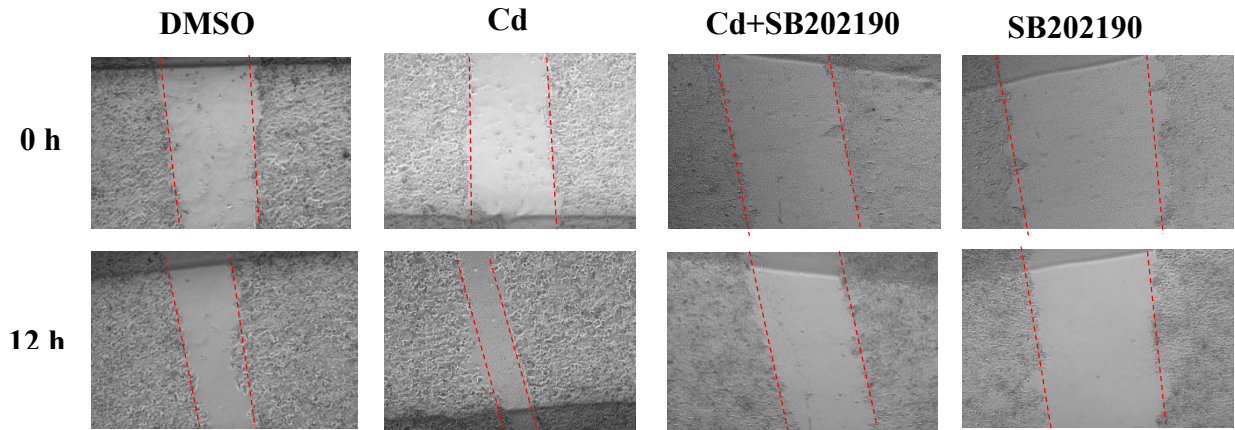
D.
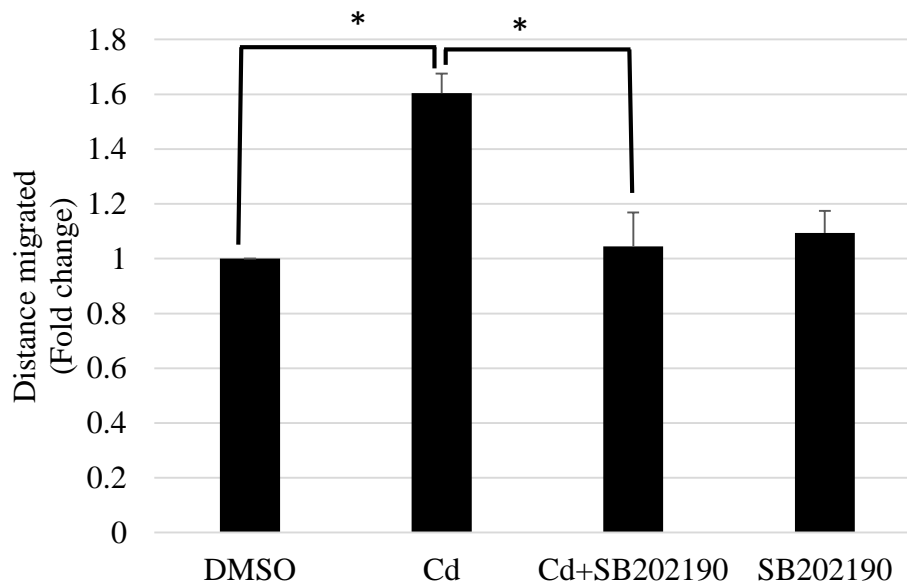
Figure 9: Cd activates p38 MAPK which is implicated in migration. *(A) and (B): HT-29 cells were treated with Cd (100 nM) for 0, 5, 10, 30 and 60 minutes and p38 MAPK phosphorylation levels were determined by western blotting. (C) and (D): Cells were*

pretreated with SB202190 (10 µM) for 30 minutes followed by Cd (100 nM) and migration was determined by wound healing assay. Values are the mean ± SEM of three replicates. * denotes p < 0.05.

5. p38 MAPK is activated by ROS

Pre-treatment with NAC significantly ($p < 0.05$) abolished Cd-induced p38 activation (Figure 10A, 10B).

A.

| Phosho-p38 |
| Total p38 |

Cd	-	+	+	-
NAC	-	-	+	+
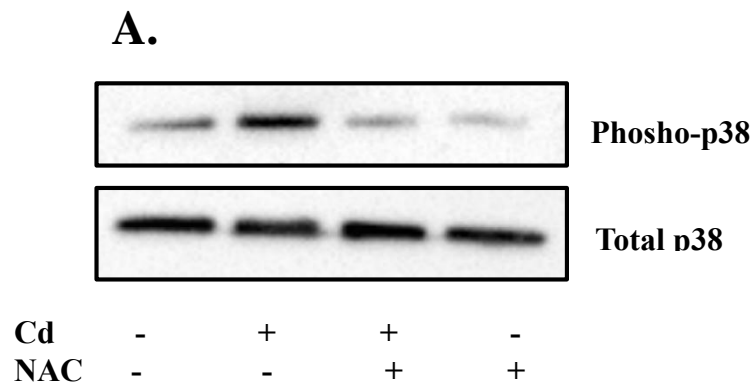
B.
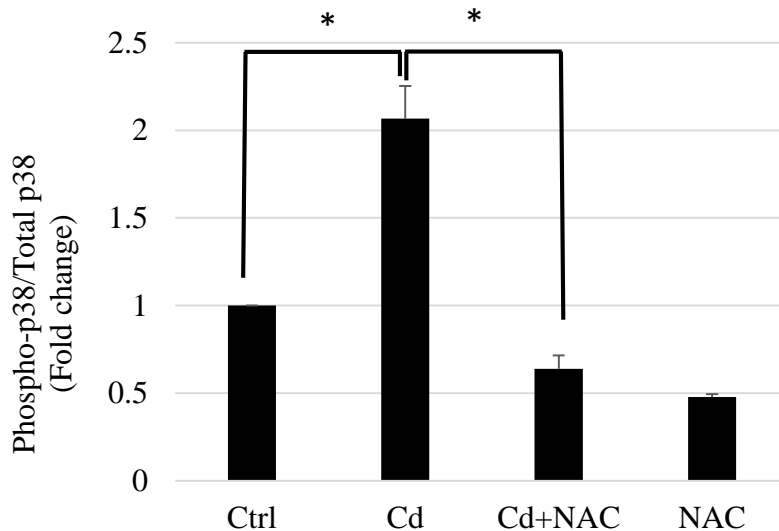
Figure 10: Cd-induced ROS is upstream p38 MAPK. *HT-29 cells were treated with Cd (100 nM) for 30 minutes in the presence or absence of NAC and p38 MAPK phosphorylation levels were determined by western blotting. Values are the mean ± SEM of three replicates. * denotes p < 0.05.*

6. Cd increases COX-2 transcription and protein expression:

Cd significantly (p < 0.05) increases COX-2 protein expression as early as 6 hours (Figure 11A, 11B) and maximally at 24 hours (Figure 11C, 11D). This result was further validated by assessing the effect of 100 nM Cd on COX-2 transcriptional activity. Consistent with the protein results, Cd caused a time dependent increase (p < 0.05) in transcriptional activity of COX-2 reporter starting at 6 hours of treatment (Figure 11E).

A.

Cd 100 nM

| Ctrl | 6 | 12 | 24 |

COX-2

β-actin

B.

C.

Cd 100 nM

| Ctrl | 24 | 48 | 72 |

COX-2

β-actin

D.

