IN VITRO STUDIES USING CURCUMIN AND CURCUMIN ANALOGUES AS CANDIDATE MITOCHONDRIA-TARGETING ANTICANCER AGENTS AFFECTING COLON CANCER CELLS

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ABSTRACT

Curcumin is one of the major curcuminoids produced by the ginger family Zingiberaceae. These curcuminoids possess pharmacological properties that include anticancer activities. We have evaluated some synthetic curcumin analogues that have shown potential as anticancer drugs. These antineoplastic agents bearing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore are electrophiles which are designed to preferentially react with sulfhydryl groups present in proteins as opposed to amino and hydroxyl groups present in DNA. In previous pilot studies, three derivatives examined in this thesis showed inhibition towards human cancer cell lines such as Molt 4/C8 and CEM T-lymphocytes. In this thesis work, I determined the cytotoxicity of these derivatives and curcumin towards human colon cancer (HCT-116) cells and also normal colon epithelial (CRL-1790) cells, and examined the possible mechanism(s) involved. I hypothesized that they act via induction of reactive oxygen species (ROS) which elicit a transient surge of mitochondrial ROS generation and a phenomenon known as ROS-induced ROS release (RIRR), along with the mitochondrial permeability transition (MPT) and mitochondrion –dependent apoptosis. I asked whether these agents react with some of the key protein thiols in the mitochondria whose oxidation/alkylation results in mitochondrion - dependent apoptosis.

NC-2109 and NC-2346 were found to be potent cytotoxic agents based on their GI_{50} values of 0.87 ± 0.38 μM and 0.90 ± 0.22 μM, respectively, and were more potent than the anticancer drug 5-fluorouracil (GI_{50} = 5.47 ± 0.55 μM) and curcumin (GI_{50} = 3.50 ± 0.36 μM). However NC-2109 was found to have a better selectivity towards cancer cells over normal cells (a selectivity index of 18.81 versus 5-FU, curcumin and NC-2346 which had selectivity indices of 1.87, 16.75 and 4.61, respectively).

In the investigations of the mechanisms involved, both curcumin and curcumin analogues were able to induce mitochondrial ROS production. Moreover, curcumin and its synthetic counterparts showed a biphasic ROS profile which is most characteristic of RIRR. Treatment with these agents also led to the

disruption of the mitochondrial membrane potential, suggesting oxidation of protein thiols and the opening of the mitochondrial permeability transition pore which is an important step to initiate mitochondria-directed apoptosis. This possibility was confirmed based on GSSG/GSH ratios, since curcumin, NC-2346 and NC-2109 all produced a higher GSSG/GSH ratio than the controls. In addition to their ability to depolarize the mitochondrial membrane in HCT-116 cells, that these molecules acted via the mitochondrial pathway were further authenticated based on their ability to induce mitochondrial swelling in rat liver mitochondria.

In another part of this thesis I evaluated the involvement of the critical thiol protein adenine nucleotide translocase (ANT), a bifunctional protein that plays a central role in mitochondrial apoptosis. ANT has four different isoforms; ANT1 and ANT3 are proapoptotic, while ANT2 and ANT4 are antiapoptotic and are overexpressed in cancer states. A combination approach using ANT2 siRNA however did not conclusively show whether these agents acted synergistically with ANT2 knockdown to potentiate mitochondria-mediated cell death. An alternative combination approach was the use of carboxyatractyloside (CAT) which binds to and retains ANT in its ‘c’ conformation, exposing thiols and potentially driving a cell towards programmed cell death. The presence of CAT enhanced the ability of curcumin and its synthetic analogs to collapse the mitochondrial membrane potential, an important step in mitochondrial-mediated apoptosis.

In conclusion, curcumin and the curcumin analogue NC-2109 were found to be cytotoxic *in vitro*, towards HCT-116 cells and also showed good selectivity. In addition, these two molecules were found to be ROS inducers, and coincidentally oxidized cellular thiols and caused depolarization of the mitochondrial membrane potential. The results support a mechanism of mitochondrial-mediated cell death upon MPT pore formation (mitochondrial swelling), perhaps involving ANT2. This conclusion was further supported by the potentiation of cell death in the presence of the ANT2 inhibitor, CAT.

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This thesis is dedicated at the lotus feet of My Dearest Baba…

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AML	Acute Myeloid Leukemia
ANT	Adenine nucleotide translocase
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BSO	l-Buthionine-(S,R)-sulfoximine
CAT	Carboxyatractyloside
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CpG	Regions of DNA those are rich in cytosine and guanosine sites alligned in a linear sequence
CRC	Colorectal cancer
DCF-DA	2',7'-Dichlorodihydrofluorescein diacetate
DCF	2',7'-Dichlorodihydrofluorescein
DLC	Delocalized lipophilic cation
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNP	Dinitrophenol
DTNB	5,5'-Dithiobis-2-nitrobenzoic acid
EGTA	Ethylene glycol bis(aminoethyl ether)-N,N,N′,N′-tetraacetic acid
FBS	Fetal bovine serum
GSH	Glutathione reduced
GSSG	Glutathione oxidized
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
GI_{50}	Concentration at which there is 50% growth inhibition
IMM	Inner mitochondrial membrane
MITOSOX	Mitochondrial superoxide
MPT	Mitochondrial permeability transition
MMP	Mitochondrial membrane potential
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
OMM	Outer mitochondrial membrane
PBS	Phosphate buffered saline
RIRR	ROS induced ROS release
ROS	Reactive oxygen species
RT	Room Temperature
SRB	Sulphorhodamine-B
siRNA	Small interference RNA
TCA	Trichloroacetic acid
VDAC	Voltage dependent anion channel
5-FU	5-Fluorouracil

1.0 INTRODUCTION

Current standard chemotherapeutic approaches for the treatment of colorectal cancers have been proven to be a major challenge due to increasing drug resistance and DNA damage (Raymond et al., 2002). Because of these problems, there is an increasing attention drawn to natural molecules such as curcumin. Curcumin is a plant-based polyphenol that is widely used as a spice, a coloring agent and as a preservative in Asian foods. Extensive *in vitro* studies on this natural molecule points to its role in decreasing cell proliferation. It is also involved in inducing cell cycle arrest and apoptosis (Sharma et al., 2005). Preclinical *in vivo* studies exhibit its effective action on neoplastic models (Perkins et al., 2002, Caroll et al., 2011). Apoptosis is a regulated process which causes activation of cell signaling molecules and initiation of cell death. This process occurs by activation of caspases and the proteins that recruit cell death which are broadly known as *Bcl2* family members (Cory and Adams, 2002). Thus induction of cell death in cancer cells may particularly be a promising approach for combating cancer (Green, 2000). Based on this background knowledge, we have particularly chosen curcumin which has multifaceted properties as both a chemopreventive and as a chemotherapeutic agent. Dr. Dimmock's group has designed molecules having similar properties (Das et al., 2008, Das et al., 2009, Das et al., 2011). These molecules have two electrophilic sites that are prone to nucleophilic addition and act in two possible ways. Typically, in the cellular systems protein thiols serve well as good nucleophiles. Both the natural and synthetic molecules may directly alkylate protein thiols, which might include those present on the mitochondrial membranes, thereby activating apoptotic signaling events. The other possible line of attack could be via an indirect route. These molecules may deplete the cellular glutathione levels resulting in the elevation of superoxide; a main cause of ROS induced ROS release (RIRR). This is a key step for apoptosis to occur. Thus, it is probable for these molecules to follow either of these routes. Other known alkylators such as oxaliplatin and cisplatin are highly potent but are highly damaging to DNA. Therefore, it would be important to search for molecules that have minimal

impact on the DNA and yet have anticancer properties. An important aspect of this project is the use of a combination approach using mitochondria based inhibitors such as carboxyatractyloside (CAT) that retains one of the mitochondrial proteins, namely adenine nucleotide translocase (ANT) in its open state (cytosolic or c' conformation). It is the 'c' state conformation of ANT which aids in mitochondrial permeability transition pore formation in collaboration with porins and cyclophilin D which is a key step for apoptosis to occur. The other part of this research work is to downregulate the expression of the ANT2 isoform by a knockdown approach. ANT2 is upregulated in cancer states. The idea of using these combination approaches is to observe a synergism in the presence of curcumin and the analogues of curcumin. Thus, in this way we can not only selectively target cancer cells over normal cells, (minimize the side effects) but also the dosage of the test agents administered would be much lesser in a combination approach when compared to test agents alone.

1.1 Hypotheses

It is hypothesized that:

1. Thiol alkylating agents namely NC-2346, NC-2109, NC-2352 and curcumin are able to induce the formation of reactive oxygen species upon depletion of glutathione (reduced form) resulting in mitochondrion directed apoptosis in an *in vitro* system such as HCT-116 cells.
2. The natural molecule, curcumin and synthetic compounds NC-2346, NC-2109, and NC-2352 show greater selectivity to cancer cells compared to healthy cells by causing oxidation of key mitochondrial thiol proteins such as ANT2, which is overexpressed in colon cancer cells such as HCT-116 cells.
3. A combination approach of the use of a synthetic molecule/curcumin and siRNA knockdown of ANT2 increases apoptosis in cancer cells as opposed to cells treated with drug alone.
4. Co-administration of CAT potentiates the process of apoptosis in colon cancer cells.

1.2 Research Objectives

1. *To determine the potency and selectivity of some thiol alkylating compounds as anticancer agents against colon cancer cells.* In order to do this, cytotoxicity screening in colon cancer cells (HCT-116) and normal colon epithelial cells (CRL-1790) was carried out by the SRB assay. Furthermore, their GI_{50} values and their selectivity indices were determined based on these cytotoxicity studies.
2. *To determine the mechanism of effects involving ROS, mitochondrial membrane potential, and protein thiol (oxidized/reduced) profiles in cancer cells.* To evaluate the mechanistic effects, rat liver mitochondria were isolated to determine if mitochondrial swelling and mitochondrial respiration took place. In addition, cellular ROS in the cancer cells were determined by use of a fluorescent probe, DCF-DA and mitochondrial superoxide levels were evaluated by MITOSOX red. The cellular glutathione levels were determined by use of the Ellman reagent in colon cancer cells.
3. *To compare a natural molecule (curcumin) effects with curcumin analogues.* It is already well established that curcumin has an important role to play in cancer therapy. It was therefore of interest to compare some of the properties of curcumin with its synthetic counterparts that have been specifically designed to target protein thiols.
4. *To assess a combination approach.* Another important aspect of my studies was a combination approach wherein the compounds could help potentiate these mitochondrial effects leading to apoptosis. For this study, two methods were adopted. One, by use of carboxyatractyloside, to force ANT into its 'c' conformation Also, knocking down the ANT-2 isoform (normally overexpressed in cancer states) by an siRNA approach in a colon cancer cell line would possibly have an additive effect in these cells. A key facet of this objective is to see if these compounds would have improved mitochondrial effects in a combination approach resulting in mitochondria-directed apoptosis. For this particular research work two approaches were adopted. First, in the presence of a small molecule, a carboxyatractyloside inhibitor was employed which would retain a key mitochondrial protein, ANT in its 'c' state conformation. Second, another novel combination approach would be to

knockdown the ANT-2 isoform (normally overexpressed in cancer states) by an siRNA approach in a colon cancer cell line.

2.0 LITERATURE REVIEW

2.1 Introduction to colon cancer

Cancer can be classified into many different types depending on the organ and the cell type (www.cancer.gov/cancertopics/what-is-cancer). Colorectal cancer (CRC) is one of the most common cancers, and is a chief cause of cancer related deaths. Yearly about 150,000 people in the United States are diagnosed with this disease, and almost one-third of these people will die (Jemal et al, 2010). The CRC threat is ~6% in the U.S, and the average age is 66 years at which CRC is diagnosed (Hawk and Levin, 2005). Based on the statistics generated by Colon Cancer Canada, it is the second most leading cause of cancer deaths in Canada. Moreover, ~300 cases are reported annually in the province of Saskatchewan (www.colorectal-cancer.ca). The etiological factors and pathogenic mechanisms causing CRC are complex as well as diverse. Agents affecting CRC include diet and day to day regimen adopted by an individual. Among the most noteworthy features, dietary and lifestyle risk factors for CRC appear to be a diet high in saturated fats and red meat, excessive alcohol consumption, and a sedentary lifestyle (Jemal et al, 2010, Hawk and Levin, 2005, Potter, 1999). Conversely, non-steroidal anti-inflammatory drugs, estrogens, calcium, statins and possibly some dietary agents such as polyphenols can help safeguard against CRC (Jemal et al, 2010, Poynter et al, 2005). Unfortunately, precise dietary and environmental features that bring about CRC in the United States and other Western countries have not been delineated. The majority of the cases of the rise of CRC are poorly defined.

At the molecular level CRC is brought about by genetic/somatic mutations. In CRC there is a decrease in the amount of DNA methylation (i.e., hypomethylation) in the cancer cells compared to normal tissues (Feinberg et al, 1988), and DNA hypomethylation has been observed in adenomatous polyps (Goelz et al, 1985). The overall pattern in CRC cells is hypomethylation, but CpG islands (these are regions on the DNA that are rich in cytosine and guanosine residues that are sequentially placed linear

to each other) at the promoter regions exhibit increased methylation that often correlates with transcriptional gene silencing modulated by promoters (Baylin et al, 1998, Kim et al, 2010). Despite the fact that hypermethylation at certain genomic sites is associated with the phenomena of aging, there are cancer-specific hypermethylated regions in CRCs. However, it is still uncertain whether cancer-specific hypermethylation stems from a selective gain brought about via transcriptional silencing of diverse genes or from a key defect in the methylation mechanism or machinery. Both possibilities seem viable. A large section of CRC gene promotors show hypermethylation and transcriptional gene silencing of potential tumor suppressor genes (Kim et al, 2010). Many other studies have been considered for their effects on the risk of CRC, but some have produced confusing results. Some contemporary experimental work focuses on the complex relation of energy balance, hormones, gut flora and inflammation in the development of CRC (Slattery and Fitzpatrick, 2009, Slattery, 2000).

Typically the development of colon cancer begins with uncontrolled cell growth and faulty repair of the colon cell lining. The cell growths, called “polyps”, are normally benign but they do have the capacity to turn cancerous. Like any other cancer types, CRC too develops via receptor activation and dysfunction of intermediary genes. This results in flawed cell behavior and function (Marinus et al, 2003, Hiraoka et al, 2010, www.cancercompass.com/colon-cancer-information/side-effects).

Oncogenes and their gene products play a key role in cellular crosstalk. Upon transmission of any signal, activation of key proteins is brought about that eventually contributes to the cancer phenotype. The function of these proteins is vital for tissue and cell homeostasis, and the control of cell division, cell differentiation and apoptosis. All the key cell signaling related genes and proteins are potential targets for anticancer drug design. Inhibition or activation of such functions could result in tumor cell eradication (Elsayed and Sausville, 2001, Christos et al, 2004).

Although an extensive analysis of the curative remedy for colon cancer is outside the scope of this

review, a few generalities should however, be noted. Though surgery is corrective in premature stages of the disease, the current known standard therapies for terminal colon cancer are predominantly palliative (Wils, 2007). Adjuvant therapies for colon cancers are very different from those of other cancers, especially rectal cancers. In colon cancer, chemotherapy remains the main adjuvant treatment, in combination with radiotherapy. However, it has not been demonstrated that it improves the outcomes (Martensen et al, 2004, Arnold and Schmoll, 2005). In such a situation, chemotherapy is used to minimize metastasis, shrink tumor size, or impede tumor growth. Chemotherapy is often useful post-surgery for cure (adjuvant) or as the principal therapy if surgery is not specified (palliative). Overall, adjuvant treatment should be considered within 6-7 weeks of surgery. Although this mode of treatment has been well recognized in stage III form of this disease, its use in stage II type is less clear. (André et al, 2004).

2.2 Anticancer drugs and drug targets

Anticancer drugs can be classified based on their interactions with different targets. A number of known chemotherapeutics target DNA transcription and/or replication or they intercalate between DNA base pairs. In addition, some of them inhibit DNA topoisomerases (Froelich-Ammon, et al, 1995). There are others that interfere with metabolic pathways by inhibition of folate or purine/ pyrimidine pathways required for DNA synthesis (Li et al, 1995). Some anticancer drugs target microtubules by reacting at various tubulin binding sites, and in this way inhibit mitosis (Mosolits, et al, 2005).

Many pathways are dysfunctional in cancers. Cancer therapy may cause a high degree of complications and possible side-effects which makes successful treatment of the disease challenging. Most cancers are highly invasive and there are problems of a relapse even after surgery, chemotherapy and radiation treatment (www.cancercompass.com/colon-cancer-information/side-effects).

Current treatments for colon cancers include a combination of 5-fluorouracil, leucovorin, and

oxaliplatin. Irinotecan has also been used with bevacizumab, which is a monoclonal antibody to the vascular endothelial growth factor (VEG-F), being used more recently during recurrence. If progression of the disease occurs during adjuvant therapy, the options are limited. Other therapies are currently being investigated like Bacillus Calmette-Guérin (BCG) as an adjuvant in combination with autologous tumor cells in immunotherapy (Mosolits et al, 2005) and introduction of exogenous genes. All of these modes of treatments hold vast prospects, though considerable practical difficulties are often encountered. Drug-based toxicity and a rise in resistance to chemotherapeutic drugs pose potential challenges to the efficacious treatment of various cancers. Effective cancer-preventative/chemotherapeutic approaches for checking colon cancer are therefore desired. One of the major issues regarding current chemotherapeutic agents is mechanism-based toxicity. Selectivity and resistance related concerns have to be seriously addressed. Therefore continuation of research in the development of anticancer agents may pave the way for adopting novel strategies. (Liu et al, 2012, Christos et al, 2004, Gray et al, 2010).

Due to their role in triggering apoptosis, mitochondria have emerged as a potential anticancer drug targets. One promising line of investigation involves the administration of thiol alkylating agents that possess a cancer-preventative activity/prooxidant property and/or growth inhibitory activity against cancer cells (Pati et al, 2009) These thiol alkylating agents may potentially act via mitochondrion-induced apoptosis.