32
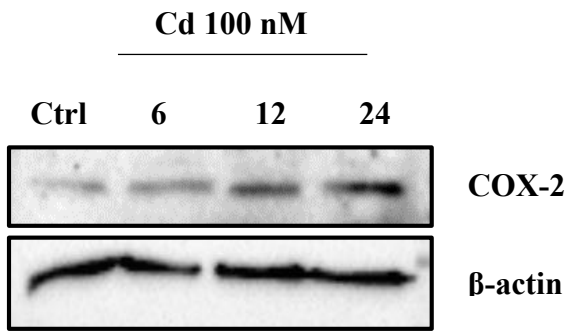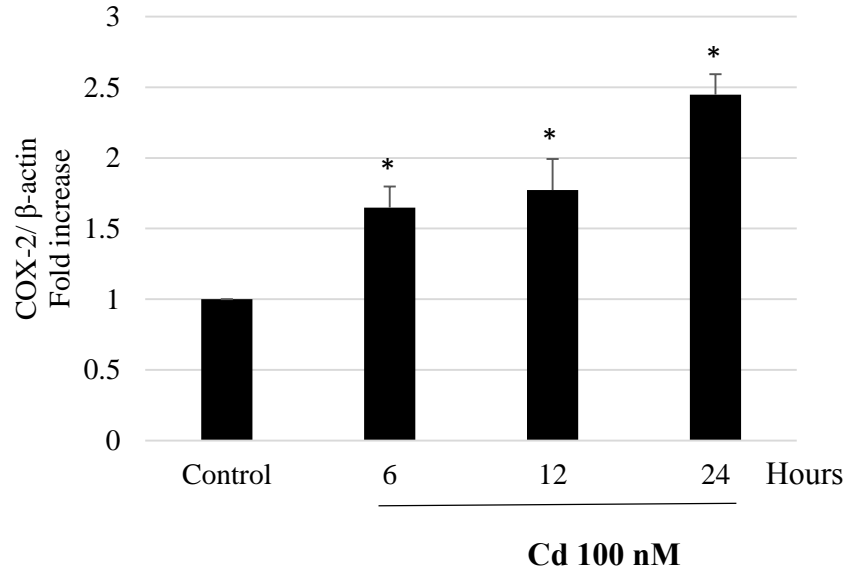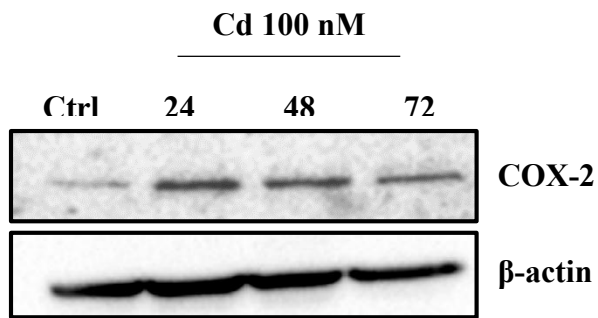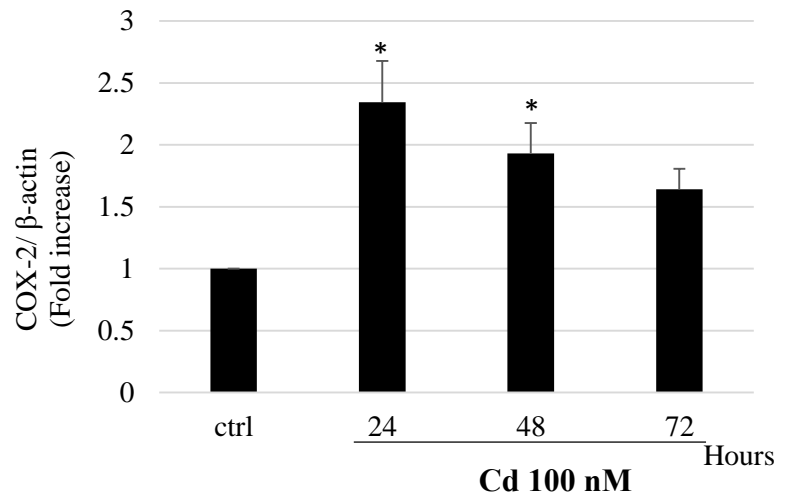
E.
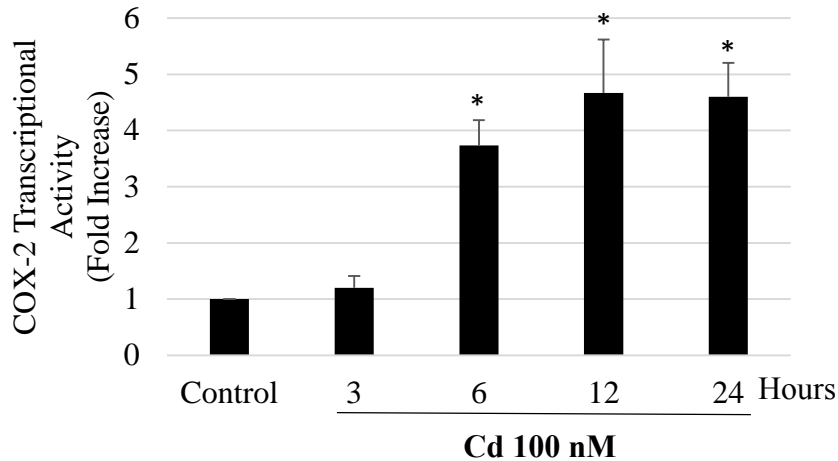
Figure 11: COX-2 protein expression and transcription are enhanced in response to Cd.

(A), (B), (C) and (D): HT-29 cells were treated with Cd (100 nM) for 6, 12, 24, 48 and 72

hours. COX-2 protein expression was assessed by western blotting. (E): Cells were treated

with Cd (100 nM) for 3,6,12 and 24 hours. COX-2 transcriptional activity was assessed

using the luciferase reporter assay. Values are the mean ± SEM of three independent

*experiments. * denotes p < 0.05.*

7. *Cd-induced migration is mediated by COX-2*

As shown in figures 12A and 12B, the selective inhibition of COX-2 with celecoxib

(CLX; 10 μM) significantly ($p < 0.05$) abolished Cd-induced migration (Figure).

A.
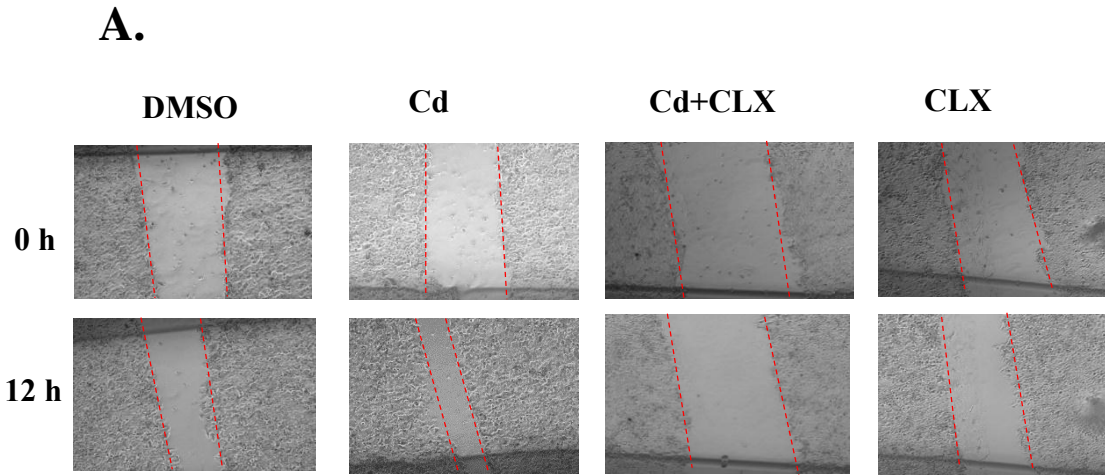
B.
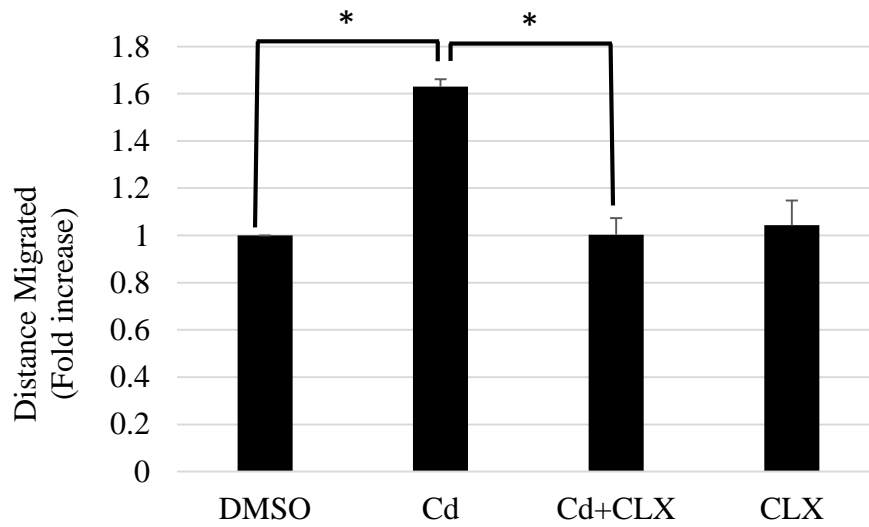
Figure 12: Cd-induced migration is COX-2 dependent. *HT-29 cells were pre-treated with CLX (10 μM) for 30 minutes followed by Cd (100 nM). Migration was determined by scratch assay. Values are represented as mean ± SEM of n=3. * denotes p < 0.05.*

8. ROS and p38 MAPK mediate COX-2 upregulation

Reducing the levels of ROS (Figure 13A, 13B) and inhibiting p38 (Figure 13C, 13D) significantly attenuated Cd-induced COX-2 upregulation. Moreover, pretreatment with SB202190 resulted in a significant ($p < 0.05$) reduction in Cd-induced increase in COX-2 transcriptional activity at 12 hours (Figure 13E).