2.3 Apoptosis

Apoptosis or ‘programmed cell death’ is a route by which process cells are prompted, by intrinsic or extrinsic signals/motions, to actively shutdown and eliminate themselves from the body. Apoptosis is an important phenomenon in metazoans and is involved in a number of physiological roles, including the removal of unwanted cells during development. In addition, it is involved in the removal of

diseased/damaged cells. Two major signaling pathways are: first, the extrinsic pathway, is brought about by ligation of death-domain-containing cell-surface receptors of the tumor necrosis factor receptor superfamily and second, the intrinsic pathway which is activated by intracellular stimuli such as DNA damage or oxidative stress. The intrinsic pathway is chiefly characterized by the release of pro-apoptotic factors from the inter-membrane space of mitochondria and seems to play a more prominent role in the apoptotic response to anticancer drugs. The *Bcl-2* family of proteins regulate it by controlling key proapoptotic polypeptides, including cytochrome *c* that activates cysteine aspartyl proteases (caspases), from mitochondria. Several *Bcl-2* family members that facilitate MPT (mitochondrial permeability transition) opening are transcriptional targets of the *p53* tumor suppressor gene, providing sufficient proof for the capacity of DNA-damaging agents to induce apoptosis (Kaufman, 2007). Thus, the two pathways controlled by the death receptor and mitochondria evidently overlap or converge upon the activation of caspases (Ellis et al., 1991).

2.4 Mitochondria-ROS cross talk and mitochondria-directed apoptosis

ROS is an expression typically used to define a number of reactive molecules and free radicals generated from molecular oxygen. These molecules include superoxide, hydrogen peroxide, the hydroxyl radical and nitric oxide. The generation of oxygen based radicals contributes to various diseased states in all aerobic organisms. Recent studies point to a central role for reactive oxygen species in tumor progression (Kumar et al, 2008; Ishikawa et al, 2008). ROS can be formed from endogenous sources, for example from mitochondria, peroxisomes, and inflammatory cell stimulation (Klaunig and Kamendulis, 2004); and exogenous sources that include drugs and chemicals released into the environment. **Figure 1** shows the possible effects of reactive oxygen species when not compensated by antioxidant defense systems of the cell. This stress due to oxidation may result in DNA, protein, and/or lipid damage, thus causing chromosome instability, genetic mutation, and/or modulation of cell growth that may bring about carcinogenesis.

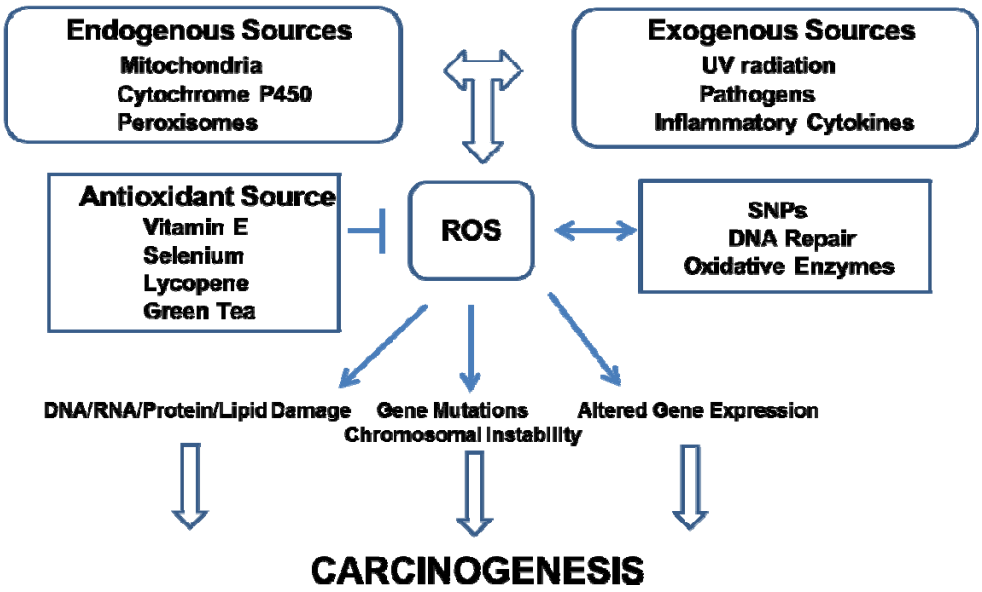


Fig 1: ***Reactive oxygen species and their role in the development of cancer***: Endogenous sources of reactive oxygen species (ROS) are primarily from mitochondria, cytochrome P450 enzyme systems and the peroxisomes. Exogenous sources of ROS include UV radiation, pathogenic organisms and inflammatory cytokines. An increase in cellular ROS results in damage to DNA/ RNA/proteins and lipids and may also cause genetic mutation such as single nucleotide polymorphisms (SNPs), which can lead to more ROS and altered gene expression. Several antioxidants such as vitamin E, selenium, lycopene and green tea bring about a balance in terms of cellular ROS. *(Adapted from Klaunig et al., 2010*)

ROS are formed as secondary byproducts during oxidative phosphorylation or by oxidoreductase enzymes and metal catalyzed oxidation, and bring about a number of harmful effects. The old school of thought was that only phagocytic cells were accountable for ROS production in host cell defense mechanisms and that ROS were always destructive. However, recent work has demonstrated that ROS have a primary role to play in events involving signal transduction that include apoptosis, gene expression and cell proliferation and that serve as intra- and inter-cellular messengers (Hancock et al, 2001). In other words, ROS accumulation inside the cell often brings about a number of events in mitochondria resulting in apoptosis (Watson et al, 2011).

ROS are often linked to tumor development and progression. For instance, ulcerative colitis is

connected to colorectal cancer and chronic gastritis, caused by infection of *H. pylori*, has been strongly linked with gastric cancer (Seril et al, 2003; Konturek et al, 2006). Also, mutation in tumor suppressor genes such as *p53* shows chronic oxidative stress being linked to different cancer states (Hwang and Bowen, 2007). Besides causing DNA, lipid, and protein damage, oxidative damage to protein-coding or -noncoding RNA may cause anomalies in protein synthesis or disturbed regulation of gene expression. This mechanism has been suggested as a basic cause of several human diseases, especially chronic neuronal degeneration (Nunomura et al., 2007). Likewise, while protein oxidation has been implicated in a number of diseases caused due to aging, most outstandingly Alzheimer's disease (Levine et al. 1994; Aksenov et al. 2001; Balcz et al. 2001), a distinct association between protein oxidation and cancer has not been firmly established. Lipid peroxidation causes the formation of reactive aldehydes that include malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE). These molecules demonstrate high reactivity with DNA and proteins that can cause mutations or changes in protein functions promoting tumorigenesis (Tuma, 2002; Kikugawa et al., 1987; Nicholls et al., 1992; Uchida and Stadtman 1993; Klaunig et al., 1998). In comparison, oxidative damage to DNA is closely associated to aflatoxin B-induced *p53* and *Ras* gene mutations in liver cancers (Shen and Ong, 1996) in UV-induced human/murine skin cancers (Nishigori et al., 2004). Furthermore, *p15INK4B* and *p16INK4*, cyclin dependent kinase inhibitors (CDKI's), are tumor suppressor genes that seem to be targets of ROS-induced renal cell carcinoma in rats (Tanaka et al., 1999).

2.5 Targeting mitochondria to induce apoptosis

Enhanced caspase-dependent apoptosis, reactive oxygen species (ROS) formation and severe mitochondrial damage are some of the occurrences which can be frequently detected in cells exposed to anticancer drug treatment. Several anticancer agents work by further boosting cellular ROS levels to circumvent the antioxidant detoxification capability of the cancer cell and prompt apoptosis. However, adaptation to cellular antioxidants can cause drug resistance. Thus there is a mammoth challenge for

medicinal chemists, in designing superior anticancer therapeutics inducing oxidative stress so as to tip the cellular redox balance to stimulate ROS-dependent cell death but without causing an increase in the antioxidant activity of the cancer cell or bringing about toxicity in normal cells (Smith et al., 2011). So far, among the agents causing an upregulation of ROS in cancer cells are some natural phytochemicals namely epigallocatechin gallate, (EGCG), curcumin, quercetin and garlic (Nakazato et al., 2005., Kang et al., 2005., Antoslewicz et al., 2008., Mertens-Talcott et al., 2005), anti-inflammatory agents like parthenolide, (Wen et al., 2002), anticancer drugs such as paclitaxel, cisplatin, and doxorubicin (Meshkini and Yazdanparast, 2012, Berndtsson et al., 2007, Ghosh et al., 2011) and hormones such as melatonin (Bejarano et al., 2011). On most occasions ROS generation correlates with apoptosis (Rasool et al., 2007). In a study using epigenetic modifiers in acute myeloid leukemic (AML) cell lines such as 5-azacytidine and histone deacetylase inhibitors (HDACi) in combination with a drug MS-275 resulted in a potentiation of the drug action due to ROS induction (Nakazato et al., 2005, Gao et al., 2008, Kim et al., 2011, Ozaki et al., 2008, Wang et al., 2007). These natural and synthetic molecules may thus possibly induce ROS and bring about mitochondrion-directed apoptosis. In consort with ROS generation, DNA damage has been generally detected, either directly or through the process of programmed cell death. These two processes, namely the increase of ROS levels and DNA damage, may occur independently or one being initiated by the other one.

ROS can activate an ephemeral increase in mitochondrial ROS generation via ROS activation of the MPT, a process widely known as ROS-induced ROS release (RIRR) (Zorov et al., 2000). ROS is believed to network with cell signaling pathways by way of alteration of key sulfhydryl groups on proteins that possess significant regulatory roles. Although substantial evidence has not been established, it is broadly thought that the MPT is made up of a few thiol containing proteins (discussed in more detail in section 4.3). Under stress conditions leading to RIRR, an upsurge in ROS causes the MPT, possibly via the oxidation of thiol groups on the adenine nucleotide translocase (ANT), which

subsequently results in the synchronized collapse of the mitochondrial membrane potential and increased ROS generation by the electron transport chain (ETC) (Zorov et al., 2000). Thus, RIRR creates a positive feedback relay for enhanced ROS production.

Constantini and coworkers (2000) have shown that thiol alkylating agents including diazenedicarboxylic acid bis 5N, N-dimethylamide (diamide), dithiodipyridine (DTDP), or bis-maleimido-hexane (BMH) have the ability to act on the adenine nucleotide translocator (ANT), one of the critical proteins within the MPT. In an *in vitro* model, ANT re-formed into artificial lipid bilayers serves to confer a membrane permeabilization response to thiol alkylating agents. Diamide, DTDP, and BMH but not tert-butylhydroperoxide (t-bOOH) or arsenite brought about the oxidation of a critical cysteine residue (Cys^{56}) of ANT. Their observations, with regard to thiol modifications within ANT were seen in intact cells, isolated mitochondria, and purified ANT. It was found that recombinant *Bcl-2* failed to avert thiol modification of ANT. In parallel, a chain of different thiol agents (diamide, DTDP, and BMH, phenylarsine oxide) but not tert-butylhydroperoxide or arsenite resulted in stimulating MPT and apoptosis irrespective of *Bcl-2* expression. Their results thus suggest that thiol alkylating agents cause an irreversible covalent modification of ANT, which is independent of *Bcl-2*.

The involvement of thiols and Ca^{2+} in the MPT was previously explored with thiol oxidizing and cross-linking agents (Kowaltowski et al, 1997). Ca^{2+} binding to the inner mitochondrial membrane accelerated far-reaching changes in the conformation of thiol groups associated with the mitochondrial pore proteins. In the presence of Ca^{2+} mitochondrial swelling (MPT) could be induced by the oxidizing agent tert-butyl hydroperoxide (t-bOOH) or the thiol cross-linkers 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) or phenylarsine oxide (PhAsO). It was seen that N-ethylmaleimide (NEM), a hydrophobic thiol mono-alkylating agent was able to impede the MPT-inducing effects of t-bOOH, DIDS and PhAsO. On the other hand, the hydrophilic thiol mono-alkylating reagent mersalyl prevented

only the effects of DIDS. The disulphide reductant dithiothreitol could restore the thiol content of mitochondria exposed to t-bOOH but not of mitochondria exposed to DIDS or PhAsO.
Thus, irreversible crosslinking of key mitochondrial protein thiols may result in changes to the MPT (and subsequent ROS generation and GSH depletion).

Growing evidence suggests that mitochondrial changes occur in tumorigenesis and tumor progression, which may be targeted in cancer treatments (Gogvadze et al., 2008). The characteristic features of cancer cells (including an unlimited potential to proliferate, impair apoptosis, enhance anabolism and reduce autophagy) have been linked to mitochondrial perturbations (Kroemer and Pouyssegur, 2008, Galluzzi et al., 2010). In addition, cancer cell mitochondria are often structurally and functionally different from their normal counterpart (Gogvadze et al., 2008, Modica-Napolitano and Singh, 2004). Also, tumor cells show a wide metabolic re-programming namely, the Warburg effect, which involves increased metabolism of glucose via the glycolytic pathway, and decreased oxidative phosphorylation (Kroemer and Pouyssegur, 2008, Bellance et al., 2009). Thus, increased aerobic glycolysis is a hallmark of most cancers. Besides altered glucose metabolism and oxidative phosphorylation, other mitochondrial changes in tumor cells are fewer cristae, higher mitochondrial membrane potential, and greater *Bcl-2*/*Bax* ratio (Luciakova and Kuzela, 1992, Gatby and Gillies, 2004, Paul and Mukhopadhyay, 2007, Bonnet et al., 2007).

Based on these foundations, mitochondria-targeting agents emerge as a means to target tumors in a selective manner. Rectifying cancer-associated mitochondrial dysfunctions and the activation of programmed cell death by pharmacophores that induce the MPT represents an attractive approach for cancer therapy. One approach may be to use thiol alkylators which react preferentially with protein thiols or act via depletion of reduced glutathione (GSH) thereby increasing the concentration of free radicals, and reactive oxygen and nitrogen species (ROS and RNS) in the form of superoxide/hydroxyl radical/peroxynitrite etc in the cell. Glutathione (GSH) exhibits a significant role in a variety of cellular

processes that include cell differentiation, cell proliferation, and programmed cell death. Any perturbations in GSH homeostasis result in progression of many human diseases such as cancer. Although GSH insufficiency, or a reduction in the GSH/glutathione disulphide (GSSG) ratio, brings about an improved predisposition to oxidative stress associated with cancer progression, increased GSH levels cause an upsurge in the antioxidant defence machinery. Also, there is a chemoresistance to oxidative stress as observed in many cancer cells (Cowan et al., 1986). The main rationale underlying the strategy of mitochondria-targeting agents for cancer treatment has been dealt with extensively in several recent publications (Pessayre et al., 2010, Armstrong, 2007, Galluzzi et al., 2006, Gogvadze et al., 2009, Zhang et al., 2010). Pathania et al. (2009) and Fulda et al. (2010) have discussed the perspectives and future developments of this area of research. Thus mitochondria-targeting compounds (mitocans) show an enormous potential for the treatment of a variety of malignancies.

Armstrong and Jones in their mini communication have suggested a probable outcome of the oxidation of mitochondrial thiol proteins upon depletion of GSH (Armstrong and Jones, 2002). Cancer cells typically have an active antioxidant system as they have high levels of ROS. Enzymes that are actively involved in maintaining the cellular redox balance are the superoxide dismutases, glutathione reductases, glutathione peroxireductases, peroxidases, peroxiredoxins, and thioredoxin reductases. These enzymes are overexpressed in cancer states (Townsend and Tew, 2003). The correlation between GSH depletion, reactive oxygen species (ROS) production, aberrant mitochondrial function and the mode of cell death in cancers remains to be resolved and offers key insights into the etiology of this disease (Mytillineou et al., 2002).

Mitochondria have emerged as a target for anticancer therapies for several reasons. The mitochondrial inner membrane and matrix holds much of the machinery at the core of cellular aerobic metabolism. Its constant exposure to fluxes of ROS such as superoxide, hydrogen peroxide and related reactive species is a common feature of mitochondria. To circumvent mitochondrial oxidative damage, there is a pool

of thiol proteins within the mitochondrial matrix. They are known to actively participate in the thioredoxin/peroxiredoxin/methionine sulfoxide reductase pathways and the glutathione/glutathione peroxidase/glutathione-S-transferase/glutaredoxin pathways. Their constant interplay with protein thiols mostly averts oxidative damage. In addition, any alteration in the redox state of many constituents of these mitochondrial thiol systems may relay redox crosstalk within and throughout the mitochondrial matrix. This is primarily to modulate the activity of biochemical processes (Murphy et al., 2009a, 2009b, 2011). Thus the mitochondrion is a major site for producing reactive oxygen species as well as programmed cell death (apoptosis) and has emerged as a novel target for anticancer therapy. This dictum is based on the studies that several known conventional and experimental chemotherapeutic agents promote the induction of MPT, a key step for apoptosis to occur. The outcome of this step, especially in cancer cells, results in the release of apoptogenic mitochondrial proteins. This capacity to involve mitochondrial-mediated apoptosis directly using chemotherapy may allow the overcoming of aberrant apoptosis regulatory mechanisms, such as mutation of *p53* and overexpression of *Bcl-2*, which are common characteristics in cancerous cells (Amadou et al., 2010;Vyas et al., 2012).

The key regulators of the mitochondrial steps in the apoptotic pathway are proteins of the *Bcl-2* family. *Bcl-2* itself predominantly serves as an inhibitor of death. *Bax* and *Bak*, which share only three domains with *Bcl-2*, promote apoptosis in most cellular contexts. (Strasser et al., 2000).