A.

COX-2

β-actin

Cd	-	+	+	-
NAC	-	-	+	+

B.

C.

COX-

β-actin

Cd	-	+	+	-
SB202190	-	-	+	+

D.

35
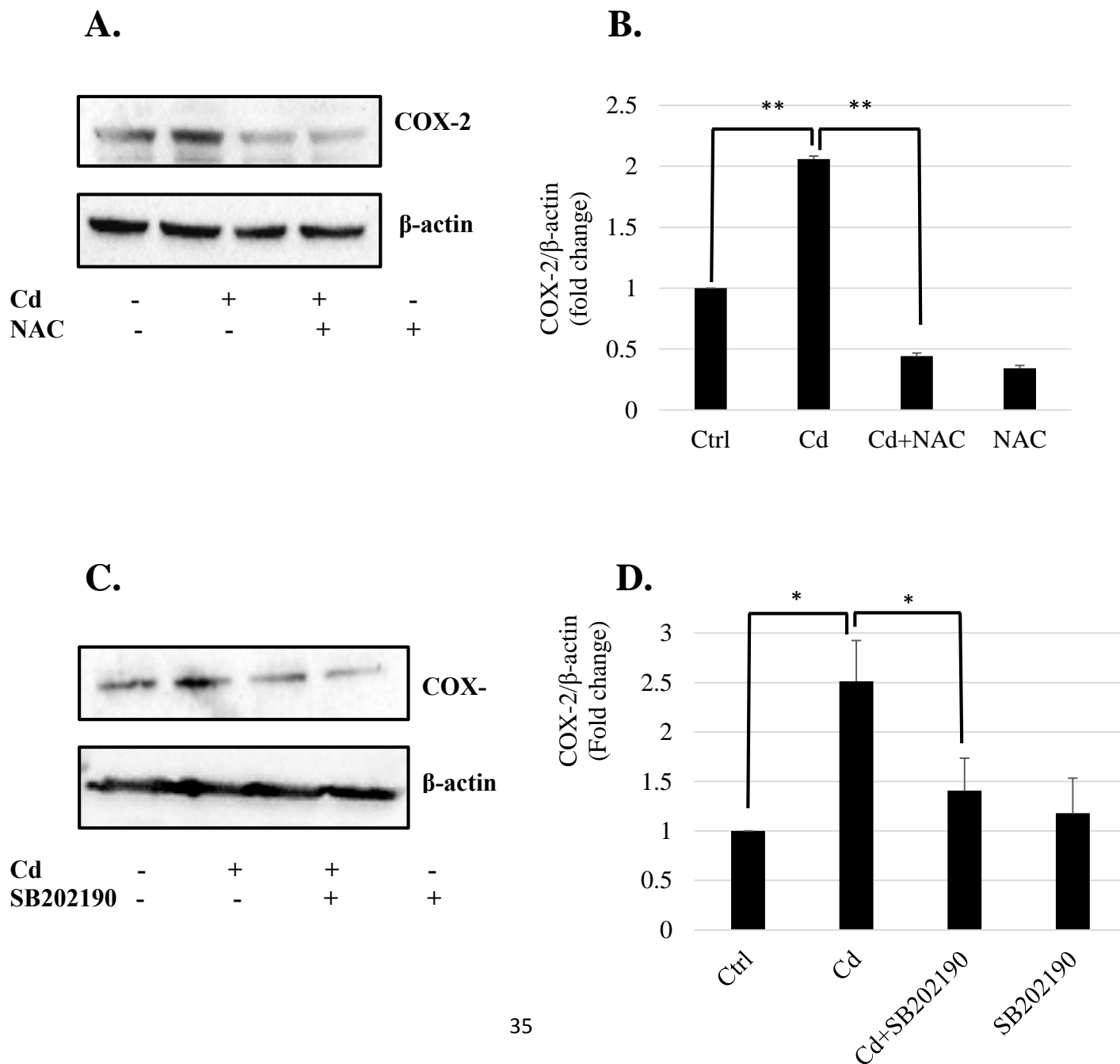
E.
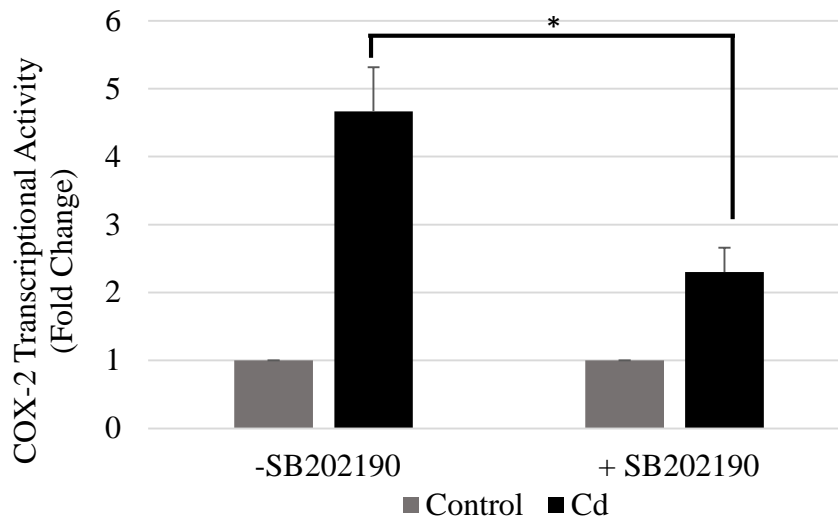
Figure 13: Cd acts through ROS and p38 to upregulate COX-2. *(A), (B), (C) and (D): HT-29 cells were treated with Cd (100 nM) for 24 hours in the presence or absence of NAC and/or SB202190. COX-2 protein levels were assessed by western blotting. (E): Cells were pre-treated with SB202190 for 30 minutes followed by Cd (100 nM) for 12 hours. COX-2 transcriptional activity was evaluated using luciferase reporter assay. Values are represented as mean ± SEM of n=3.* denotes p < 0.05, ** denotes p < 0.01.*

9. PGE$_2$ potentiates migration and its production is enhanced in response to Cd

Exogenous PGE$_2$ significantly ($p < 0.05$) increased HT-29 migration (Figure 14A). Moreover, except for the 10 nM concentration, Cd at all the tested concentrations caused a significant ($p < 0.05$) increase in PGE$_2$ production. Interestingly, 100 nM Cd was associated with the highest rise in PGE$_2$ levels (Figure 14B) and caused a time dependent increase ($p < 0.05$) in PGE$_2$ production starting as early as 1 hour (Figure 14C).

A.
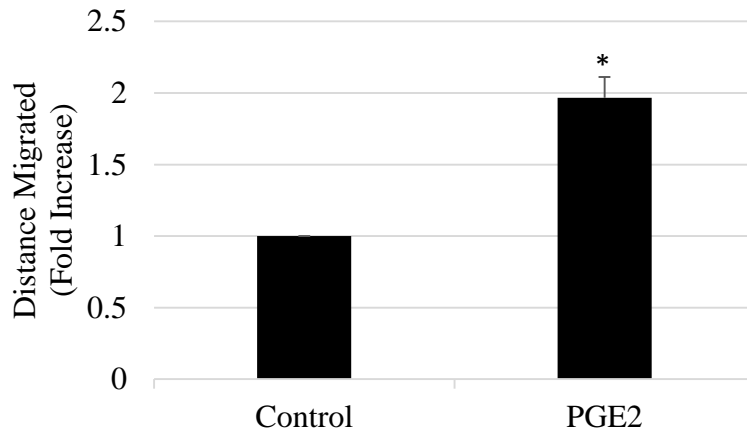
B.
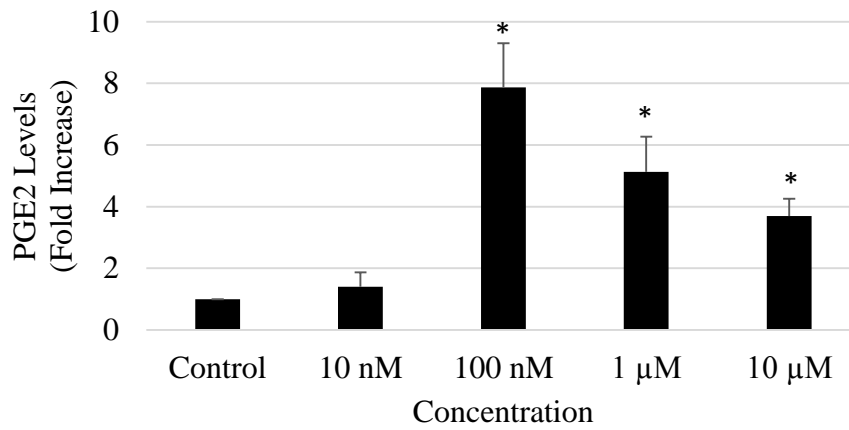
C.
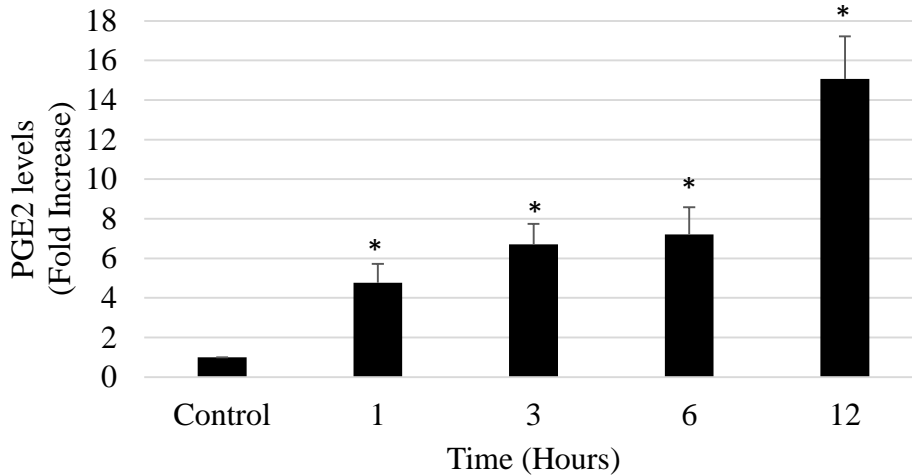
Figure 14: PGE$_2$ enhances migration and its levels are increased in response to Cd.

*(A): Cells were treated with 1 μM PGE$_2$ for 12 hours and migration was assessed by scratch assay. (B): HT-29 cells were treated with increasing concentrations of Cd (10 nM, 100 nM, 1 μM and 10 μM) for 6 hours and PGE$_2$ levels were measured using ELISA kit. (C): Cells were treated with Cd (100 nM) for 1, 3, 6 and 12 hours and PGE$_2$ production was assessed using ELISA kit. Values are represented as the mean of fold increase ± SEM of three independent experiments. * denotes p < 0.05.*

10. ROS, p38 MAPK and COX-2 are implicated in Cd-induced PGE$_2$ production

Reducing cellular levels of ROS and blocking p38 or COX-2 activity was associated with a significant reduction ($p < 0.05$) in Cd-induced PGE$_2$ production at 6 hours (Figure 15).
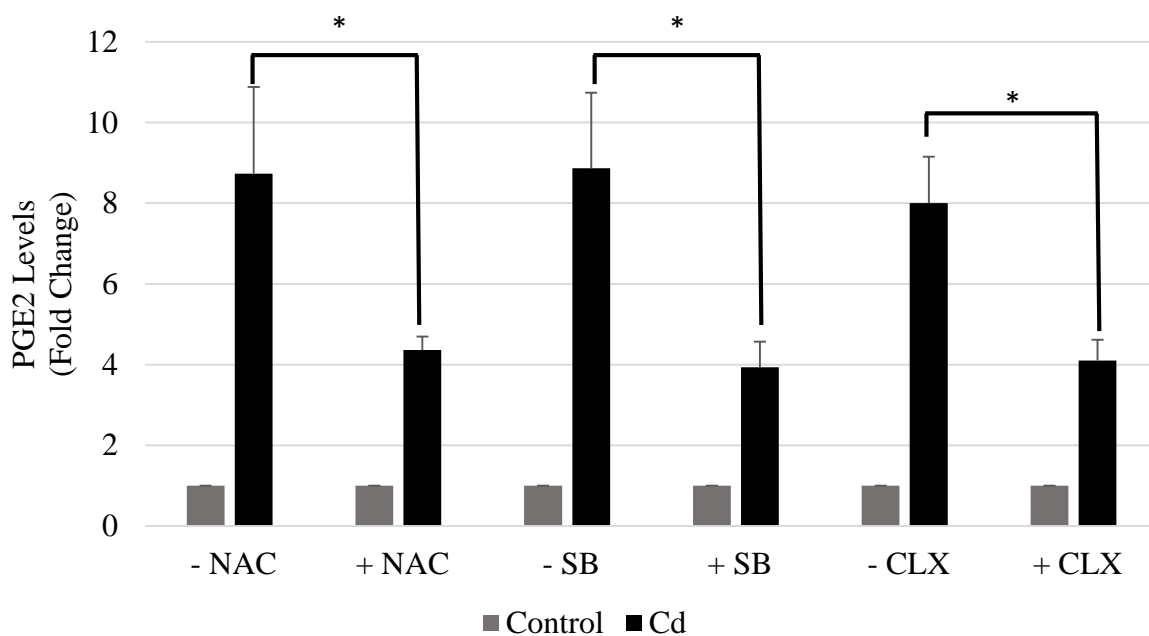
Figure 15: Cd increases PGE$_2$ levels in a ROS, p38 and COX-2 dependent pathway. *HT-29 cells were pre-treated with NAC, SB202190 or CLX for 30 minutes followed by Cd (100 nM) for 6 hours. PGE$_2$ levels were measured using ELISA kit. Values are represented as the mean of fold change ± SEM of n=3. * denotes p < 0.05.*

11. Cd activates Akt in a ROS dependent pathway

Cd caused a significant increase in Akt phosphorylation, 30 and 60 minutes following treatment (p <0.05 and 0.01 respectively) (Figure 16A, 16B). Moreover, pre-treatment with NAC significantly attenuated Cd-induced Akt phosphorylation at 60 minutes (Figure 16C, 16D).

A.

B.

C.

D.