By stimulating endogenous mitochondrial-mediated apoptosis mechanisms, certain chemopreventive/chemotherapeutic thiol alkylating agents may block the progression of premalignant cells to malignant cells or the distribution of malignant cells to remote organ sites as a means of curbing tumorigenesis *in vivo*. In our *in vitro* study in HCT-116 colon cancer cells, we have examined cancer chemotherapy with respect to mitochondrial directed apoptosis, by using various thiol alkylating agents. Our studies in HCT-116 cancer cells strongly indicate that upon ROS generation, there is a

depletion of reduced glutathione leading to the oxidation of key mitochondrial protein thiols. Upon oxidation of the protein thiols, there is a collapse of the mitochondrial membrane potential and subsequent release of cytochrome *c* resulting in mitochondrion-mediated apoptosis. It would now be possible to build a hypothetical model supporting the notion that the mitochondria and its key thiol proteins are novel targets for the chemoprevention/chemotherapy of cancer. Thus, these thiol alkylators may directly alkylate the protein thiols present on the mitochondrial membrane proteins leading to their irreversible alkylation. **Figure 2** shows models depicting differences between normal and cancer cells and the possible action of thiol alkylating agents.With respect to ANT, it would then go through a change of conformation namely the 'c' state that would result in the induction of the MPT opening. Conversely, the action of these thiol alkylators could be indirect by bringing about an irreversible alkylation of glutathione. This would then increase the superoxide levels causing the oxidation of key thiol proteins (possibly ANT) thereby bringing about a change in the conformation to a 'c'state leading to MPT opening (Armstrong and Jones 2002).

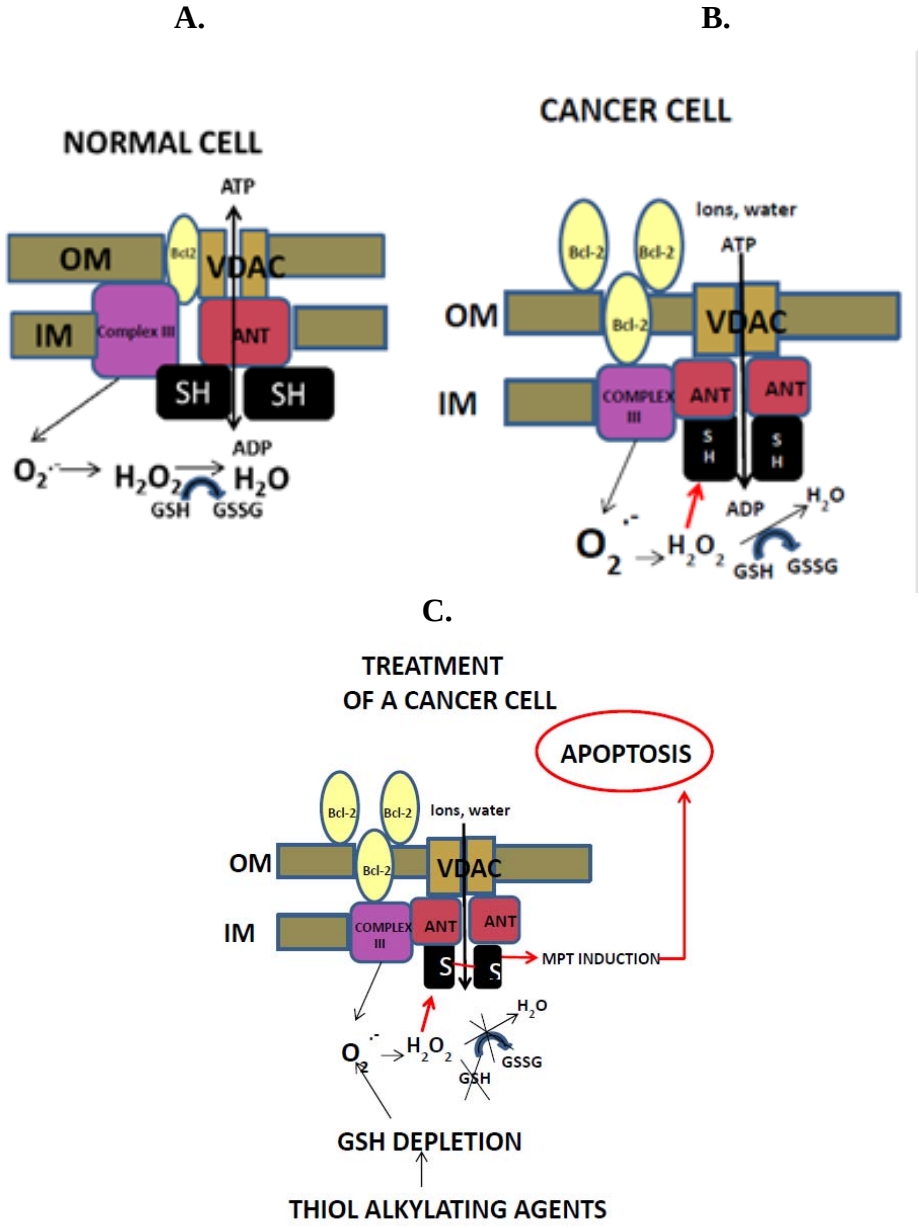


Fig 2: ***Model for the mechanism of action for curcumin and the curcumin analogues:*** **A.** a typical way by which the reducing environment of a cell is maintained in normal cell mitochondria. The "m'state of ANT allows the *Bcl-2* to bind onto the VDAC thus preventing apoptosis. In addition there is its regular intake of ADP and sufficient output of ATP. **B.** a cancer cell where there is an anomaly by way of utilization of ATP by the mitochondrion. Also, in cancer states there is an overexpression of *Bcl-2*. Hence, even though the cell has an anomalous mechanism it is unable to undergo apoptosis. **C.** the possible mechanism by which thiol alkylators may act. (*Images adapted from Armstrong and Jones 2002*).

2.6 Targeting tumor mitochondria

In addition to having overexpressed *Bcl-2* antiapoptic protein, tumor mitochondria often have a higher membrane potential compared to normal cell mitochondria (Huang, 2002). For this reason, delocalized lipophilic cations may be used to selectively target tumor mitochondria (Modica-Napolitano and Singh, 2004, Zhang et al., 2010).

Delocalized lipophilic cations (DLCs) are cationic molecules that are easily permeable across membranes, and are taken up by mitochondria due to the mitochondrial membrane potential (negative inside). For example, studies describing a series of antioxidants conjugated to DLC molecules have shown potential as mitochondrial protectors (Fulda et al., 2010). Due to the higher mitochondrial membrane potential in cancer cells they may accumulate a lipophilic cation to a greater extent than do normal cells. An attractive approach of improving the selectivity for cancer mitochondria is by use of a charged derivative. This would mean that there would be lesser side effects upon chemotherapy (Zhang et al., 2010). However, normal myocardial cells have also shown a similar mechanism for a propensity for DLC drug retention. Thus, cardiac toxicity due to DLC molecules cannot be ruled out (Zhang et al., 2010). Nevertheless, in this way they tend to selectively accumulate in the mitochondria of the cancer cells (Calabrese et al., 2008, Modica-Napolitano and Singh, 2004).

However, the selective uptake of lipophilic cations by tumor mitochondria may not be a simple relationship with membrane potential. Kowaltowski et al. (2002) showed that in *Bcl-2* overexpressing cells the apparent higher mitochondrial membrane potential may be due to improved uptake or accumulation of membrane potential probes such as safranin and rhodamine 123, independent of the mitochondrial membrane potential. Furthermore, their findings were supported by the existence of equal respiratory rates in *Bcl-2*$^{+}$ and *Bcl-2*$^{-}$ cells. Structural changes in *Bcl-2*$^{+}$ mitochondria have been suggested to possibly account for the enhancement of the dye uptake such as an increase in

mitochondrial volume and also increased structural complexity (Kowaltowski et al., 2002). Nevertheless these differences may be used to selectively target tumor mitochondria.

2.7 ANT as a key mitochondrial thiol protein

There are several protein thiols on the surface of the mitochondrial membrane and their irreversible crosslinking through oxidation or alkylation results in MPT pore formation (Pessayre et al., 2010, Kowaltowski et al., 2001); a critical step in apoptosis. The ANT is mainly involved in exporting mitochondrial adenosine triphosphate (ATP) to the cytosol. ANT is functionally bound to the voltage dependent anion channel, (VDAC) on the outer mitochondrial membrane while towards the inner mitochondrial membrane; ANT is bound to cyclophilinD (CyD) and respiratory complex V, the F_1F_o-ATP synthase (Boekema and Braun, 2007). The involvement of ANT as a key component of the MPT was first proposed by Hunter and Haworth (Hunter and Haworth, 1979). Later on other groups provided convincing evidence (LeQuoc and LeQuoc, 1988, Halestrap and Davidson, 1990). The sensitivity of the MPT to physiological ADP levels and other MPT inducing ligands determines its conformational "c" (cytosolic) and "m" (matrix) states. Previous work by Beyer and Nusher (1996) and Halestrap and Brenner, (2003), show that oxidation of mitochondrial membrane protein thiols is involved in the MPT (LeQuoc and LeQuoc, 1988, Halestrap and Davidson, 1990). ANT is known to have three cysteine residues that show disparity in terms of reactivity towards various thiol/oxidizing reagents in a conformation dependent manner (Majima, et al., 1993a, Majima, et al., 1994, Majima et al., 1995). These cysteines on ANT regulate the function of CyD, which is another protein (with peptidyl prolyl cis -trans isomerase activity) involved in the MPT pore formation. In addition, these cysteines are also involved in the inhibitory effects of ADP on MPT and the mitochondrial membrane depolarizing effects of ANT (Halestrap et al., 1997). These protein thiols could serve as key targets for irreversible alkylation in the presence of low concentrations of reagents such as carboxyatractyloside (CAT) that stabilizes ANT in its "c" state conformation. This would result in exposing a critical

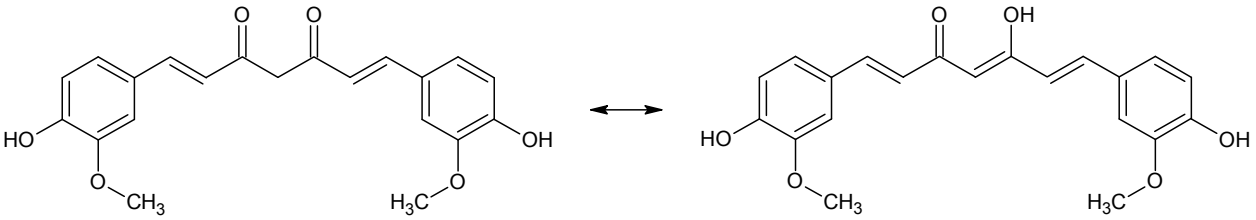
cysteine^{56} (cys^{56}) residue. The oxidation of the cys^{56} residue is an important step in MPT formation (Pestana et al., 2009). Peptidyl prolyl cis -trans isomerase activity of CypD on proline^{61} present on ANT, increases the mobility of cys^{56} (Pestana et al., 2009). In this way the "c" state conformation of the ANT would be maintained and in the presence of a thiol alkylating agent it would be much more prone to alkylation due to the exposed cys^{56} residue. Thus a combination approach would help in accelerating mitochondrion-induced apoptosis due to synergism exhibited by both carboxyatractyloside and the thiol alkylating agents.

The literature indicates that human ANT has four known isoforms namely ANT1, ANT2, ANT3 and ANT4. ANT1 and ANT3 exhibit a proapoptotic activity while ANT2 and ANT4 show antiapoptotic activity. Interestingly many proliferative and undifferentiated cancer cell lines (eg HT-29 and HeLa) show a greater expression of the ANT2 isoform. Thus ANT2 can potentially serve as a mitochondrial target for anticancer biotherapy (Chevrollier et al., 2011, Sharaf et al., 2011). In addition, an siRNA approach to knock down the ANT2 (antiapoptotic) isoform would help in accelerating the MPT formation and eventually lead to cell death mediated by mitochondria. A combination approach by the use of siRNA ANT2 knockdown in the presence of thiol alkylating agents may aid in enhanced mitochondrion-directed apoptosis. One important aspect of chemo- and radio-therapy is that the cancer stem cells (CSCs) are highly resistant to them, and can persist to regenerate new tumors. This is a central reason why various types of anticancer therapies are unsuccessful in fully eradicating the tumors, though they often kill and eliminate the majority of cancer cells. Jang et al., (2008) through some elegant experiments showed that ANT2 knockdown could also be effective in stimulating apoptosis in breast cancer stem-like cells. In their study they used the adeno-ANT2 shRNA virus to inhibit ANT2 expression. Their findings clearly show that ANT2 expression was elevated in both stem-like cells and non-stem-like cells of MCF7 and MDA-MB-231 cells, and was stimulated and overexpressed by mesenchymal transdifferentiation in MCF10A cells (MCF10A^{EMT}). ANT2

knockdown by adeno-shRNA virus proficiently caused stimulation of apoptotic cell death in the stem-like cells of MCF7 and MDA-MB-231 cells, and MCF10A^{EMT}. Stem-like cells of MCF7 and MDA-MB-231, and MCF10A^{EMT} cells showed very high resistance to the drug doxorubicin, and also overexpressed a multi-drug resistant related molecule, ABCG2. Adeno-ANT2 shRNA virus strikingly made the stem-like cells of MCF7 and MDA-MB-231, and the MCF10A^{EMT} cells highly sensitive to the drug, doxorubicin. This was also complemented by decreased expression of ABCG2. Thus, these results strongly suggest that ANT2 knockdown is an efficient way to bring about induction of apoptosis and intensify the chemosensitivity of stem-like cells (Jang et al 2008).

2.8 Curcumin and related thiol-alkylating drug candidates

The rhizome of the plant *Curcuma longa* contains the phytochemical curcumin as well as the structurally related compounds demethoxycurcumin and bisdemethoxycurcumin (Kuichi et al., 1993). The curcumin molecule undergoes keto-enol tautomerism and both the diketone and enol tautomeric structures are portrayed in **Figure 3**. The olefinic carbon atoms adjacent to the aryl rings are electrophilic and have the capacity to interact with thiols.



Curcumin (keto form) Curcumin (enol form)

Fig 3: ***Chemical structures of curcumin showing the keto and enol forms***

The anticancer properties of curcumin have received much attention, both as chemo-preventive and chemotherapeutic agents. Animal and cell culture studies show that curcumin regulates numerous cellular pathways involved in carcinogenesis including activating or inducing transcription factors

(such as NF-κB, NRF-2, HIF-1, STAT2), kinases (such as ERK, JAK, EGFR), cytokines (such as IL, TNF) and enzymes (such as GST, iNOS) (Goel, et al., 2007). Thus curcumin has the potential to act as a multitargeted ligand (MTL) and some of the advantages of MTL's in cancer chemotherapy are as follows. (1). This approach is likely to be successful in diseases in which a number of genes have mutated. (2). The toxicity of a MTL may be lower than an agent having a single target. (3).The therapeutic effects of interactions with multiple targets may be synergistic. (4). By using a single compound in contrast to combination chemotherapy, the pharmacokinetic differences between various compounds will be avoided. Hence, some of the biological properties of curcumin which relate to its potential as an anticancer agent are now presented.

In a recent study (Cai et al., 2012), curcumin dose-dependently modified thioredoxin reductase 1 (TrxR1) and was involved in the transfer of electrons from NADPH to oxygen resulting in superoxide generation. Also, it was able to significantly down-regulate Trx1 protein expression and its enzymatic activity in HeLa cells. This action resulted in decreasing intracellular free thiols and disturbing the intracellular redox balance to a more oxidative state. Furthermore, when the HeLa cell line was pretreated with curcumin, the cells were susceptible to oxidative stress. Knockdown of TrxR1 brought about a sensitization of HeLa cells to curcumin cytotoxicity, stressing the physiological importance of targeting TrxR1 by curcumin. Taken together, these results reveal an unidentified prooxidant property of curcumin in cells, and offer key insights in understanding its mechanism *in vivo*.

Ravindran et al (2009), showed the ability of curcumin to modulate several cell signalling pathways which include cell proliferation (cyclin D1, c-myc), cell survival (*Bcl-2*, *Bcl-*$X_{L,}$ cFLIP, XIAP, c-IAP1), caspase activation (caspase-8,-3, -9), tumor suppressor (*p53, p21*) death receptor pathway (DR4, DR5), mitochondrial, and protein kinase (JNK, Akt, and AMPK) pathways. In addition, curcumin has shown differential specificity towards cancer cells over normal cells. However it is not fully established as to why curcumin preferentially kills tumor cells over normal cells but a few reasons have been suggested.

Tumor cells overexpress NF-κB which mediates their survival (Sethi et al., 2008) and curcumin is known to suppress the expression of NF-κB (Zaidi et al., 2009). In addition, absorption and fluorescence spectroscopic studies reveal that the cellular uptake of curcumin is enhanced in cancer cells over normal cells (c.f. Ravindran et al., 2009).

The literature strongly points to curcumin's role as an anti-tumorigenic, cancer-preventive or antibacterial agent, although different results have also been shown in a long term *in vivo* study. Short-term studies performed in animals and humans point out that it is safe to take curcumin when administered orally. No toxicity was reported in 25 subjects taking curcumin at concentrations up to 8000 mg/day (l15 mg/kg/day) for a period of 3 months (Cheng et al., 2001). Likewise no carcinogenic effects were seen at the same concentration in mice treated with turmeric extract for 3 months.

However, after a period of 2 years, carcinogenic effects were observed in mice fed with concentrations of turmeric that were equivalent to doses of curcumin of approximately 200 mg/kg/day (National Toxicology Program, 1993). This study therefore points to a conclusion that we cannot establish that oral consumption of curcumin is safe without performing long-term studies in humans, as dietary supplements containing high concentrations of curcumin may cause cancer-like effects when ingested on a long-term basis (c.f. Lopez-Lazaro., 2008). Nevertheless, other *in vitro* studies and clinical trials substantiate that curcumin may serve as a chemopreventive molecule preventing the formation of colorectal cancers in humans (Johnson and Mukhtar, 2007). Preliminary work has shown curcumin's role in the overexpression of a nuclear receptor PPAR γ. Stimulation of PPAR γ results in cell differentiation and apoptosis. In addition, PPAR γ ligands have also shown to be effective inhibitors of angiogenesis, a phenomenon obligatory for tumor growth and metastasis, and preventive against cellular transformation (c.f.Villegas et al, 2008).