Phospho-Akt

Total Akt

Cd	-	+	+	-
NAC	-	-	+	+
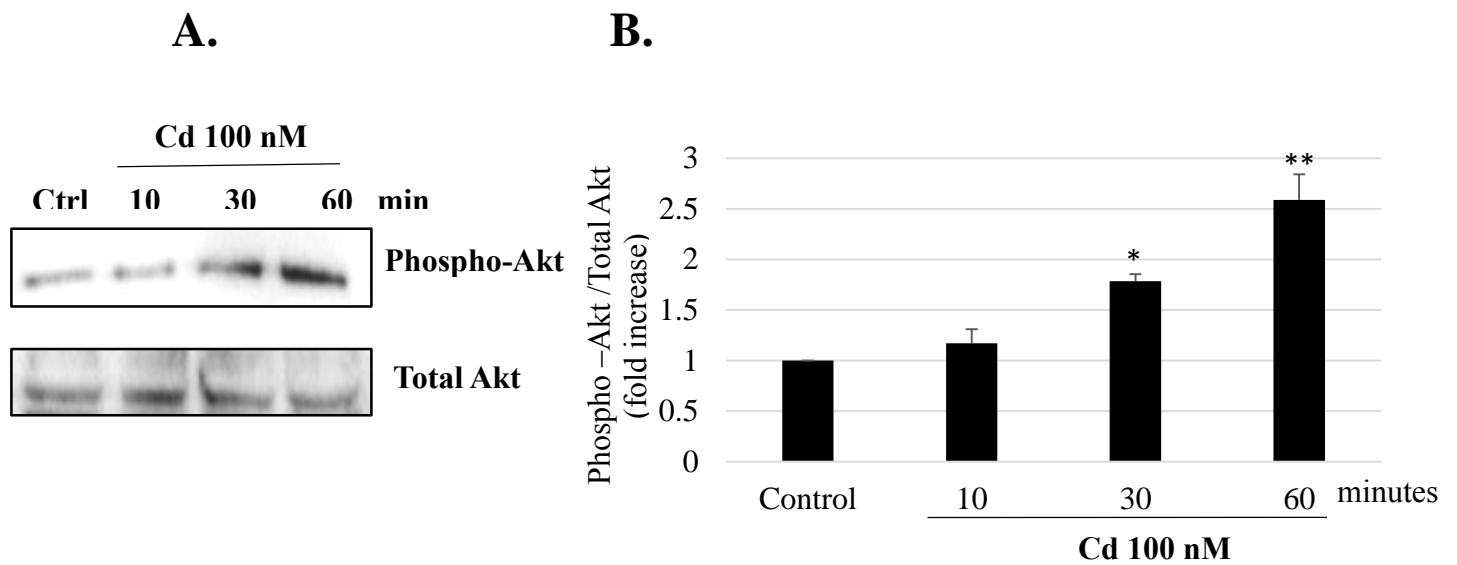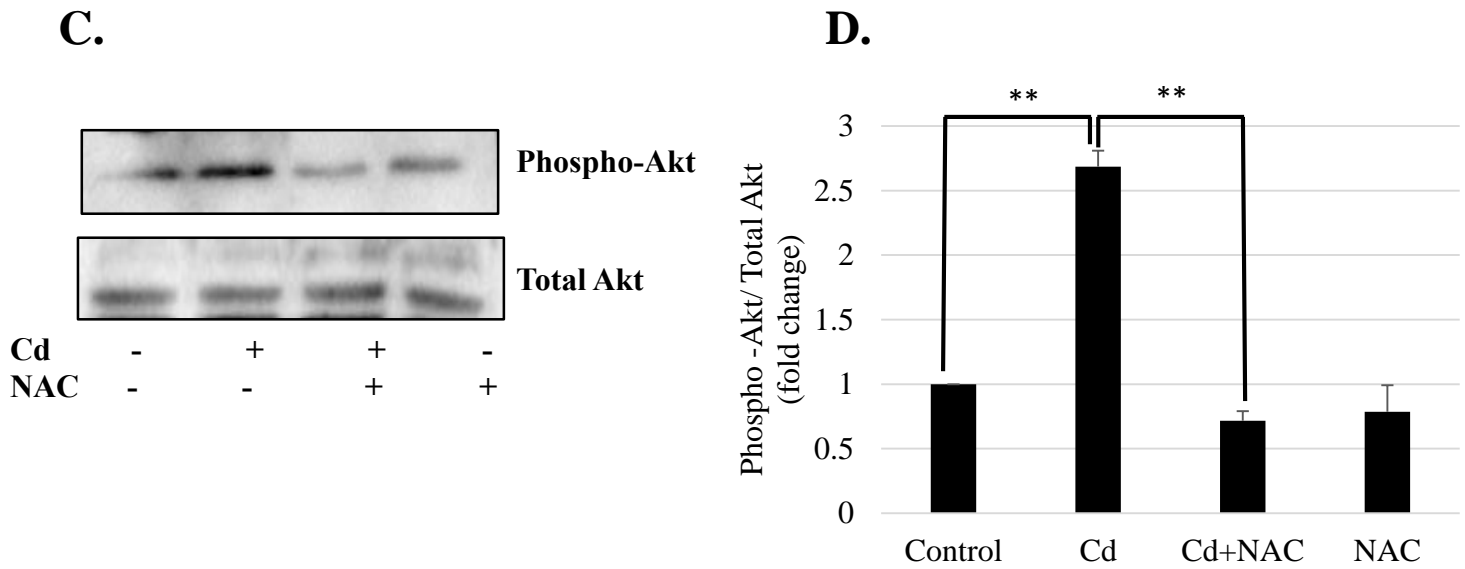
Figure 16: Cd activates Akt in a ROS mediated pathway. *(A) and (B): HT-29 cells were treated with Cd (100 nM) for 0, 10, 30 and 60 minutes and Akt phosphorylation levels were assessed by western blotting. (C) and (D): cells were pre-treated with NAC (10 mM) for 30 minutes followed by Cd (100 nM) for 60 minutes and Akt phosphorylation levels were determined by western blotting. Values are represented as mean ± SEM of n=3. * denotes p < 0.05, ** denotes p < 0.01.*

12. Akt is a crucial mediator of Cd-induced migration

The inhibition of PI3K and thereby Akt significantly ($p < 0.05$) abrogated Cd-increased migration. Pre-treatment with both CLX and LY294 002 didn't provide additional decrease in migration.
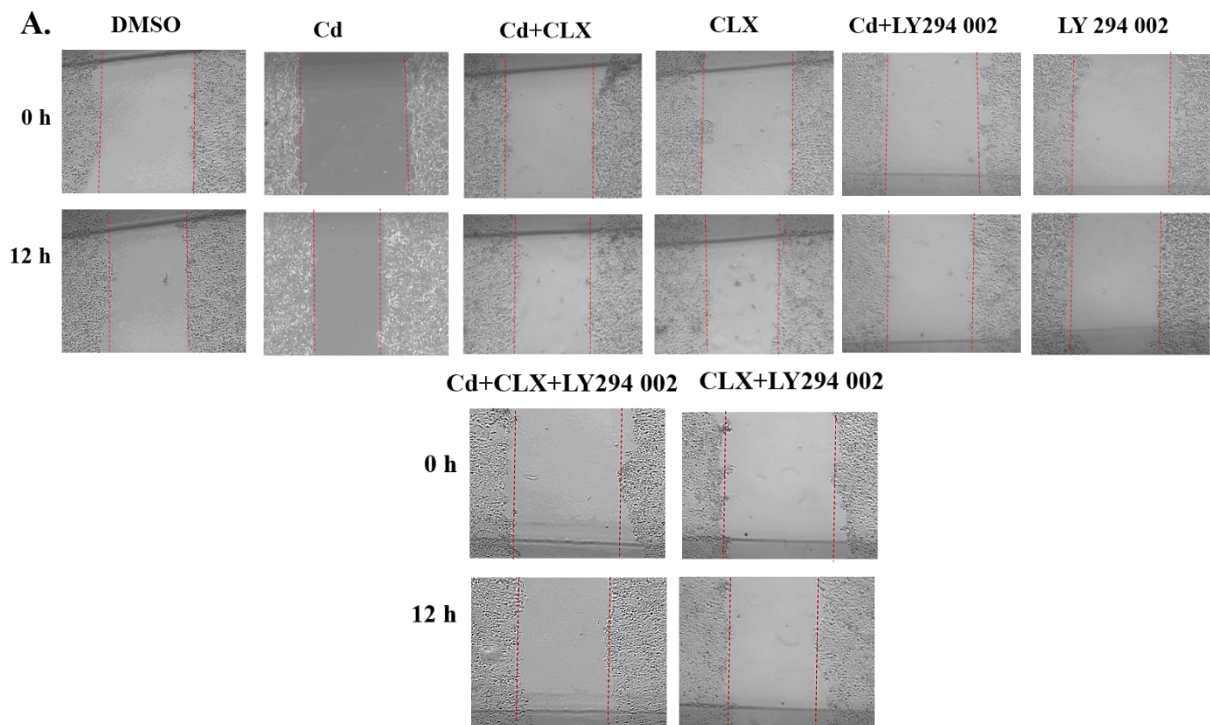
B.
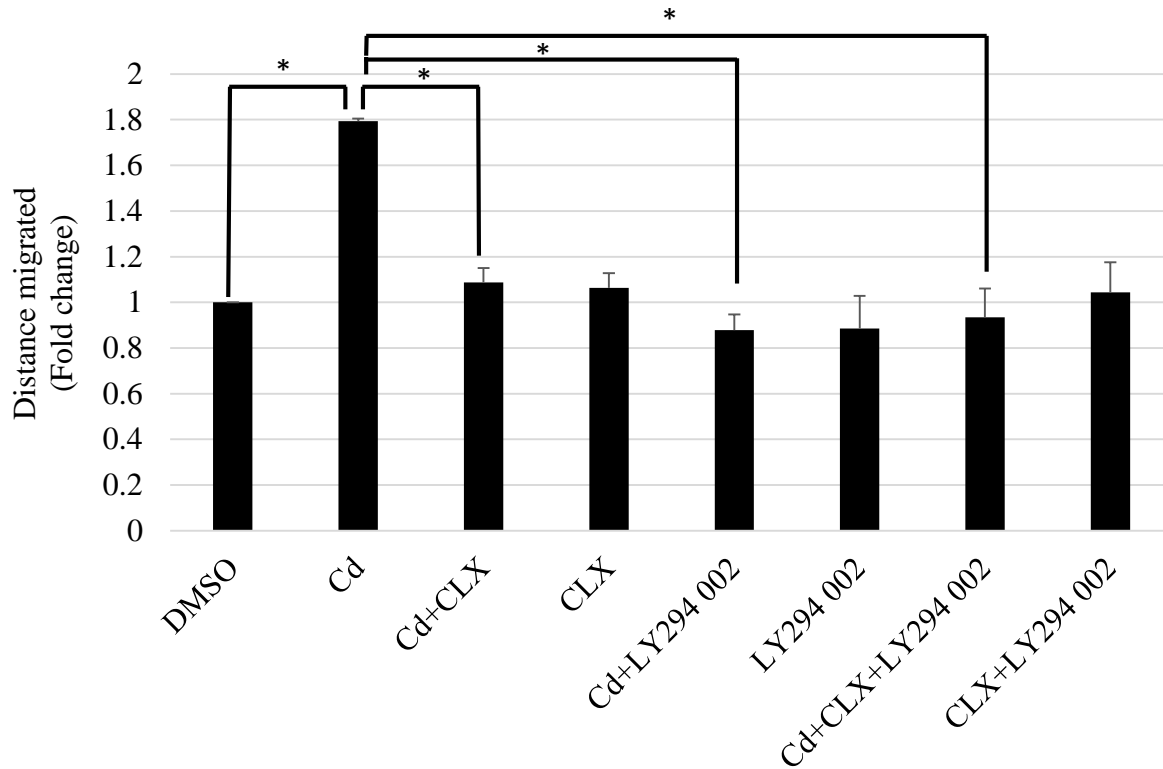
Figure 17: Cd-induced Akt activation is implicated in migration. *HT-29 cells were treated with Cd (100 nM) in the presence or absence of LY294 002 (10 μM), a PI3K inhibitor and/or CLX. Migration was evaluated using scratch wound healing assay. Values are represented as mean ± SEM of n=3.* denotes p < 0.05.*