Curcumin has also shown promise as an inhibitor of β-catenin/ T-cell factor-lymphoid enhancer factor

(TCF-LEF) and hence reduced the β -catenin/TCF signaling in various cancer cell lines (c.f. Shehzad et al, 2010). In addition, it has been suggested that curcumin stimulates cell cycle arrest and apoptosis through *Wnt* signaling, and the cell–cell adhesion pathway. The antitumor property of curcumin has also been exhibited in intestinal tumors by lowering the expression of β -catenin in animal models of familial adenomatous polyposis. Furthermore, recent studies have shown that curcumin induces apoptosis and cytotoxicity through stimulation of caspase-3 in human colon cancer cells, helps in the overexpression of *Bax*, cytochrome *c*, and *p53*, and inhibits the expression of *Bcl-2* and *Bcl-*X_L. Curcumin also displays chemotherapeutic activities against cancer cells. Several studies show its antineoplastic role by preventing tumor proliferation in cell lines (Simon et al, 1998) and animal models (Huang, et al., 1994). In *in vivo* experiments of both colon and gastric cancer, curcumin was able to retard the growth of cancerous/ precancerous lesions prompted by N-methyl-N'-nitro-N-nitrosoguanadine (MNNG), a well-known mutagen (Ikesaka et al., 2001). In this study, MNNG was provided in drinking water at a concentration of 100 ppm for 8 weeks. Following which, 0.2% or 0.5% of curcumin was given as a feed to the rats for 55 weeks. It was observed that the amount of atypical hyperplasia in curcumin-treated rats was reduced compared to the control groups. Similarly, the curcumin analog bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione impeded the tumorigenic effect of 1,2-dimethylhydrazine in rats (Devasena et al., 2003).

A few studies have suggested an influence of curcumin on mitochondria. Studies performed on rat liver mitochondria suggest that the anticancer property of curcumin may be due to the induction of apoptosis of tumor cells (Ligeret et al., 2004a, 2004b). This could be by increasing the permeability of the mitochondrial membrane. Furthermore, curcumin induced mitochondrial swelling, depolarizing the mitochondrial membrane and causing eventual release of cytochrome c. Morin et al., (2001) suggest that the pore induction by curcumin may involve the oxidation of membrane bound thiol proteins in the presence of low levels of Ca^{2+} concentrations, leading to MPT formation. They evaluated the effect of

thiol reagents on curcumin-induced mitochondrial swelling in rat liver mitochondria. Their studies show that thiol oxidation using a cross linker NEM increased the prospects of MPT opening, whereas thiol substitution or disulfide reduction prevented such an effect (Morin et al., 2001). Thus, these results show the importance of the thiol groups in the MPT formation as shown in **Figure 2**.

The competition between its role as an antioxidant, and its ability to stimulate MPT which is a direct basis of apoptosis, could possibly force a cell to choose between life and programmed cell death. Interestingly, curcumin in the keto form has two thiol alkylating sites as shown in **Figure 3**. Fang et al., (2005) through some elegant experimental work showed the inhibition of thioredoxin reductase (TrxR) activity by curcumin. Further, curcumin not only caused irreversible thiol alkylation of the enzyme but also conferred a prooxidant role to it.

Recent work by Marrache and Dhar (2012) investigated the potential of entrapping curcumin or other agents in triphenylphosphonium ion- conjugated nanoparticles for the diagnosis and targeted delivery of therapeutics for mitochondrial dysfunction-related diseases. Their study essentially shows that the nanoparticles conferred with a surface charge let them pass effortlessly through the mitochondrial membrane which is otherwise highly selective. Their cytotoxicity studies in HeLa cells using nanoparticles entrapping both a glycolysis inhibitor, lonidamine and a tumor selective drug, α-tocopherol succinate, were able to exhibit a ~5 fold lower IC_{50} as opposed to drugs or nanoparticles alone. The rationale behind such a potent effect could be due to the fact that the nanoparticles that had a positive charge were able to easily cross the inner mitochondrial membrane due to a charge difference (Marrache and Dhar, 2012). Thus in this case, there was an easy delivery of biodegradable nanoparticles containing drugs to mitochondria.

The synthetic derivatives 1,5-diaryl-1,4-pentadienyl-3-oxo compounds, with structural similarities to curcumin are shown in **Figures 5a, 5b,** and **5c.** These organic molecules which have a dienone moiety

have shown antineoplastic properties (Das et al., 2007, Das et al., 2009). Several of these compounds are highly effective cytotoxins having micromolar/submicromolar IC_{50} values towards a broad range of neoplastic and transformed cell. On occasions greater specificity in terms of toxicity towards neoplasms than normal cells was exhibited. Many of these compounds have exceptional tolerability in rodents (Dimmock et al., 1990, Das et al., 2008a,). These organic molecules have been designed specifically by our group to have a greater affinity for sulfhydryl groups as opposed to amino and hydroxyl groups present in nucleic acids. Therefore the compounds are expected to target thiol proteins and enzymes and not DNA and are not likely to be genotoxic as exhibited by a number of current anticancer drugs. Initial cytotoxicity studies as shown in **Table 1** (unpublished data) with curcumin analogues containing the 1,5-diaryl-1,4-pentadienyl-3-oxo pharmacophore (as shown in **Figure 4**) have been carried out using cell lines such as Molt4/C8 (human lymphoblastic leukemic), CEM (human myeloid leukemic) and L1210 (mouse lymphocytic leukemic) cells as shown in **Table 1.**

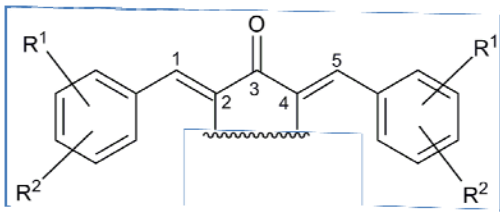


Fig.4: ***A basic structure of the 1,5-diaryl-1,4-pentadienyl-3-oxo pharmacophore.***

NC-2346, one of the members of the series contains a dimethylaminomethyl hydrochloride group (as shown in **(Figure 5b)** has shown potent cytotoxicity. The absence of the dimethylaminomethyl hydrochloride group on NC-2109 (**Figure 5a**) reduced the potency based on its IC_{50} values. Further addition of two more dimethylaminomethyl hydrochloride substituents to NC-2346 yielded NC-2352 **(Figure 5c).**

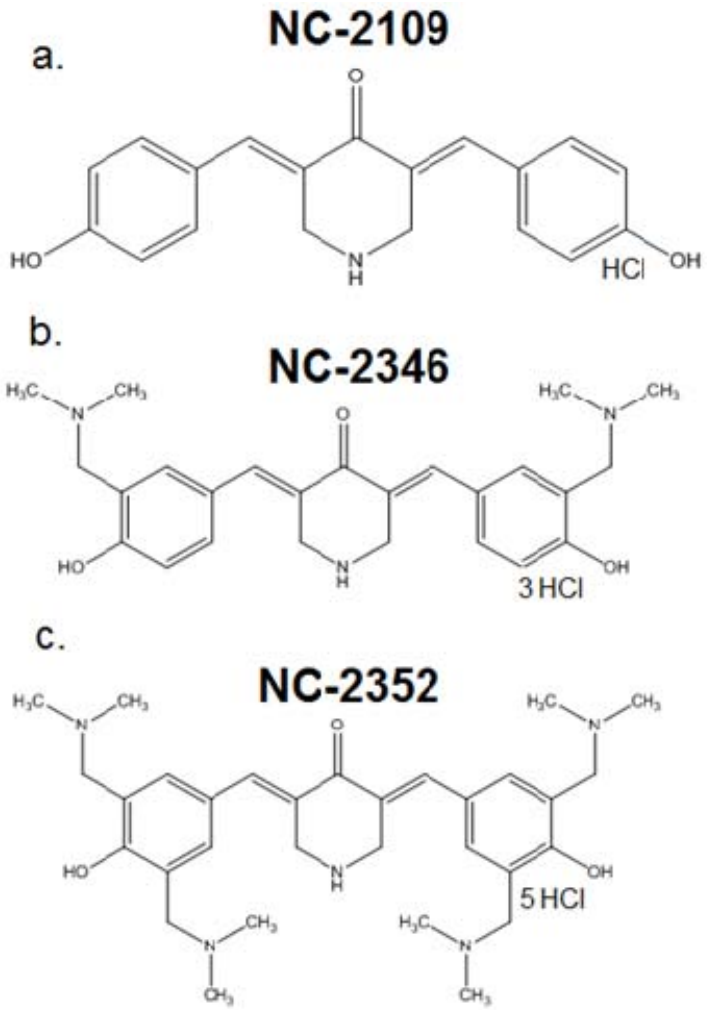


Fig-5: ***Structures of curcumin derivatives:*** These synthetic derivatives of curcumin showing the hydroxyl group (NC-2109), two dimethylaminomethyl functional groups (NC-2346) and four dimethylaminomethyl functional groups (NC-2352).

IC_{50} (µM)

Compound	**Molt 4/C8**	**CEM**	**L1210**
N.C. 2346	0.428 ± 0.006	0.64 ± 0.097	1.28 ± 0.18
N.C. 2109	5.02 ± 2.74	13.2. ±9.20	96.8 ±0 34
N.C. 2352	>500	>500	>500
Curcumin	6.46±1.41	8.16±1.66	15.1±1.60
Melphalan*	3.24±0.79	2.47±0.30	2.13±0.038

Table 1: ***Evaluation of curcumin analogs against Molt4/C8, CEM and L1210 cells*****:** The values for the synthetic derivatives NC-2346, NC2109 and NC2352, are part of previous unpublished work and those of curcumin are from Kong et al. (2009) and Hashim et al. (2012). *The values for melphalan are from Das et al.,2011.

These synthetic derivatives had not been compared previously for their *in vitro* cytotoxicity toward colon cancer cells and normal colon epithelial cells. Following up on the structures synthesized by the Dimmock group (Pati et al., 2009, Das et al., 2008a, 2008b), Simoni and coworkers (2010) have produced 3,5-bis(arylidene)-4-piperidone derivatives conjugated to polyamine moieties to serve as a vehicle to target mitochondria. These conjugated analogues showed tremendous potential as neuroprotectant lead molecules. In addition, they also served as effective antioxidants in mitochondria as well as fibroblasts. In particular, by inserting a polyamine functional group into the molecule it exhibited improved intracellular uptake and mitochondria-directed targeting. It also brought about a significant decrease in the cytotoxic effects.

Another study on 3,5-bis(arylidene)-4-piperidone (DAP) compounds (Kalai et al., 2011) showed a significant decrease in cell viability in a number of human cancer cell lines. The research work by this group demonstrates structure-activity relationships of a number of DAPs that were either N-alkylated or acylated with nitroxides in order to serve as potent antioxidants. The cytotoxic properties of the modified DAPs were tested in several cancer cell lines such as MCF-7, A2780, and H9c2. Cytotoxicity

studies in these cell lines showed a significant loss of cell viability, and these analogues were more toxic to cancer cells than normal cells.

Thus both the synthetic analogues and curcumin have remarkable potential as anticancer drugs. Curcumin's cytotoxicity towards a plethora of cancer cell lines (HT-29, HCT-116, and SW620 among others) has been well established. However, its role in *in vivo* studies poses a few challenges due to solubility issues. Of the three synthetic molecules, NC-2109 and NC-2346, due to their attractive GI_{50} values, provide several possibilities for use and potential as anticancer agents.

Since mitochondria and ROS tightly govern cellular homeostasis by regulating fundamental cell-death and cell survival ROS production may provide the cellular signal in directing cells towards life or death. It is clear that many key mitochondrial thiol proteins may play a significant role in affecting important apoptotic proteins. Although there is a positive correlation between mitochondrial ROS and cancer, it has been a subject of debate for more than four decades now. Recent discoveries of the prooxidant roles of curcumin, its mechanism of action via depletion of reduced glutathione and its influence on conformational changes in mitochondrial thiol proteins, highlight the significance of investigating ROS dynamics and show their continuance as attractive drug research targets.

2.9 Combination approach

One important aspect of chemo- and radio-therapy is that the cancer stem cells (CSCs) are highly resistant to them, and can persist to regenerate new tumors. This is a central reason why various types of anticancer therapies are unsuccessful to fully eradicate tumors though they often kill and eliminate the majority of cancer cells. Jang et al., (2008) through some elegant experiments showed that ANT2 knockdown could also be effective in stimulating apoptosis in breast cancer stem-like cells. In their study they used the adeno-ANT2 shRNA virus to inhibit ANT2 expression. Their findings clearly show that ANT2 expression was elevated in both stem-like cells and non-stem-like cells of MCF7 and MDA-

MB-231 cells, and was stimulated and overexpressed by mesenchymal transdifferentiation in MCF10A cells ($MCF10A^{EMT}$). ANT2 knockdown by the adeno-shRNA virus proficiently caused stimulation of apoptotic cell death in the stem-like cells of MCF7 and MDA-MB-231 cells, and $MCF10A^{EMT}$. Stem-like cells of MCF7 and MDA-MB-231, and $MCF10A^{EMT}$ cells showed very high resistance to the drug doxorubicin, and also overexpressed a multidrug-resistant related molecule, ABCG2. Adeno-ANT2 shRNA virus strikingly made the stem-like cells of MCF7 and MDA-MB-231, and the $MCF10A^{EMT}$ cells highly sensitive to the drug, doxorubicin. This was also complemented by decreased expression of ABCG2. Thus, these results strongly suggest that ANT2 knockdown is an efficient way to bring about the induction of apoptosis and intensify the chemosensitivity of stem-like cells (Jang et al 2011). The distribution of chemotherapeutic agents that aim at precise molecular pathways has been the trademark of drug development in the field of oncology over the past decade or so (Murgo et al., 2008). In a very few of the explicit research studies conducted so far, a single small molecule or antibody directed against an oncogenic target that inhibits tumor growth and improves survival has shown to be most effective offering for a long-term control of a specific disease state. Recent studies carried out by Zhang and coworkers showed that polyclonal anti-ovarian cancer antibody was able to prevent cancer cell proliferation in rabbits that were previously immunized with human ovarian cancer cell line SKOV3 (Zhang et al., 2014). In addition, Tai et al., have shown that anti-B-cell maturation antigen antibody-drug conjugate (GSK2857916) is able to selectively kill multiple myeloma cells (Tai et al., 2014). Some of the well-known drugs in the clinic include the use of imatinib (Gleevec, Novartis) for the treatment of chronic myelogenous leukaemia (Druker, 2009) or gastrointestinal stromal tumor (Joensuu et al., 2001) and trastuzumab (Herceptin; Genentech/Roche) for cancer of the breast that overexpresses human epidermal growth factor receptor 2 (HER2) gene (Vogel et al., 2002). Nevertheless, the genetic complexity of most of the malignant tumors implies that ablation of any particular target or pathway crucial for tumor proliferation is not likely to produce continued inhibition of growth. Hence, therapeutic intervention with a variety of diverse, although interrelated, growth

control tools by multiple drugs or MLTs has been presumed to be required to improve the achievement of treatments that use molecularly targeted anticancer agents (MTAs).

Thus, based on the literature background provided, the principal aims of this project were designed as follows: First, to see if the natural molecule curcumin and its synthetic analogues namely, NC-2346, NC-2109, and NC-2352 follow a mitochondrion-directed apoptosis in an *in vitro* system such as HCT-116 colon cancer cells. In addition, their selectivity towards cancer cells over normal colon epithelial cells will also be evaluated. Second, whether these test agents in the presence of a small molecule inhibitor such as CAT and an siRNA approach are able to potentiate mitochondrion-directed apoptosis as opposed to treatment with test agents alone.

3.0 MATERIALS AND METHODS

3.1 Materials

All materials, unless otherwise stated were obtained from Sigma Aldrich. ANT2 siRNA was purchased from Santa Cruz Biotechnologies. The cell lines, HCT-116 and CRL-1790 were obtained from the ATCC and also with their respective media from the same source. The NC-compounds were obtained from the research group of Dr. Dimmock. The % of DMSO (used as a vehicle for dissolving curcumin and its synthetic analogues) in cell culture treatments was maintained at 0.5% (v/v).

3.2 Protein estimation

The protein content in all the mitochondrial samples was determined by the Biuret method. This method is based on the development of a purple colored complex between the copper salts and protein molecules containing two or more peptide bonds in a basic environment. The procedure involved subjecting the samples to vigorous vortexing in the presence of the Biuret reagent (32 mM potassium sodium tartarate, 12 mM copper sulfate, 30 mM potassium iodide, 0.2M NaOH) in a ratio 1 part of the sample to 9 parts of the Biuret reagent (vol/vol). This mixture was incubated for 20 min at RT and the absorbance was read at 550 nm. Bovine serum albumin was used as protein standard (Layne, 1957).

3.3 Isolation of rat liver mitochondria

Wistar rats weighing ~200 g were sacrificed by decapitation. Isoflurane anaesthesia was used during this procedure. The animal use for the proposed research has been approved by the UCACS Animal Research Ethics Board The livers were instantaneously excised, sliced and placed in 50 mL ice-cold homogenization buffer (250 mM sucrose, 10 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) - HEPES, 1 mM ethylene glycol bis(aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), pH

7.2). The liver tissue was chopped finely with scissors with 2-3 washes to remove excess blood. Homogenization of the liver was carried out in a glass-teflon Potter-Elvehjem homogenizer using 2-3 passes with a loose pestle and one pass with a tight pestle. The homogenate was then centrifuged at 1000 x g, 4^{o}C, for 8 min to remove nuclei and cell debris. The supernatant was centrifuged at 10,000 x g, 4^{o}C, for 10 min.

The mitochondrial pellet obtained following the centrifugation step was resuspended in 30 mL of cold 250 mM sucrose, 10 mM HEPES, 0.3 mM EGTA, pH 7.2, and recentrifuged at 10,000 x g, 4^{o}C, for 10 min. The final mitochondrial pellet was resuspended in 1 mL of cold 250 mM sucrose, 10 mM HEPES, pH 7.2. Mitochondrial protein concentrations in aliquots of these suspensions were determined by the Biuret assay and the concentrations ranged between 50-80 mg protein/mL. Mitochondrial preparations were used within 3-4 hrs. Mitochondrial preparations displayed respiratory control ratios of 5-7.

3.3.1 Mitochondrial swelling

Mitochondrial swelling is the gradual expansion of the inner mitochondrial membrane called the cristae (Blondin, and Green, 1967). It most often results from the formation of the MPT pore. As a result the normally opaque mitochondria become transparent and have a tendency to scatter light. Thus this process can be evaluated by the decrease in the absorbance at 520 nm. Mitochondrial swelling was estimated by the decrease in the apparent absorbance at 520 nm of a mitochondrial suspension (1 mg protein/mL) exposed to the test agents followed by Ca^{2+}.

The cytotoxic agents being tested for mitochondrial swelling were added to mitochondria energized with 5 mM malate, 5 mM pyruvate in a standard medium consisting of 125 mM sucrose, 65 mM KCl and 10 mM HEPES-KOH, pH 7.4, at 30 °C. 100 µM of Ca^{2+} was added 1 min after the reaction started and the extent of swelling was evaluated after 20 min and compared to the tracings of the test agents (Kowaltowski et al., 1997).

3.3.2 Mitochondrial respiration

Mitochondria from rat liver were isolated by differential centrifugation (the protocol has been optimized by our laboratory). Briefly, rat liver mitochondria were isolated from male Wistar rats (150-250g) by differential centrifugation as described by Savage et al. (Savage et al., 1991) Mitochondrial total protein was measured by the Biuret method with bovine serum albumin (BSA) as a standard (Layne. 1957). All experiments were carried out with fresh mitochondria on the day of isolation. Freshly isolated mitochondria (in 10 mM HEPES, 250 mM sucrose (pH 7.2) were kept at 4 ºC on ice until mitochondrial respiration was measured.

To test the integrity of the mitochondria, its respiratory control ratios (RCRs) were measured, and the effects of the different compounds on mitochondrial respiration were evaluated. This experiment was monitored by the amount of oxygen consumption with a Clark electrode using 2 mM pyruvate and malate substrates in the respiratory control buffer in the presence and absence of ADP and the potential inhibitor or uncoupler (in this case the piperidone analogs). Mitochondrial respiration was measured for 2 min before and 4 min after the addition of the test agent using a Clark-type oxygen microelectrode fitted to an oxygen monitoring system (Hansatech). Mitochondria (1 mg/mL) were incubated in 1 mL of respiratory control buffer. Dinitrophenol (20 μM) which is a mild uncoupler was used as a positive control (Estabrook, 1967).

3.4 In vitro studies in HCT-116 cells

3.4.1 Assessment of cytotoxicity by sulforhodamine B assay.

The cytotoxicity of the compounds against the HCT-116 and CRL-1790 cell lines was determined using the sulforhodamine B (SRB) assay in 96-well plate format using the optimized protocol of Vichai and Kirtikara (Vichai and Kirtikara, 2006). Briefly, the assay is used for the determination of cell

density on the basis of the measured cellular protein content.

Cells suspended in 100 μL of growth medium at a concentration of 2 x 10^4cells/mL were added to the wells, and the plate was incubated in a 5% CO_2 incubator for 24 h at 37 ^oC. Briefly, the compounds were dissolved in DMSO and added to RPMI 1640 medium at three replicates per concentration and incubated for 48 h. Subsequently the media was removed from the plates and cells were fixed by addition of 100 μL of ice-cold 10 % (w/v) trichloroacetic acid (TCA) in water and kept at 4^oC. After overnight incubation, TCA was decanted by washing the plates six times with distilled water, and the plates were allowed to dry at room temperature for 24 h. A solution of sulforhodamine B, sodium salt (100 μL of 0.057% w/v solution dissolved in 1% acetic acid) was subsequently added to every well, and the plates were incubated at room temperature for 30 min. Following this step, unbound sulforhodamine B was removed by several washings with 1% acetic acid. Sulforhodamine B bound to proteins was freed by the addition of 200 μL of 10 mM Trizma base, and the absorbance of each well was measured at 510 nm on a plate reader after 20 min of incubation at room temperature. A total of nine replicates (in 3 independent experiments) of each compound were added at 10 different concentrations ranging from 100 μM to 5 nM in a 96-well plate. Each plate contained positive (known anticancer agent, 5-fluorouracil) and negative (DMSO vehicle) controls for the calibration of the percentage of cell death observed in each well, which were used to construct a dose-response curve and calculate a GI_{50} for the each test and standard compound. GI_{50} is defined as the concentration at which there is 50% of growth inhibition. This concept was recently introduced by NCI, and has been renamed from IC_{50} (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html). The GI_{50} for the test sample is calculated as follows:

$(T - T_0)/(C - T_0) \times 100 = 50$

Where:

T is the optical density of the test well that is exposed for 48 h to the drug to be tested.

T_0 is the optical density of the untreated well at day 0.

C is the optical density of the well containing the negative control.

The GI_{50} evaluates the cell growth inhibition of the test agent. The IC_{50} values of the compounds to Molt4/C8, CEM and L1210 cells were determined by the Rega group.

3.4.2 Mitochondrial membrane potential

Cells were plated at 20,000 cells/well (giving a density of 62.5 cells/mm^2) on clear black 96-well optical bottomed plates (Nunc) and incubated at 37 °C, 5% CO_2 and 95% humidity for 24 h to allow proper attachment of the cells. The following day, the medium was aspirated from the plates, and fresh medium was added. To these plates, GI_{50} concentrations of the compounds were added namely 0.90 μM (NC-2346), 0.87 μM (NC-2109), 9.07 μM (NC-2352), 3.50 μM (Curcumin), 5.47 μM (5-fluorouracil). Compound-treated plates were incubated at 37 °C for 48 h. Post-incubation, the compounds were aspirated from the plates and the plates were washed three times with PBS. A RPMI medium (without phenol red) containing 11 mM glucose was added to the plates, following which JC-1 was added to a final concentration of 2 μM. The plates were incubated for 60 min at 37 °C. After incubation with the dye, it was aspirated from the plates and the plates were washed three times with cold PBS. Fluorescence was measured first at excitation/emission 530 nm/580 nm ('red') and then at excitation/emission 485/530 nm ('green') using a plate reader (Chen. 1988).

3.4.3 Reactive oxygen species determination by the fluorescence probe DCF-DA

Cellular reactive oxygen species (ROS) are commonly measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA), which is hydrolyzed by intracellular esterases and can be oxidised to highly fluorescent dichlorofluorescein (DCF) by intracellular ROS. In error, the oxidised probe 2',7'-dichlorofluorescein diacetate had been purchased previously and was used in these experiments instead of the reduced form 2',7'-dichlorofluorescin diacetate. However since the probe gave results which

may be related to the intracellular redox state, the experiments are reported herein.

In these experiments the endogenous ROS/redox state was detected by use of the cell permeable probe, 2',7'-dichlorofluorescein diacetate (DCF-DA). This fluorescent probe in the presence of cellular esterases is cleaved intracellularly to DCF. That the initial fluorescence right after treatment of the cells was very low which suggests that the probe had been reduced intracellularly to 2',7'-dichlorofluorescin (DCFH). The subsequent changes in fluorescence therefore would be due to changes in the relative rates of reduction and oxidation (presumably by ROS).

HCT-116 cells were used for this experiment. These cells were maintained at 37° C in a humidified atmosphere of 95% air-5% CO_2 for 24 h in 96-well optical bottomed plates at a seeding density of 20,000 cells/well. The GI_{50} concentrations of each compound (at different time points, 15 min, 20 min, 30 min, 60 min, 120 min, 240 min and 480 min) were added to these cells. Hydrogen peroxide (5 μM) was used as a positive control. Following these incubation times, the cells were loaded with 2',7'-dichlorofluorescein diacetate (DCF-DA) (10 μM) and incubated at 37º C for 30 min in the dark. The cells were then washed, resuspended in PBS and analyzed on a plate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively. ROS was expressed in terms of the fold difference with respect to the control (Qin et al., 2008).

3.4.4 Determination of mitochondrial superoxide by MitoSOX red

HCT-116 cells were cultured at 37° C in a humidified atmosphere of 95% air-5% CO_2 for 24 h in 96-well plates at a seeding density of 20,000 cells/well. For detection of the acute effects of the test agents on mitochondrial superoxide levels, the cells in the medium were loaded with the test agents (at their GI_{50}'s) and the fluorogenic probe MitoSOX Red (Molecular Probes) at 2 μM for 15 min. MitoSOX red is a fluorescent probe that detects superoxide levels in live mitochondria Antimycin A (5 μM) was used

as a positive control. The cells were then washed three times with pre-warmed phosphate buffered saline and the fluorescence was measured at 0-105 minutes time points. Detection was carried out at the excitation and emission wavelengths of 500 nm and 590 nm, respectively, using a fluorescence microscope and quantified by a microplate reader (Robinson et al., 2006).

3.4.5 Evaluation of GSH/GSSG ratios

Based on Hu's protocol (Hu, 1994), GSH and GSSG were measured. Glutathione reductase was used to measure GSSG, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). GSH serves as an intracellular antioxidant which reacts with free radicals and organic peroxides. Furthermore, it acts as a substrate for other detoxification enzymes such as glutathione peroxidase and glutathione S-transferase. The activity of glutathione reductase is an important measure of the antioxidant status of the cell. Glutathione reductase requires NADPH for catalysis, resulting in the reduction of GSSG to GSH and the corresponding oxidation of NADPH to $NADP^+$. This assay is typically based on evaluating the loss in absorbance at 340 nm due to the conversion of NADPH to $NADP^+$. In regard to the GSH measurement, the sulfhydryl group of glutathione reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces the yellow colored 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of reduced glutathione in a sample.

3.5 Combination approach in HCT-116 cells:

3.5.1 Curcumin and curcumin analogs in the presence and absence of carboxyatractyloside by qualitative and quantitative analyses

In these experiements, 20,000 cells/well were plated on clear 6-well plates and incubated at 37 °C, 5% CO_2 and 95% humidity for 24 h to allow adherence of the cells. The following day, the medium was aspirated from the plates, and fresh medium was added. To these plates, the GI_{50} concentrations of the compounds were added in the presence /absence of CAT (1 μM). The compound and CAT-treated plates were incubated at 37 °C for two time points namely 8 h and 16 h. Post-incubation, compounds were aspirated from the plates and the plates were washed three times with PBS, trypsinized and centrifuged to sediment the cells. Cells were re-suspended in PBS, following which JC-1 to a final concentration of 2 μM was added. The cells were incubated for 30 min at 37 °C. After dye incubation, the cells were washed with cold PBS and centrifuged twice at 1100 rpm for 5 min at 4 °C. The cells were finally suspended in PBS and 100μL of each was added on to an optical bottomed plate. Fluorescence images were taken using a fluorescence microscope (Olympus IX71), and fluorescence was quantified at the excitation/emission 530 nm/580 nm ('red') and then at excitation/emission 485/530 nm ('green') using a plate reader.

For the quantitative analysis by microplate reader, 20,000 cells/well were plated on optical bottomed 96-well plates. An overnight incubation was carried out at 37°C, 5% CO_2 and 95% humidity so as to permit cell adherence. The following day, the media was removed, and fresh medium was added. Curcumin, a curcumin derivative, NC-2109 and a known anticancer drug, 5-fluorouracil using their GI_{50} concentrations were added in the presence /absence of CAT (1 μM). The compound and CAT-treated cells were incubated at 37 °C for 16 h. Following this incubation period, the compounds were removed from the plate and three PBS washings were given. Cells were suspended in RPMI-1640

media (minus phenol red) following which JC-1 to a final concentration of 2 μM was added. The cells were incubated for 30 min at 37°C. After dye incubation, the cells were washed with cold PBS and centrifuged twice at 1100 rpm for 5 min at 4°C. The cells were finally suspended in RPMI-1640 media. Microplate readings were taken at excitation/emission 530 nm/590 nm ('red'or the aggregate form) and then at excitation/emission 485/530 nm ('green' or the monomeric form) using a microplate reader.

3.5.2 Curcumin and curcumin analogs in the presence and absence of ANT2 siRNA

A pilot experiment was conducted with ANT2 siRNA to understand the mechanism by which the natural molecule and its synthetic derivatives follow, that is whether they are able to induce mitochondrion-directed apoptosis either by alkylating or oxidizing any of the mitochondrial thiol proteins, the chief player being ANT. The cancer cells overexpress the ANT2 isoform which is primarily antiapoptotic. Hence, it was hypothesized that ANT2 knockdown in the presence of the test agents would have an additive effect in bringing about mitochondria- driven apoptosis. Briefly, 20,000 cells/well were plated on clear 6-well plates and incubated at 37°C, 5% CO_2 and 95% humidity overnight to allow adherence of the cells. The following day, the medium was aspirated from plates and fresh medium was added. Following this step, transfection was carried out (the transfection reagent was provided by the Santa Cruz Biotech kit, directions, as per the kit). Following 6 h incubation with the transfection reagent, the transfection reagent was aspirated out carefully and fresh media containing no FBS was added. Upon acclimatization of the cells to this environment for 8-10 h, fresh media was added to these cells and the GI_{50} concentrations of the drugs were used to treat the cells. Following a 48 h incubation period, the cells were processed for JC-1 staining. Readings were taken at excitation/emission 530nm/580 nm ('red') and then at excitation/emission 485/530 nm ('green') using a plate reader (Jang et al., 2008). Based on the fluorescence results obtained for determining the mitochondrial membrane potential, treatment with ANT-2siRNA did not produce any additional benefit with NC-2109 or curcumin **(Appendix 1 Figure 18A and 18B)** so the experiments were not pursued

further. Subsequent analyses of cell numbers however showed further inhibition of cell growth in the presence of curcumin **(Appendix 1 Figure 18A and 18C)**. Also, ANT2siRNA augmented the effects of 5-FU on both mitochondrial membrane potential and cell growth **(Appendix Figure 18)**. These results are discussed further in section 10.1.1.and Future Directions.

4.0 STATISTICAL ANALYSIS

The results were either expressed as the means $\pm$ SD or SEM. SD was used for replicates of a single experiment and SEM was used when three independent experiments were carried out; each experiment having three replicates. Inferential statistics were carried out using either one-way or two-way ANOVA, and depending on the comparisons desired, with either Dunnett's or Tukey's post-hoc analyses for means comparison. For the combination approach experiments, a two-way ANOVA was carried out. The alpha level for statistical significance was set at $p < 0.05$.

5.0 RESULTS

5.1 Cytotoxicity of curcumin and its analogues in HCT-116 colon cancer cells and normal colon epithelial cells, CRL-1790

In order to compare the relative anti-colon cancer potential, cytotoxicity studies were carried out by the sulforhodamine B (SRB) colorimetric assay. Based on the calculated GI_{50} values as seen in **Table 2** both NC-2346 (GI_{50} of 0.90 μM) and NC-2109 (GI_{50} of 0.87 μM) were significantly more cytotoxic ($p<0.05$) to colon cancer cells than was the standard anticancer agent 5-fluorouracil. On the other hand, NC-2352 (GI_{50} of 9.07 μM) was found to have a higher GI_{50} value in comparison to the other synthetic and natural molecules.

SRB Cytotoxicity Screening	Mean GI_{50} (μM) in HCT-116 Colon Cancer Cells	Mean GI_{50} (μM) in Normal Colon Cells	Selectivity Index
NC-2109	0.87±0.38	16.37±2.64	18.81
NC-2346	0.90±0.22	4.15±1.08	4.61
NC-2352	9.07±1.10	535.24±52.84	59.01
Curcumin	3.50±0.36	58.64±10.14	16.75
5-Fluorouracil	5.47±0.55	10.26±0.22	1.87

Table 2: ***GI_{50} (μM) values of the test compounds (NC-2346, NC-2109, NC-2352 and curcumin) and positive control (5-fluorouracil):*** The term GI_{50} is used which is the concentration at which there is 50% growth inhibition. The cytotoxicity screening was carried out in CRL-1790 normal colon epithelial cells and HCT-116 colon cancer cells by the sulforhodamine-B assay. The GI_{50} values were determined from 3 separate experiments, with the concentrations tested in triplicate in each experiment. The selectivity index is the ratio of the GI_{50} for the cancer cells over the normal cells.

With regard to the cytotoxicity toward normal colon epithelial cells (CRL-1790 cells), NC-2346 was the most cytotoxic (GI_{50} of 0.90 μM), being more potent than the standard anticancer agent, 5-fluorouracil. Both 5-fluorouracil and NC-2346 had low selectivity indices. NC-2352 showed lower cytotoxicity effects towards CRL-1790 cells, and based on the selectivity index, NC-2352 had the greatest value, but it was not very potent toward the cancer cells. Curcumin, on the other hand, displayed low potency towards CRL-1790 cells, and so was found to be selectively cytotoxic to the cancer cells over normal cells. NC-2109 had a good selectivity index, which was similar to that of the natural curcumin molecule, but it was substantially more potent.

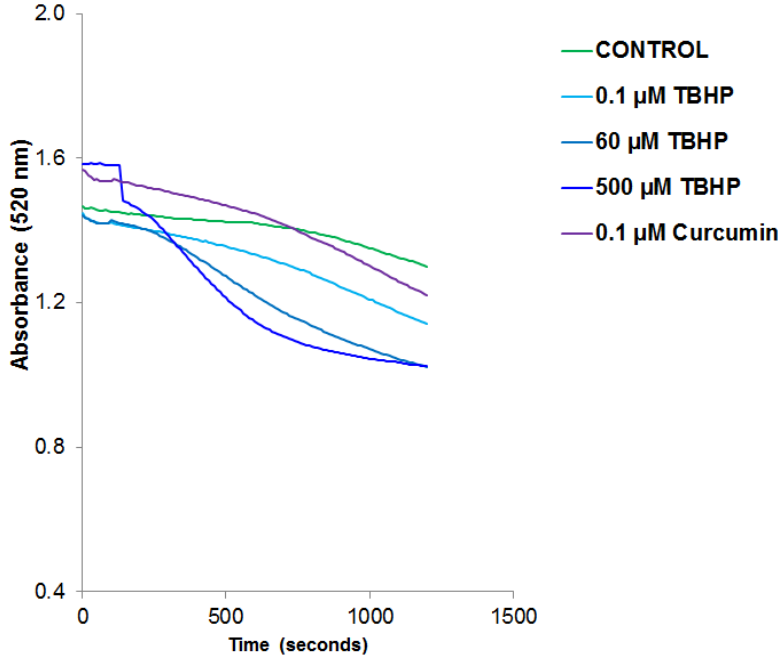
5.2 Studies on Mechanisms of Action

5.2.1 Effects on rat liver mitochondria

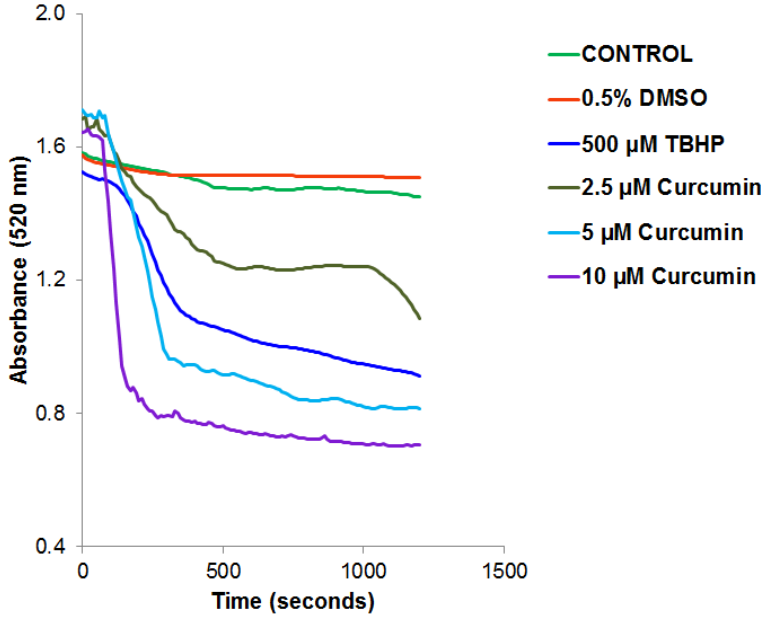
5.2.1.1 Mitochondrial swelling

To test whether these anticancer agents were able to induce the MPT, we measured mitochondrial swelling in rat liver mitochondria. This was mainly performed in order to evaluate the mechanistic effects of these agents, i.e., whether they were able to induce mitochondrial swelling which is an important step for the initiation of mitochondrial-directed apoptosis. All of the test agents except NC-2352 were able to induce mitochondrial swelling in rat liver mitochondria (**Figure 6**).