B. Proliferation:

1. *Cd reduces HT-29 viability:*

Cells were treated with increasing concentrations of Cd relevant to those detected in human serum [35] (1, 10 or 100 nM) for different time points (24, 48 and 72 hours). As shown in Figure 18, Cd caused an unexpected decrease in HT-29 proliferation.
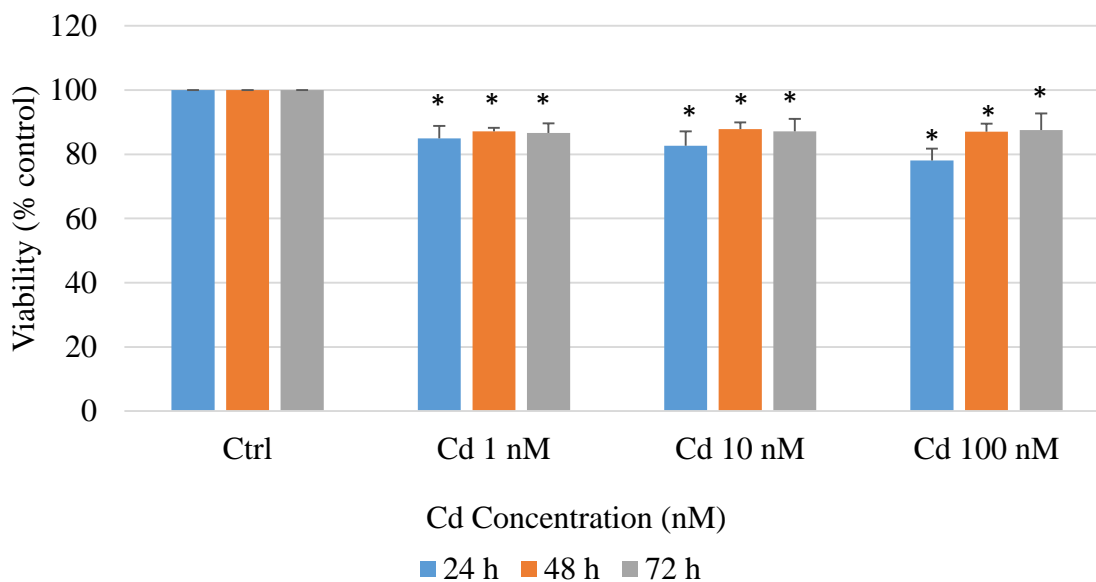
Figure 18: Cd caused a paradoxical decrease in HT-29 proliferation. *HT-29 cells were treated with increasing concentrations of Cd (1, 10 or 100 nM) for 24, 48, and 72 hours. Viability was determined by MTT assay. Viability values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of n=3. * denotes significance (p < 0.05) to its relative control.*

2. *Cd-induced anti-proliferative effects are ROS mediated*

Pre-treatment with NAC significantly abolished Cd-induced anti-proliferative effect at 24 hours, suggesting that Cd acts through ROS to reduce cell viability (Figure 19).
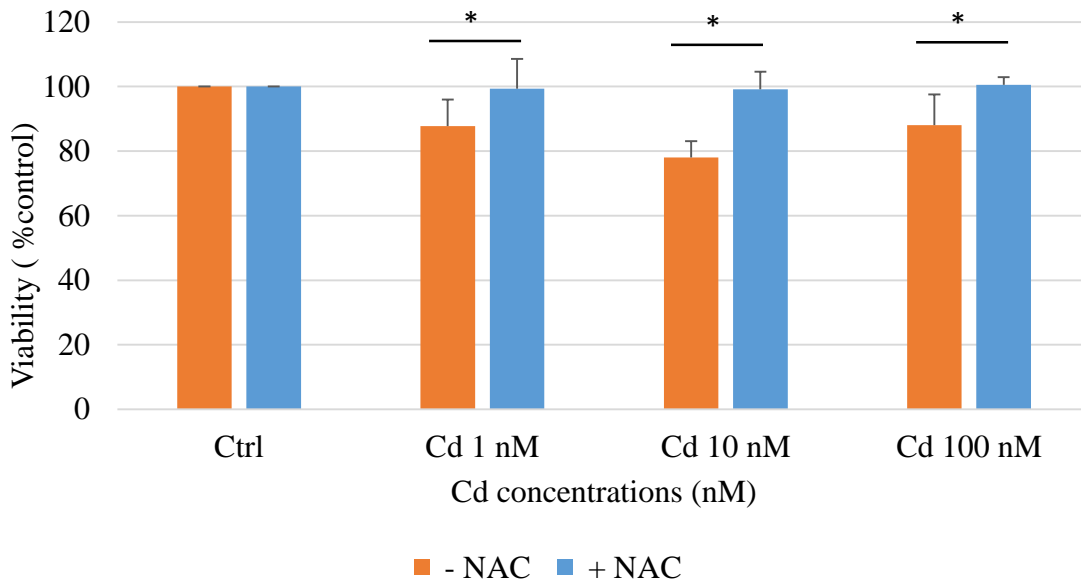
Figure 19: Oxidative stress contributes to Cd cytotoxicity. *HT-29 cells were treated with increasing concentrations of Cd (1, 10 or 100 nM) for 24 hours in the presence or absence of NAC. Viability values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of n=3.* denotes $p < 0.05$.