A.


2.0
1.6
1.2
0.8
0.4
Absorbance (520 nm)
0
500
1000
1500
Time (seconds)
CONTROL
0.1 µM TBHP
60 µM TBHP
500 µM TBHP
0.1 µM Curcumin

B.


2.0
1.6
1.2
0.8
0.4
Absorbance (520 nm)
0
500
1000
1500
Time (seconds)
CONTROL
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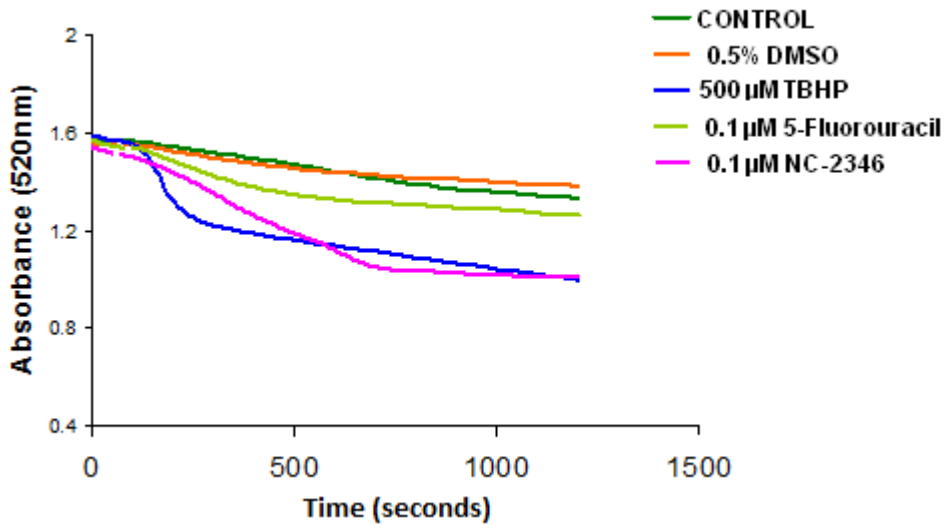
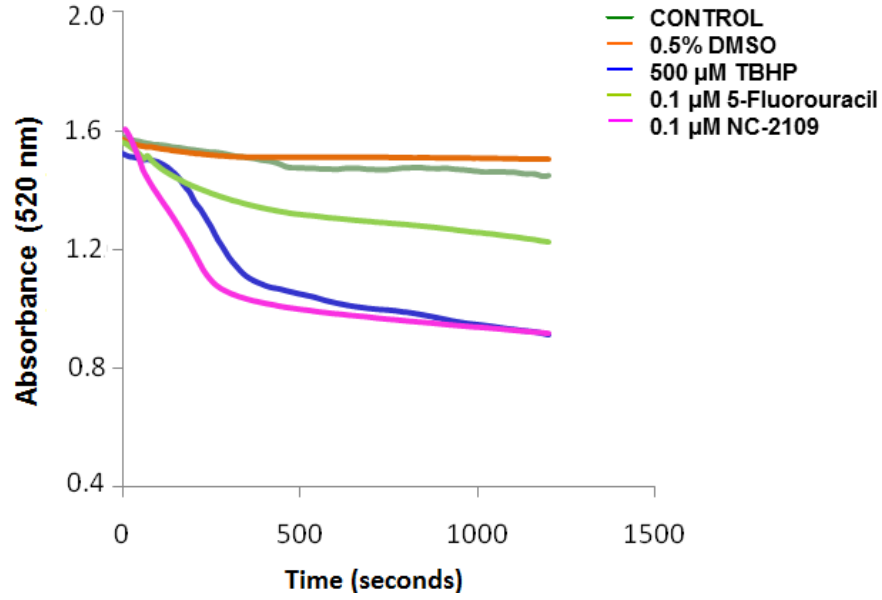
500 µM TBHP
2.5 µM Curcumin
5 µM Curcumin
10 µM Curcumin

C.


CONTROL
0.5% DMSO
500 µM TBHP
0.1 µM 5-Fluorouracil
0.1 µM NC-2346
Absorbance (520nm)
2
1.6
1.2
0.8
0.4
0
500
1000
1500
Time (seconds)

D.


CONTROL
0.5% DMSO
500 µM TBHP
0.1 µM 5-Fluorouracil
0.1 µM NC-2109
Absorbance (520 nm)
2.0
1.6
1.2
0.8
0.4
0
500
1000
1500
Time (seconds)

E.

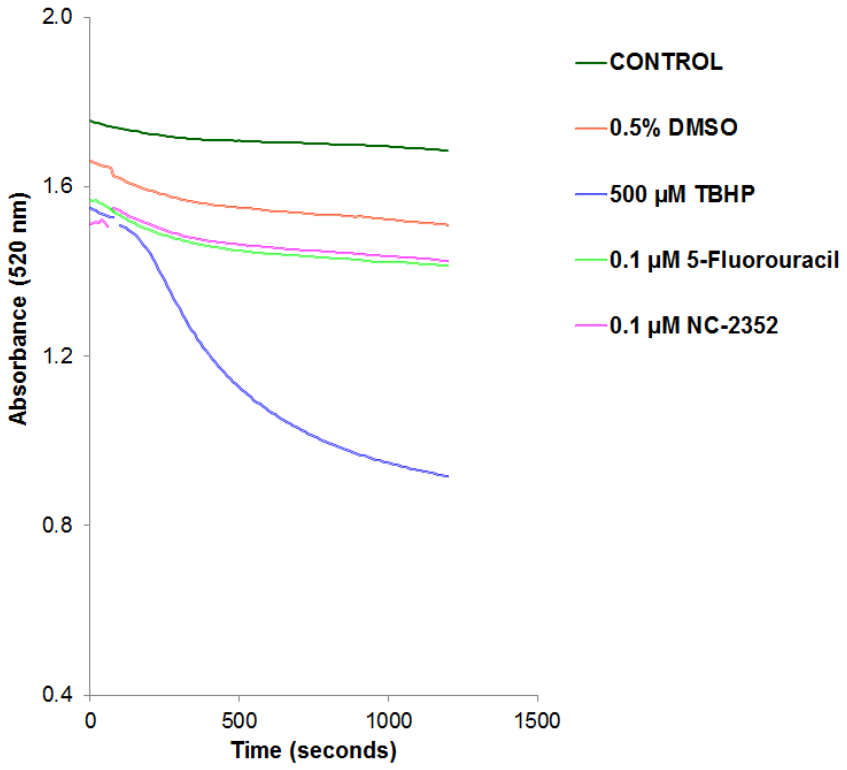


Fig. 6: ***Effects of curcumin, NC-2346, NC-2109 and NC-2352 on mitochondrial swelling:*** Rat liver mitochondria (1 mg protein/mL) were preincubated in the respiratory control buffer containing 2 mM pyruvate and 2 mM malate (pH 7.2). Swelling was induced by the addition of either *tert*-butyl hydroperoxide (TBHP, a standard oxidizing agent), the test agent (at 0.1 µM) or DMSO (used as a vehicle for curcumin and the synthetic derivatives). (A), Curcumin at 0.1 µM compared to TBHP at three different concentrations, 0.1 µM, 60 µM and 500 µM, (B) Curcumin, (C) NC-2346, (D) NC-2109, (E) NC-2352: The figures show representative traces of experiments done in triplicate.

Figure 6A shows the dose response of TBHP as a control pro-oxidant for mitochondrial swelling experiments. Furthermore, it also shows that curcumin at 0.1 µM was able to induce mitochondrial swelling to a similar extent to 0.1 µM *tert*-butyl hydroperoxide (TBHP).

Curcumin, as shown in **Figure 6B** was able to cause increased swelling at 2.5 µM, and at 5 µM and 10 µM it was found to be more rapid than 500 µM TBHP. **Figures 6C** and **6D** show the comparable effectiveness of the test compounds, NC-2346 and NC-2109 with that of TBHP even at a very low concentration (0.1 µM). **Figure 6E** shows negligible swelling with respect to NC-2352.

In summary, curcumin, NC-2346 and NC-2109, but not NC-2352 show swelling in rat liver mitochondria. This supports the belief that programmed cell death could occur via a mitochondrial route. NC-2109 was able to induce rapid mitochondrial swelling while NC-2346 progressively induced swelling over a period of time.

5.2.1.2 Mitochondrial respiration

The effects of the different agents on mitochondrial respiration were measured in order to test the mechanistic effects of these thiol alkylating agents, i.e., whether they are cytotoxic due to inhibiting mitochondrial respiration. None of the compounds inhibited mitochondrial respiration over this time period **(Figure 7).** Compared to ADP, DNP and DMSO, a mild and concentration-dependent increase in state 4 oxygen consumption was observed with the test compounds NC-2109 and NC-2352. NC-2109, NC-2352 and 5-fluorouracil could serve as mild uncouplers similar to dinitrophenol, a standard mild uncoupler.

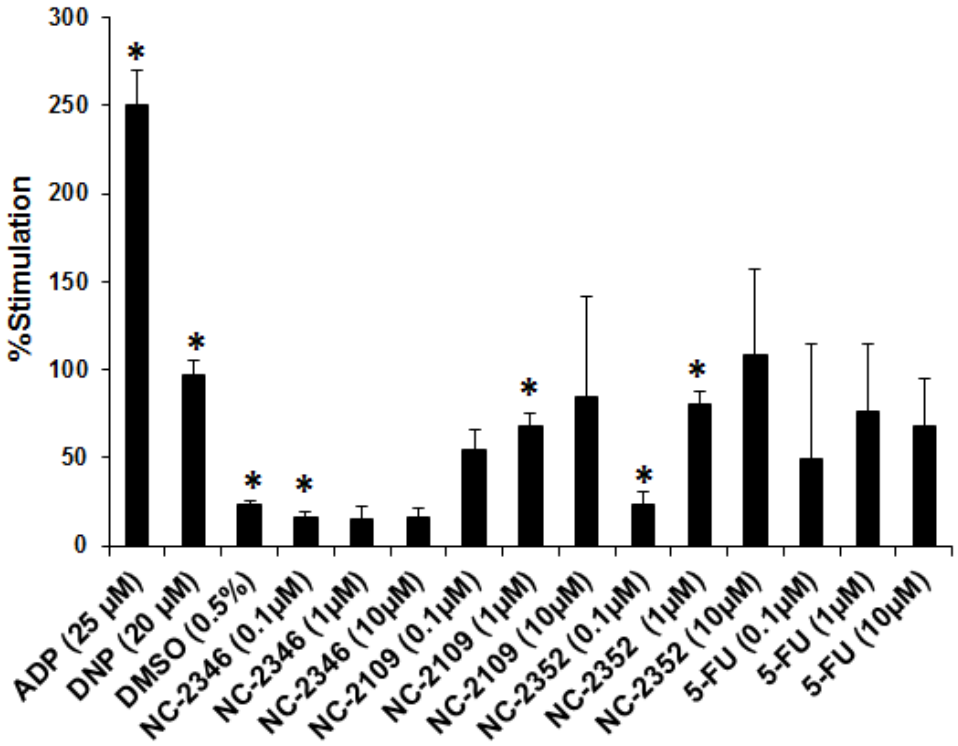


Fig 7: ***Effect of synthetic compounds and 5-fluorouracil on mitochondrial respiration:*** Rat liver mitochondria (1mg/mL) were incubated in a respiratory control buffer containing 2 mM pyruvate plus 2 mM malate (pH 7.2), and oxygen consumption was measured polarographically before and after the addition of the indicated compounds, ADP (25 µM), DNP (20 µM) or DMSO (0.5%).*Significantly different from control based on one-way ANOVA with Dunnett two-sided post-hoc analysis and a p value < 0.05.

5.2.2 Effects on HCT-116 colon cancer cells

5.2.2.1 Mitochondrial membrane potential in HCT-116 cells

The ability of the test agents to cause a decrease in the mitochondrial membrane potential which is, a possible indication of mitochondrial swelling, was tested in HCT-116 cells using the JC-1 dye. The microscopic images of the cells stained with JC-1, show depolarization of the mitochondria by the uncoupler CCCP (positive control) and curcumin indicated by a green fluorescence, and partial depolarization by NC-2109 as shown by red and green fluorescence **(Figure 8).**

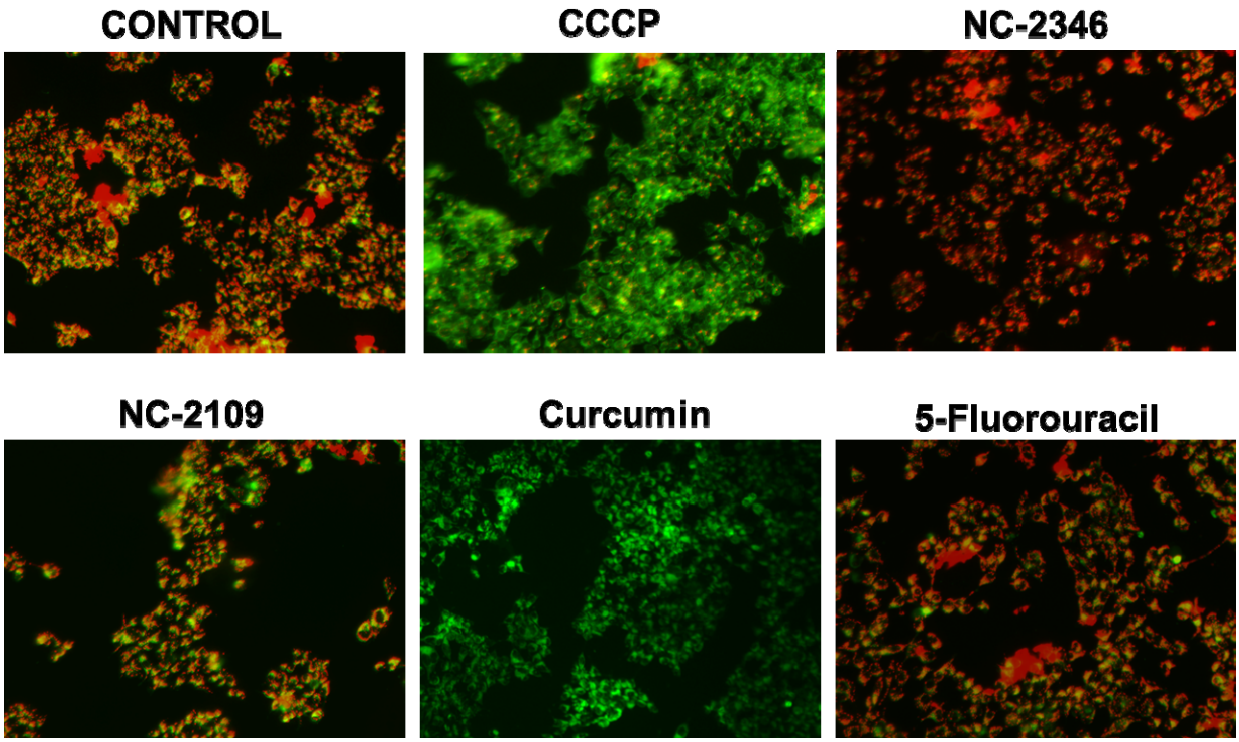
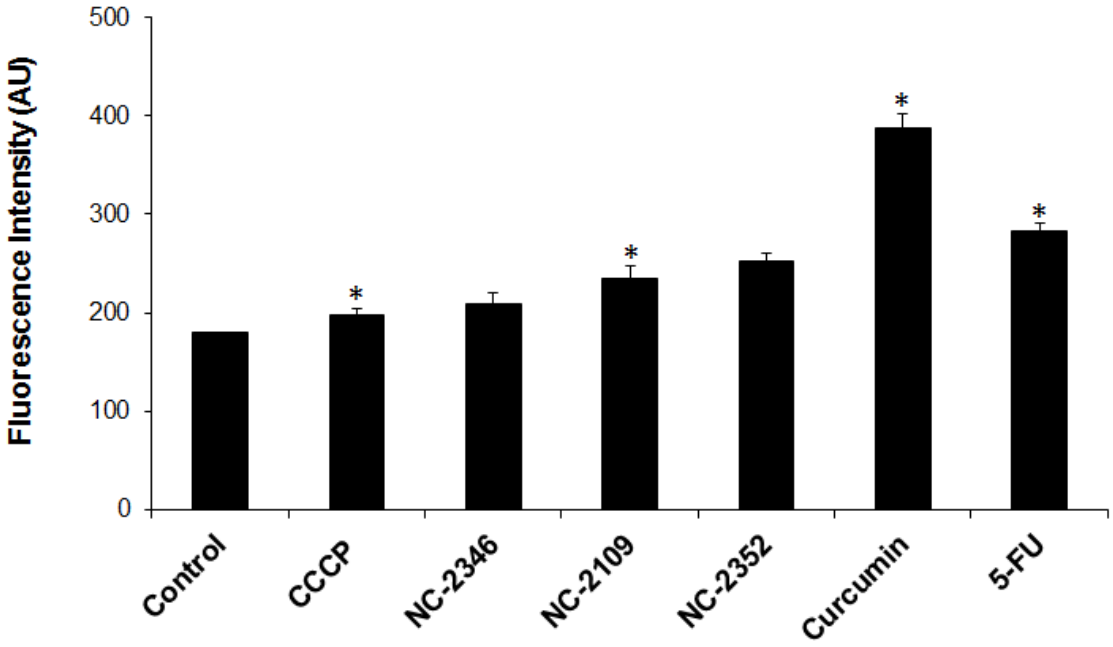


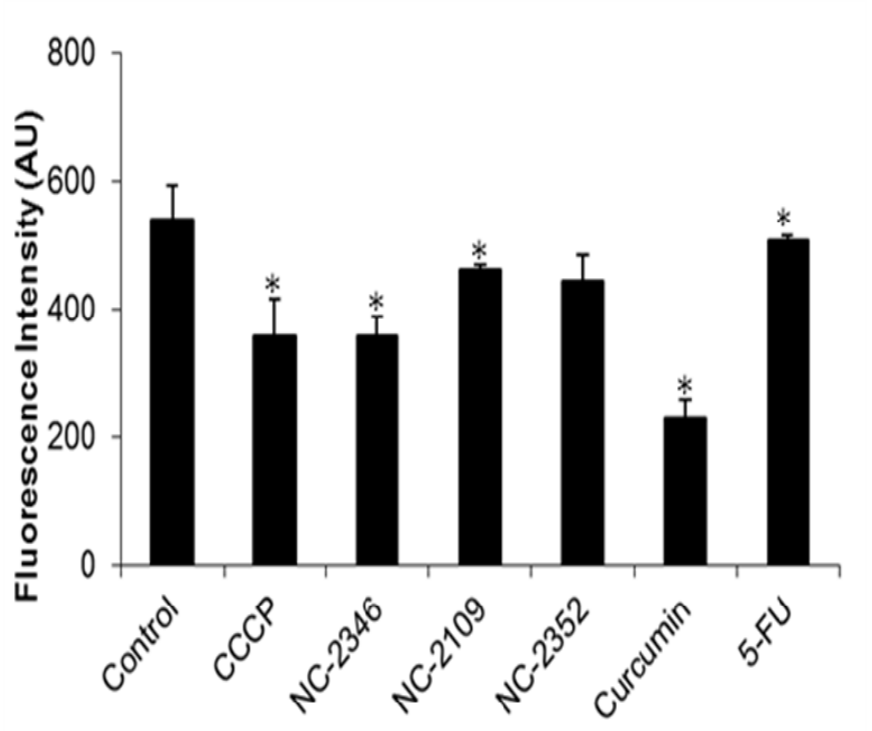
Fig 8: ***Fluorescence microscope images of JC-1-stained HCT-116 colon cancer cells post-48 h treatment with the test agents.*** is the images are 1 of 3 representative wells of the experiment and are an overlay of two images. Red color represents the aggregate form of the JC-1 dye while the green color represents the monomeric form. The images were taken at 100x magnification.

The fluorescence detected from the experiment shown in **Figure 8** was also measured quantitatively in a plate reader (**Figure 9**). **Figures 9A** and **9B** show that curcumin brought about an increase in the monomeric form of JC-1 (green), and caused low levels of the aggregate form (red) of the dye**. Figures 9A** and **9B** shows that NC-2109 and NC-2352 also significantly increased the monomeric form of the JC-1 dye, while NC-2109 and NC-2346 significantly decreased the dye aggregate level. When expressed as a ratio, which corrects for any differences in cell number, **Figure 9C** shows that curcumin, and NC-2109 were able to bring about a significant decrease in the mitochondrial membrane potential in comparison to the untreated control.

A. Monomer (485 nm/530 nm)



B. Aggregate (530 nm/590 nm)



C. Aggregate /Monomer Ratio

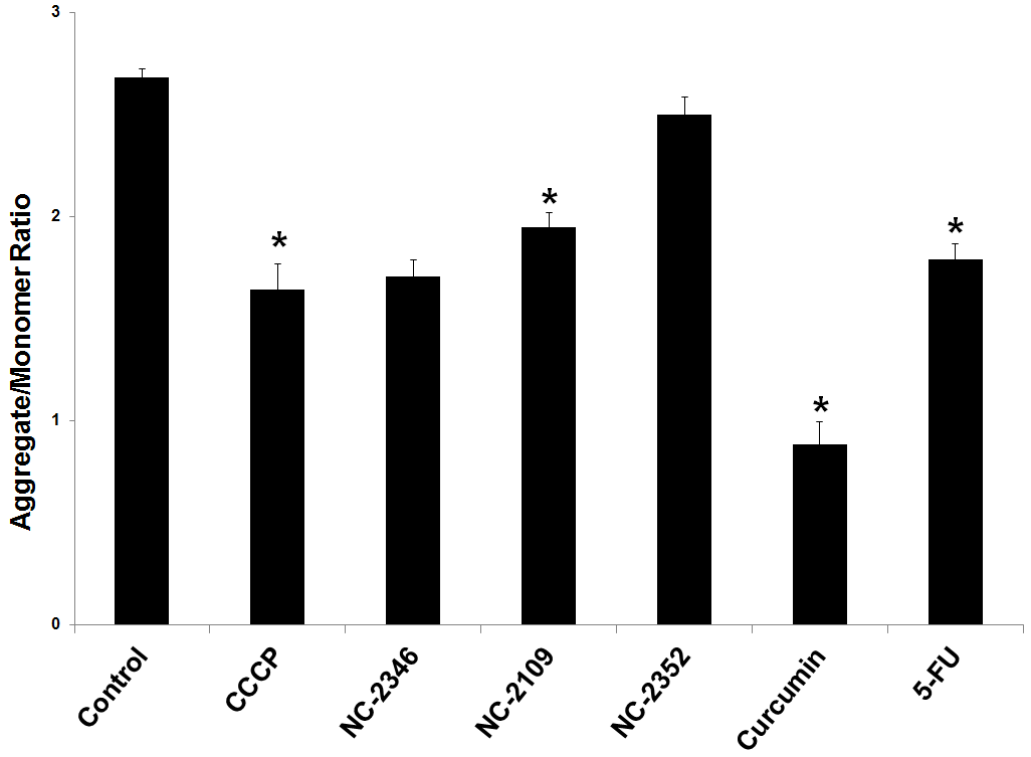


Fig 9: ***The effects of curcumin and its analogues and their ability to destabilize the mitochondrial membrane potential:*** The experiment depicted in **Figure 7** was also quantified by fluorescence measurements in a plate reader. Measurements of the mitochondrial membrane potential with JC-1 dye were conducted after 48 h treatment of HCT-116 cells with GI_{50} concentrations of the thiol alkylating agents, NC-2346, NC-2109, NC-2352 and curcumin. The uncoupler CCCP (16 μM) added to control cells 5 min before JC-1 was used as a positive control, relative to control (untreated) HCT-116 colon cancer cells. In addition, a standard anticancer agent, 5-fluorouracil (5-FU) at its GI_{50} concentration was used to see its effect on the mitochondrial membrane potential. **Panel A** shows fluorescence intensity of the monomeric form of the dye, which is retained in the cytosol. **Panel B** indicates the fluorescence intensity from the aggregate form of the JC-1 dye, which forms in the mitochondria dependent on the mitochondrial membrane potential. **Panel C** shows the ratio of aggregate to monomer as an index of mitochondrial membrane potential (independent of cell number). The bars show means ± SEM of three independent experiments with three replicates per experiment. *Significantly different from control based on one-way ANOVA with Dunnett two-sided post-hoc analysis and a p value < 0.05.

5.2.2.2 Thiol status in HCT-116 cells

In order to test if the candidate anticancer agents are able to deplete the cellular reduced glutathione levels, the thiol status was determined in HCT-116 colon cancer cells. Based on the results shown in **Figure 10** curcumin gave a 9 fold increase glutathione oxidized/reduced ratio, while NC-2346 and NC-2109 gave modest (6 and 3 fold) increased levels of the oxidized/reduced glutathione significantly

higher compared to the negative control. Furthermore, the standard anticancer agent 5-FU in comparison to all of the test agents showed a low GSSG/GSH ratio and was not statistically significant from the control.

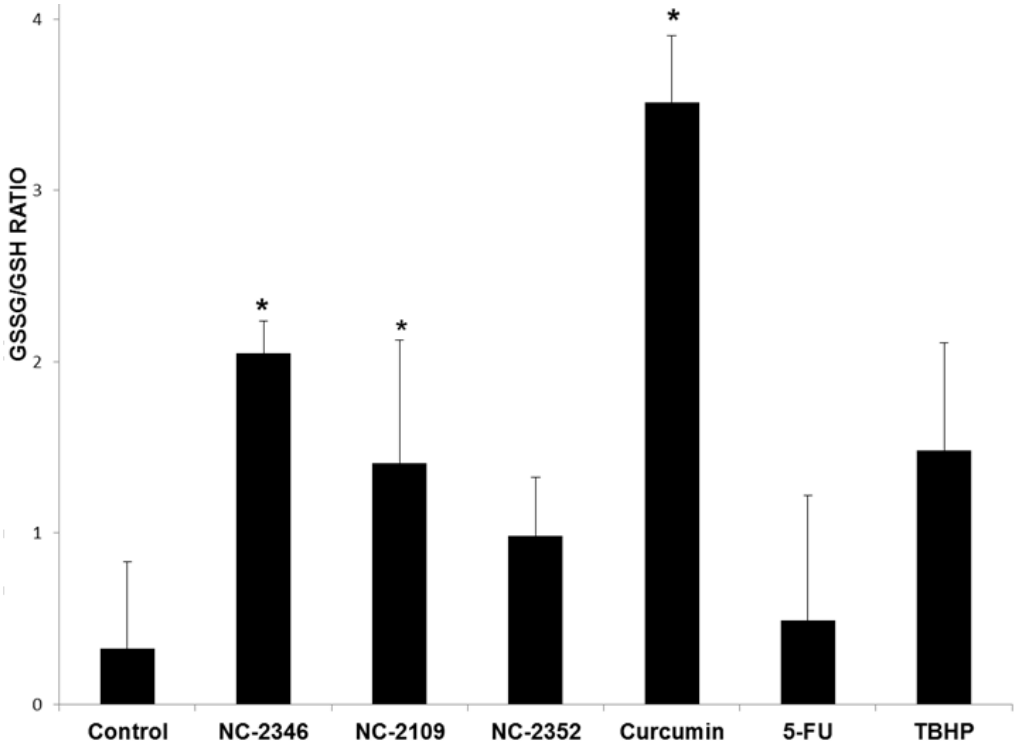


Fig 10: ***Thiol status in HCT-116 colon cancer cells treated with test agents:*** Cells were treated with test agents using their GI_{50} concentrations for 48 h, and GSSG and GSH measured as described in Materials and Methods. The oxidant tert-butyl hydroperoxide (TBHP) was used at a concentration of 500 µM as a positive control. The bars represent means ± SD of two independent experiments with 6 replicate treatments per experiment. * indicates significantly different ($p < 0.05$) from control based on one-way ANOVA with Dunnett (two-sided) post-hoc analysis.

5.2.2.3 Reactive oxygen species generation

In order to investigate the possible mechanism of cytotoxicity, the early time course of ROS generation (or a change in redox status) induced by the test agents was measured using DCF-DA. **Figure 11** shows that the natural molecule curcumin as well as the synthetic derivatives and 5-fluorouracil show a biphasic profile in terms of ROS release. There was an initial 2- to 3-fold increase in fluorescence

(from the oxidized probe) in the first 30 min followed by a decline and a slowly developing second phase of fluorescence. The pro-oxidant H_2O_2 also gave such a biphasic response. It should be noted that the amount of ROS released by curcumin was lower than that of NC-2109.

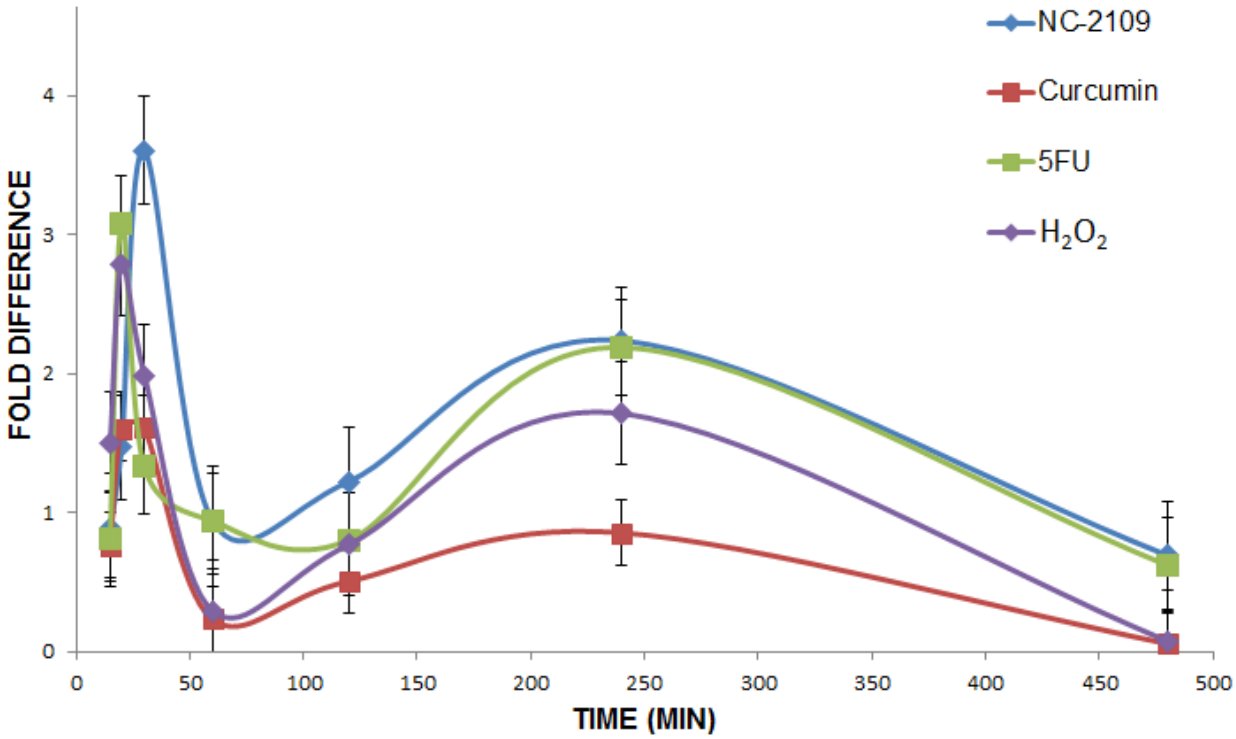


Fig 11: ***Time course of reactive oxygen species generation in HCT-116 cells using DCF-DA as a fluorescent probe:*** The cells were treated with curcumin and curcumin analogues using their GI_{50} values and incubated for a series of time points namely 15 min, 20 min, 30 min, 1 h, 2 h, 4 h and 8 h in cells plated in different plates. Following the incubation period, 10 µM of DCF-DA (upon replacement with the fresh media containing FBS and phenol red) was added & incubated for 15 -20 min. 2-3 PBS washes were given and the plate was read at 485 nm (excitation) and 520 nm (emission). Fold difference was calculated with respect to the control as a function of time. The points represent means ± SD of three replicates.

5.2.2.4 Mitochondrial superoxide production in HCT-116 cells

It is seen in **Figure 12A** all of the synthetic molecules bring about an increase in the mitochondrial superoxide levels (red fluorescence), a pattern similar to that of the positive control, antimycin A treated HCT-116 cells. While on the other hand, **Figure 12B** shows that the synthetic molecules are able to cause mitochondrial superoxide production which is similar to that of the antimycin A- (positive

control) treated cells. It was also observed that the untreated cancer cells have some basal endogeneous levels of mitochondrial superoxide.