CHAPTER IV

DISCUSSION

Although Cd is a known human carcinogen, the underlying signaling pathways implicating it in colorectal malignancy are not well characterized. In this report, the effect of Cd on CRC cells migration and proliferation were investigated. Herein, we show that Cd increases the migratory capacity of CRC cells via activating two inter-connected pathways: ROS-p38-COX-2-PGE$_2$ pathway and the ROS-Akt pathway. Moreover, we report that Cd-induced migration is associated with a paradoxical reduction in cell viability, also via a ROS-mediated pathway.

It is widely accepted that inflammation plays an important role at different stages of tumor development [76, 77]. Overwhelming evidence shows that the cyclooxygenase-mediated pathway is a primary pro-inflammatory signaling cascade implicated in colorectal malignancy [90]. In fact, expression of COX-2 was reported to be increased in human colorectal cancer mucosa when compared to the normal tissues [92]. Epidemiological evidence demonstrates that chronic aspirin use is associated with 40- 50% reduction in CRC relative risk [136]. Indeed, the pharmacological inhibition of COX-2 using NSAIDs is associated with reduced cancer cells proliferation [108], migration, invasion [103] and angiogenesis [137]. Here, we report that Cd causes a significant increase in cellular migratory capacity. To investigate the underlying mechanisms of Cd-induced cell migration, we looked at COX-2 expression. Interestingly, Cd caused a time dependent increase in the transcription and protein expression of COX-2. The fact that Cd upregulated COX-2 as early as 6 hours

most likely indicates a post-transcriptional regulation, which could be explained by either increased COX-2 translational efficiency or mRNA stability. To assess whether Cd-induced COX-2 upregulation is positively implicated in migration, cells were pre-treated with CLX. Indeed, blocking COX-2 activity abolished Cd-induced migration, clearly indicating that Cd, via increasing COX-2 expression, is enhancing migration. This is consistent with several lines of evidence which established that COX-2 plays a crucial role in promoting migration of cancer cells [138, 139].

It is well known that CLX and other NSAIDs reduce carcinogenesis via inhibiting COX-2 activity. Nevertheless, COX-2 independent anti-tumor effects of NSAIDs have been also reported [140]. For instance, CLX was reported to reduce membrane fluidity and hence metastatic potential of CRC cells in a COX-2 independent mechanism [141]. Conversely, COX-2 positive but not COX-2 negative CRC cells treated with CLX showed a significant reduction in EMT, strongly implying a COX-2 dependent anti-tumor mechanism [105]. Moreover, it has been reported that CLX tends to accumulate in the hydrophobic interior of plasma membrane of different human colorectal cancer cells, and thus a higher intracellular concentration is needed to observe its anti-carcinogenic effects [142]. Accordingly, the concentration of CLX used in the present study is 10 µM, which is higher than the maximal plasma concentration achieved (3-5 µM) following the administration of 800 mg/day CLX [143].

A huge body of evidence indicates that ROS plays an important role in different types of cancer including CRC [144]. Several signaling pathways have been implicated in ROS-mediated carcinogenicity. In fact, ROS mediates the activation of Src kinases, PI3K/Akt, and

MAPKs, all of which promote malignancy [145]. Previous findings support the notion that ROS could promote EMT [146] as well as tumor cell migration and invasion [147]. It is not surprising then, that reducing cellular levels of ROS using NAC is associated with the inhibition of migration of several cancer cells [148, 149]. The present study established that Cd induces ROS production as early as 5 minutes. This is in agreement with literature which indicates that Cd is an oxidative stressor [49]. Moreover, it has been suggested that ROS plays a crucial role in Cd-induced carcinogenicity [67]. To explore whether oxidative stress is involved in Cd-induced migration, the ROS scavenger NAC was used. Indeed, pre-treatment with NAC significantly attenuated Cd- increased cell migration. Interestingly, ROS was reported to upregulate COX-2 in CRC [144]. In line with this, our results show that reducing oxidative stress led to a significant decrease in Cd-induced COX-2 expression, clearly implicating ROS as a COX-2 positive modulator.

It is important to note that in addition to its tumor promoting role, a tumor suppressive role of ROS has been also reported [150]. It is now accepted that low levels of ROS support cells proliferation, migration and survival whereas high levels are generally cytotoxic [144, 151]. This might explain the reason behind the failure of some clinical trials that were based on antioxidants as potential anticancer therapy. Contrary to our findings, *Piskounova* et.al demonstrated that NAC promoted metastasis in melanoma human xenografts [152]. Similarly, NAC markedly increased migration and invasion of human malignant melanoma cells [153]. Moreover, dietary supplementation with antioxidants as NAC or Vitamin E was shown to accelerate the progression of lung cancer in mice [154].

Aberrant MAPK signaling plays a crucial role in the development and progression of different malignancies [71]. Three main groups of MAPKs have been identified in mammals: ERK1/2, JNK 1/2/3 and p38 MAPK [155]. The MAPKs are activated by variety of stimuli such as growth factors, pro-inflammatory cytokines, hormones and oxidative stress [156]. Indeed, being an oxidative stressor, Cd was reported to activate MAPKs [157]. Contextually, herein we report that Cd significantly increases p38 MAPK phosphorylation in a ROS mediated pathway. Previous studies indicate that p38 is implicated in several human diseases including cancer [158]. In fact, elevated levels of phosphorylated p38 have been associated with various malignancies [159]. A recently published study shows that p38 MAPK is involved in the migration of human breast cancer cells [160]. A positive association has been reported between high endogenous p38 MAPK activity and cancer cells invasiveness [161]. Additionally, pro-proliferative [162] and anti-apoptotic effects were correlated with p38 activation [163, 164]. The fact that SB202190 abolished Cd-induced HT-29 migration is consistent with reports implicating active p38 as a mediator of cancer cells migration [162]. Moreover, we have shown that Cd-induced increase in COX-2 transcriptional activity and protein expression was significantly attenuated by SB202190. This is in agreement to previous findings suggesting that the activation of p38 could contribute to cancer progression via upregulating COX-2 in different tumors [159]. It is noteworthy that besides its tumor promoting effect, a tumor suppressive role of p38 has been also described [165]. In fact, increasing evidence highlights the pro-apoptotic roles of p38 [165]. Remarkably, p38 activity appears to be essential for some chemotherapeutic drugs induced apoptosis. The alkylating agent Cisplatin for example, was shown to induce a p38 mediated tumor cell death [166].

Importantly, the function of p38 MAPK in cancer appears to depend on the cell type, the stimuli and/or the isoform that is activated [164].

Because PGE_2 is thought to be the major pro-tumorigenic metabolite of COX-2, we next explored its effect on HT-29 migration. Our findings indicate that the addition of PGE_2 significantly potentiated migration. These results support the notion that PGE_2 is an important mediator of colon cancer cells migration [124, 125] . Previous studies yielded conflicting results regarding the catalytic activity of COX-2 in HT-29 cell line. Parker *et al.* demonstrated that the addition of AA simulates the production of PGE_2 in HT-29 [135]. On the contrary, other investigators have shown that although COX-2 is expressed in HT-29 cells, it is catalytically inactive and is therefore unable to convert AA to PGs [167]. Our results indicate that Cd at most of the tested concentrations causes a significant rise in PGE_2 production, clearly suggesting that the Cd-induced de novo synthesized COX-2 is enzymatically active and is probably implicated in Cd-increased migration. Interestingly, 100 nM Cd was associated with the highest increase in PGE_2 levels. This might explain why the 100 nM Cd and not the 1000 nM caused a more significant increase in cells migration. The present study further established Cd-induced PGE_2 production was significantly attenuated by NAC, SB202190 and CLX pre-treatment. These findings support the fact that PGE_2 is major COX-2 product and that ROS and p38 are critical mediators of COX-2 expression.

Substantial evidence indicates that the PI3K/Akt pathway is implicated in cancer [122]. In fact, this pathway plays an important role in promoting cell proliferation, survival, invasion, metastasis and angiogenesis [122]. The selective PI3K inhibitor LY294002 was shown to significantly decrease ovarian cancer cells proliferation [168], invasion and

metastasis [169]. As a matter of fact, aberrant PI3K/Akt pathway occurs in several malignancies including CRC [122]. Interestingly, when compared to normal colonic mucosa, colorectal adenomas and carcinomas frequently overexpress Akt, the major downstream player of PI3K [170]. Importantly, *Turečková* et al. established that Akt strongly stimulates the migration of colorectal cancer cells via phosphorylating focal adhesion kinase (FAK) in a Src mediated pathway [171]. In line with this, our results indicate that Cd significantly increases the phosphorylation of Akt. Furthermore, inhibiting Akt significantly abolished Cd-increased migration, clearly implicating Akt as a mediator of migration. Our findings further demonstrate that inhibiting both COX-2 and Akt wasn't associated with any additional decrease in migration. The exact explanation remains to be fully elucidated, however the following could be postulated: Cd-induced PGE_2 production before 1 hour could actually be activating the PI3K/Akt pathway. Previous reports have shown that PI3K/Akt pathway is an important downstream mediator of the COX-2/PGE_2 pathway. In fact, PGE_2 was reported to increase the growth, migration and invasion of colorectal cancer cells in PI3K/Akt mediated pathway [123].

It is well established that the PI3K/Akt pathway could be activated by ROS [172]. Herein, we have shown that pre-treatment with NAC significantly attenuated Cd-induced Akt phosphorylation, strongly suggesting that ROS is upstream Akt.

The present study further established that besides increasing migration, Cd causes a paradoxical decrease in cell viability. In an attempt to explain these observed effects, the following could be hypothesized: (1) Cd-induced PGE_2 production could be anti-proliferative. Although the bulk of the literature indicates that COX-2 and thereby PGE_2 play an important

role in cancer cell proliferation, there are studies which showed that PGE_2 could produce anti-proliferative effects in colon cancer cells [132, 135]. In fact, it has been postulated that these conflicting results are attributed to different experimental conditions including PGE_2 concentration, proliferation time frame, method, inclusion or exclusion of serum and others (Figure. 5) [132]. (2) Cd mediated activation of p38 could be pro-apoptotic. In fact, accumulating evidence indicates that active p38 could induce apoptosis and cell death [163]. Contextually, several studies have shown that Cd induces apoptosis via activating p38 in different cell lines [173, 174]. (3) Cd-induced ROS formation might be cytotoxic. Several lines of evidence indicate that ROS is involved in multiple aspects of carcinogenesis. However, it is now well known that while low ROS levels contribute to cell survival, high levels are generally cytotoxic [151]. Remarkably, Cd was reported to decrease the viability of breast cancer cells in a ROS dependent manner [175]. Induction of apoptosis was mediated by oxidative stress and consequent p38 activation in HT-29 colorectal cancer cell line [176]. In the present study, pre-treatment with NAC abolished Cd-induced cytotoxicity, suggesting that the decrease in viability is ROS mediated. However, since the inhibition of ROS will ultimately inhibit p38 activation, we can't rule out the role of p38 in Cd-induced anti-proliferative effects. Therefore, whether the decrease in viability is a direct effect of ROS or not remains to be fully elucidated. It is important to note that although hyperproliferation is a cancer hallmark, increasing evidence now suggests that decreased proliferation might actually be a requisite to enhanced migration [177]. Therefore, the anti-proliferative effect seen here might be an important part of the migratory phenotype induced by Cd.
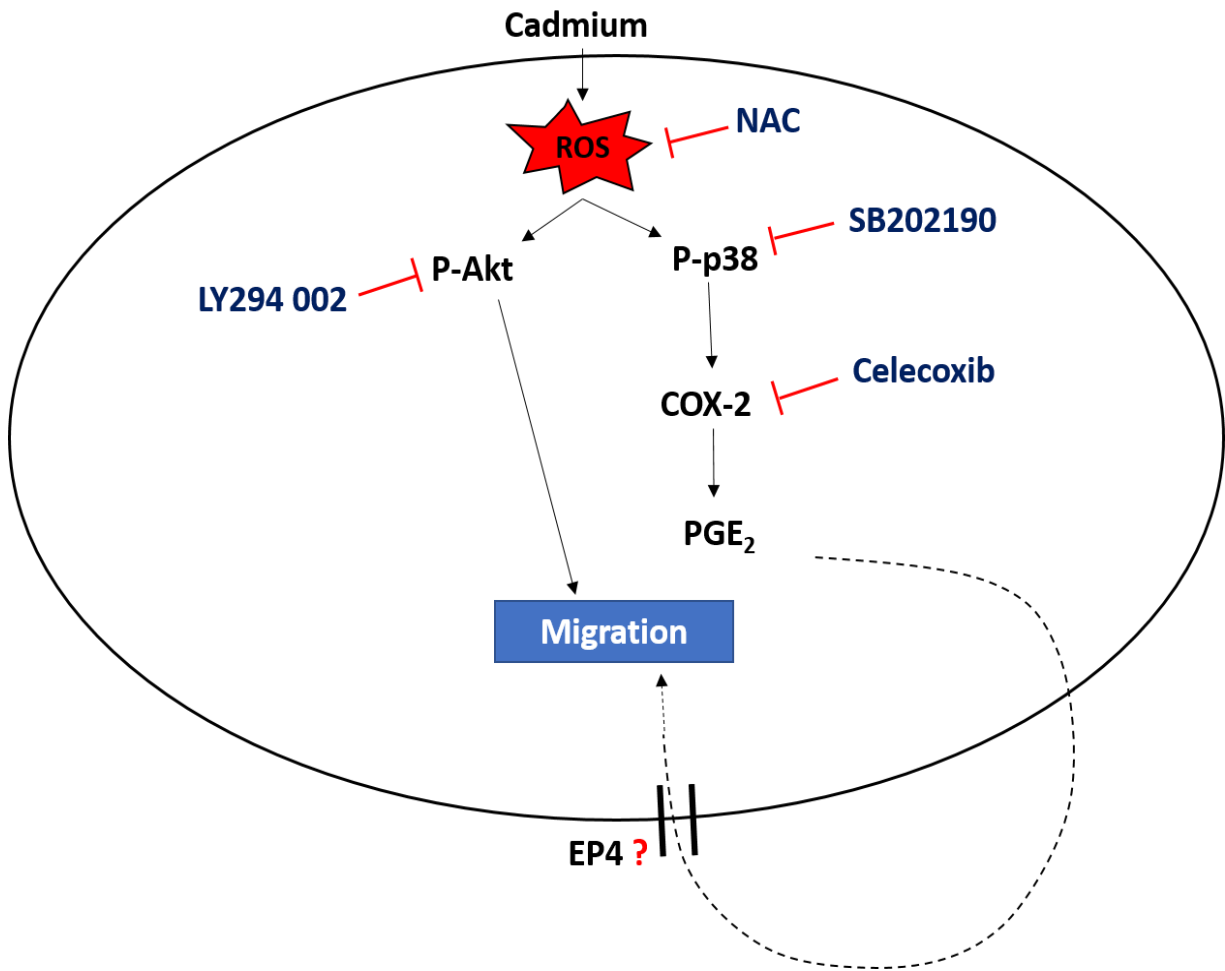
Figure 20: The suggested signaling pathway of Cd-induced CRC migration

CONCLUSION

Findings from the present study show that exposure to low Cd levels enhances migration of CRC cells via induction of two inter-related pathways: ROS-p38-COX-2-PGE$_2$ as well as ROS-Akt pathway. Therefore, COX-2, PGE$_2$ receptors or Akt may represent potential targets in treatment of CRC, specifically in patients exposed to Cd.

LIMITATIONS AND FUTURE PERSPECTIVES

In the present study, the HT-29 human colorectal cancer cell line was only used. Therefore, to further confirm the role of Cd in colorectal malignancy, our future studies will investigate the effect of Cd on different CRC cell lines. Moreover, we have shown that Cd caused a surprising decrease in HT-29 viability. This is in contrast to what is well known that hyperproliferation is a cancer hallmark. Thus, it would be interesting to explore the effect of Cd on other cell lines proliferative capacities. Another limitation is the fact that we didn't used a selective EP blocker to validate that COX-2 derived PGE$_2$ is implicated in Cd-induced migration. Furthermore, this study has only addressed the effect of Cd on migration and proliferation. Hence, our upcoming studies will aim to depict the role of Cd on other cancer hallmarks such as angiogenesis and invasion. Finally, this study investigated the effect of Cd in cultured colorectal cancer cells and not in an *in vivo* setting. Therefore, future work will focus on studying the role of Cd on colorectal tumorigenesis *in vivo*.

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