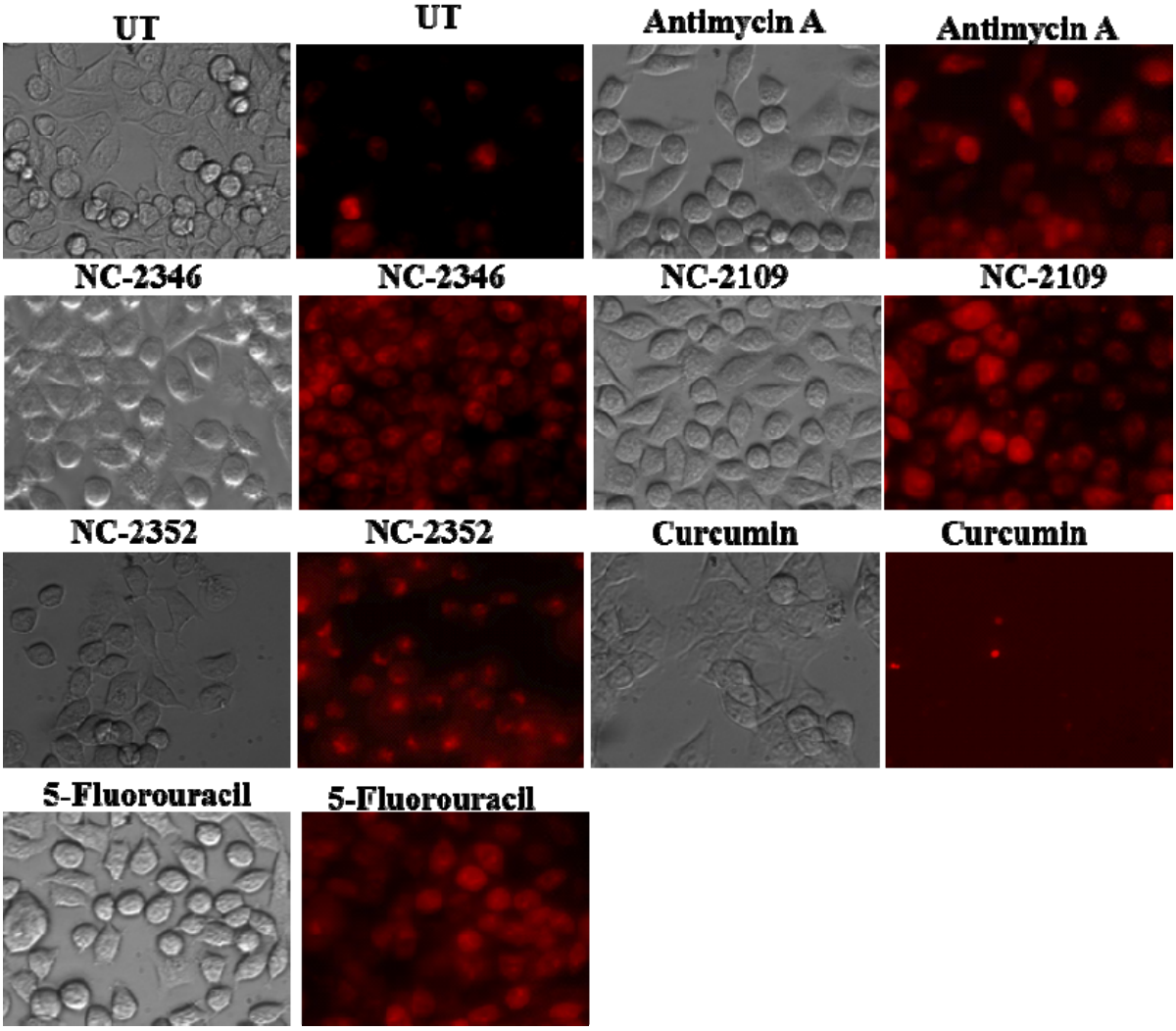


Fig 12A: ***Detection of mitochondrial superoxide generation in HCT-116 colon cancer cells treated with the test agents in the presence of an indicator MitoSOX red:*** A positive control, antimycin A (2 μM) was used to generate mitochondrial superoxide. GI_{50} concentrations were used to monitor the effects of the test agents.UT is the untreated HCT-116 colon cancer cells showing the endogenous superoxide levels. The fluorescence images were taken at 2 h timepoint after addition of the test agent and probe for 15 min, and washing thrice with PBS. The images were taken at 400x magnification, and are representative of one of three replicate wells.

In the experiment shown in **Figure 12A** the effects of the test agents (NC-2346, NC-2109, NC-2352, curcumin and 5-FU) on mitochondrial superoxide generation were also measured at early time points after addition of the agent, to evaluate the early response. As shown in **Figure 12B** NC-2109, curcumin and 5-fluorouracil, as well as the positive control, antimycin A, mildly elevated superoxide levels over

the first 105 min. NC-2346 and NC-2352 however, showed a rapid fall in the superoxide levels in comparison to other test compounds.

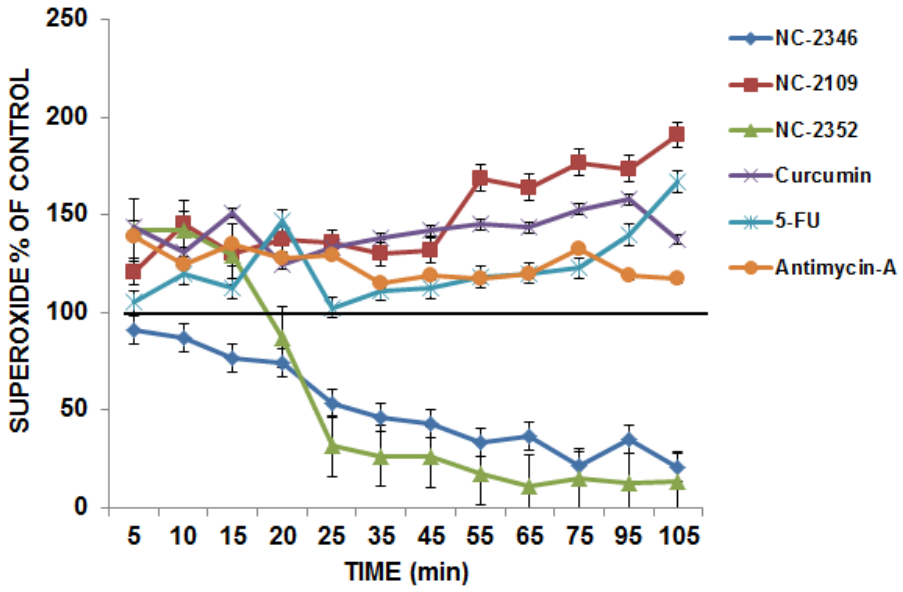


Fig 12B: ***Mitochondrial superoxide generation in HCT-116 colon cancer cells treated with the test agents:*** Mitochondrial superoxide generation was determined in the presence of an indicator MitoSOX red, added at the indicated times after addition of the test agent and incubated at 37^{o}C for 15 min. A positive control, antimycin A (2 µM) was used as an agent known to increase mitochondrial superoxide generation. Curcumin, curcumin analogs, and 5-fluorouracil were added into growth medium at their GI_{50} concentrations. After washing the cells 3x with PBS, superoxide levels were detected using a microplate reader. Fluorescence was measured at excitation/emission 500 nm/530 nm. Points represent means ± SD of three replicates for the experiment.

5.3 Combination approach

5.3.1 Effects in the presence/absence of carboxyatractyloside in HCT-116 cells

5.3.1.1 Effect on mitochondrial membrane potential

The effect of CAT was carried out to see whether it was able to potentiate the mitochondrial effects of curcumin and its analogues. As CAT retains ANT in its ‘c’ state conformation, there is a greater possibility of exposing the cysteines present on ANT than when in the ‘m’ state. While in previous experiments the drugs alone were incubated for 48 h, experiments on possible potentiation by CAT were conducted at earlier time points to reveal possible acceleration of the effect of the test agents.

After 16 h treatment, curcumin alone, and to a lesser extent NC-2109 and 5-FU already showed a strong decrease in the membrane potential (green fluorescence), so no additional benefit of CAT was observed (**Figure 13**).

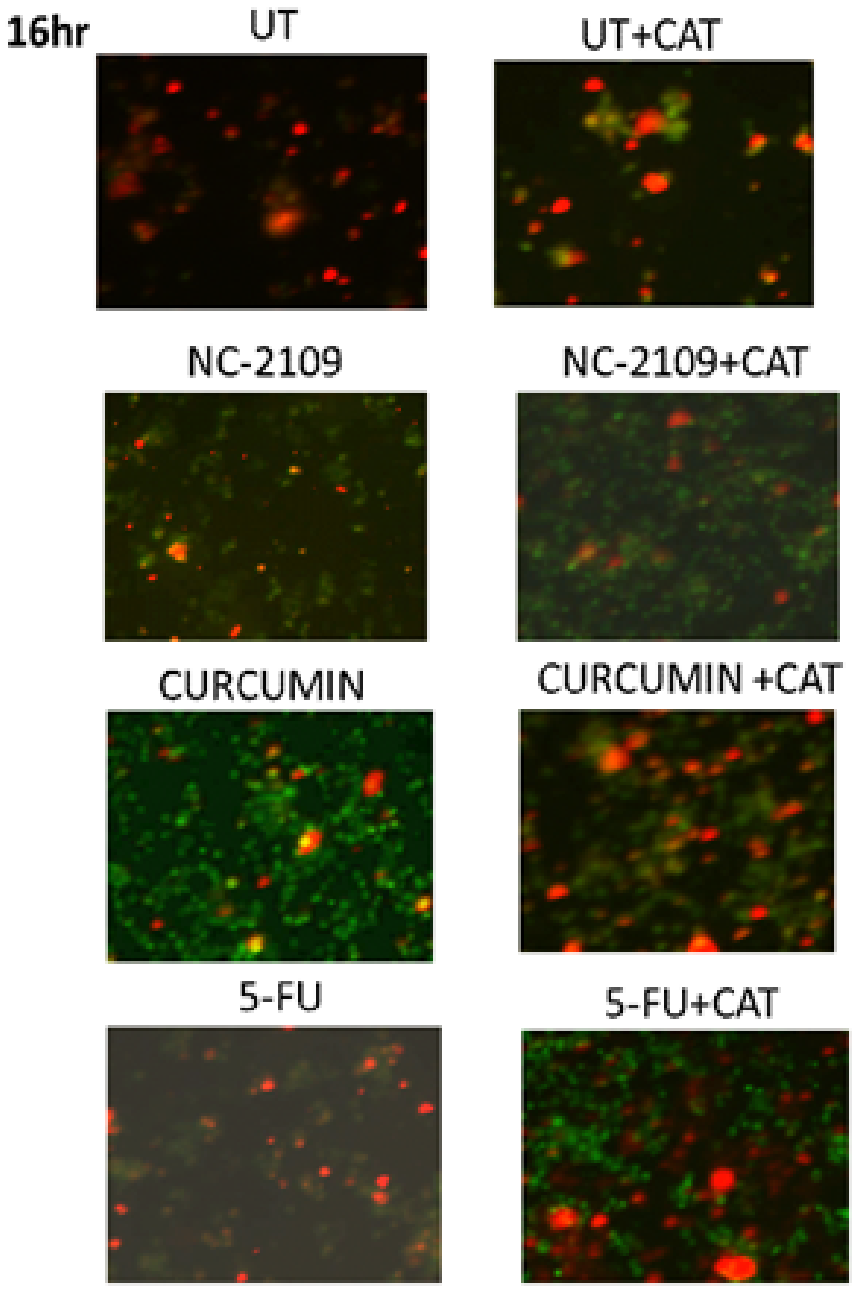


Fig 13:-*Effect on mitochondrial membrane potential of HCT-116 colon cancer cells treated with /without carboxyatractyloside (CAT) in the presence of test agents for16 h:* UT-Untreated; 5FU- 5-fluorouracil. The images are one representative out of three wells taken at 100x magnification.

The experiment was therefore repeated and measured after 8 h (**Figure 14**). **Figure 14** shows the fluorescence and bright field images. At this time point it was observed that CAT alone only slightly depolarized the mitochondrial membrane potential, but it greatly potentiated the effect of curcumin and less so 5-fluorouracil on membrane potential indicated by the monomeric form of JC-1 (green fluorescence) **(Figure 14).** Although the membrane potential was strongly affected, the bright field images showed little effect of CAT on cell density (**Figure 14**).

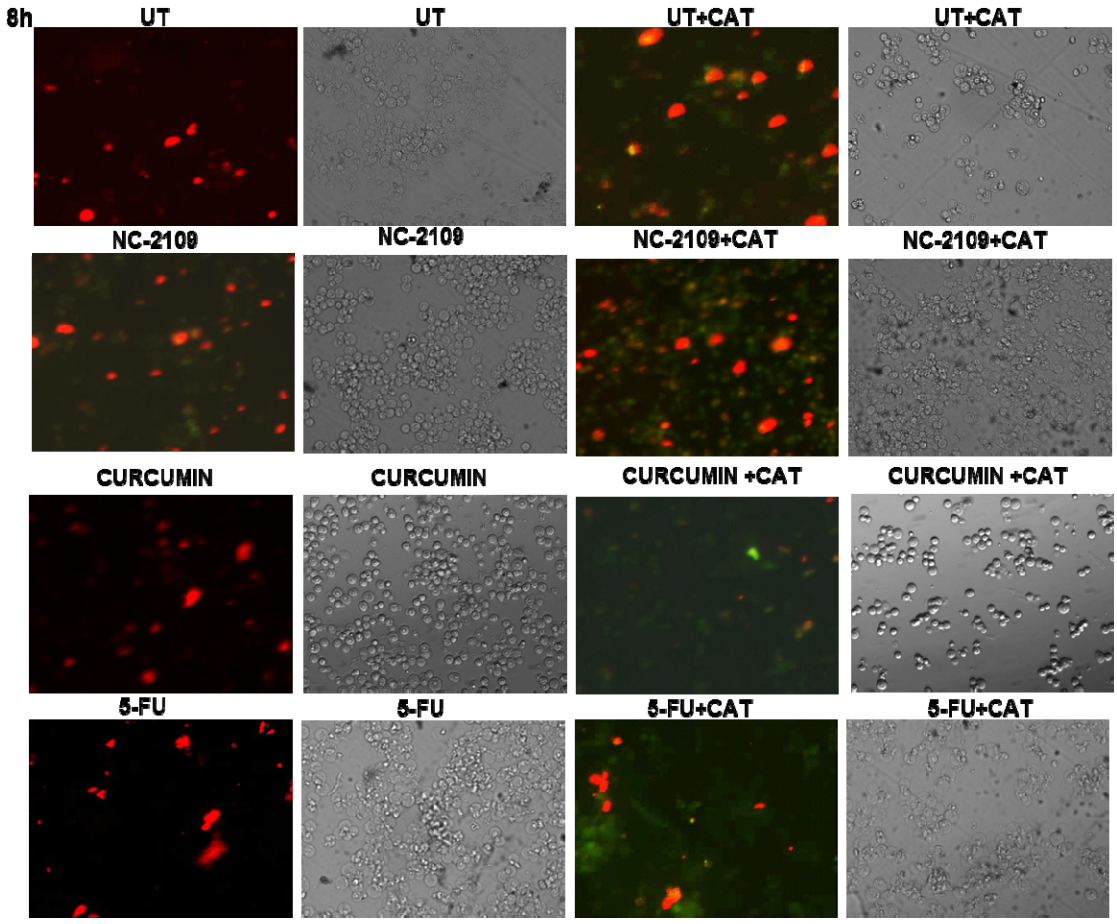


Fig 14: ***Effect on mitochondrial membrane potential and cell growth of HCT-116 colon cancer cells treated with /without carboxyatractyloside (CAT) in the presence of test agents for 8 h:***. UT-Untreated; 5FU- 5-fluorouracil. Shown are fluorescence (overlayed red and green) and bright field images taken at 100x magnification. The image is one representative out of three wells.

Additional experiments on potentiation by CAT were conducted using the same parameters shown in **Figures 13** and **14,** and the effects on mitochondrial membrane potential were both visualized under

the microscope and measured quantitatively with a plate reader (which was unfortunately unavailable in the previous experiment) **(Figure 15).** The results at both 16 h and 8 h showed that CAT potentiated membrane depolarization in the presence of curcumin. However with NC-2109 or 5-FU, CAT had no significant effect. At 16 h, NC-2109 and curcumin had already shown strong mitochondrial depolarization, while in this experiment 5-FU did not give depolarization. At the moment no explanation has been felt appropriate for the result obtained with respect to 5-FU in this experiment.

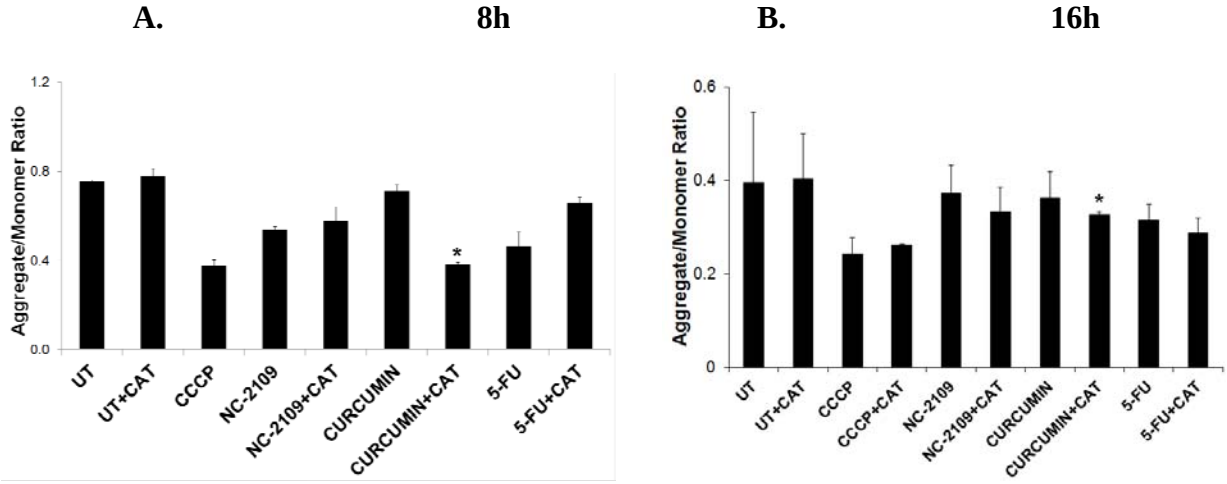


Fig 15: ***Effects on mitochondrial membrane potential of a combination approach in which HCT-116 colon cancer cells were exposed to in the presence and absence of carboxyatractyloside (CAT) upon treatment with test agents.*** While in **panel A** the HCT-116 cells were treated with the test agents in the presence and absence of CAT at 8h time point, in **panel B** they were treated for 16h. UT-Untreated; 5FU- 5-fluorouracil. The fluorescence image is one representative out of three wells, taken at 100x magnification. UT-Untreated; 5FU-standard anticancer drug; Curcumin is the natural molecule being compared with its synthetic counterpart, NC-2109. * Statistically significant with respect to that of curcumin alone ($p < 0.05$) based on two-way ANOVA with Tukey's post-hoc analysis. Bars represent means ± SEM of two independents experiment carried out each in triplicate.

5.3.1 Cell growth results:

In addition to the mitochondrial membrane potential experiments, a separate experiment on the influence of CAT on cell growth, measured by the SRB method was carried out. This was done in order to understand whether there was a lower cell growth in the presence of CAT in HCT-116 cells that were treated with these anticancer test agents. As shown in **Figure 16**, the cells that were treated for 8 h with NC-2109 and curcumin in the presence of CAT reduced cell growth further by ~50% when

compared to cells that were treated with NC-2109 and curcumin alone from 42% and 56% to 19% and 35% respectively. Inhibition of cell growth by 5-FU was relatively unaffected by CAT (53% versus 45%).

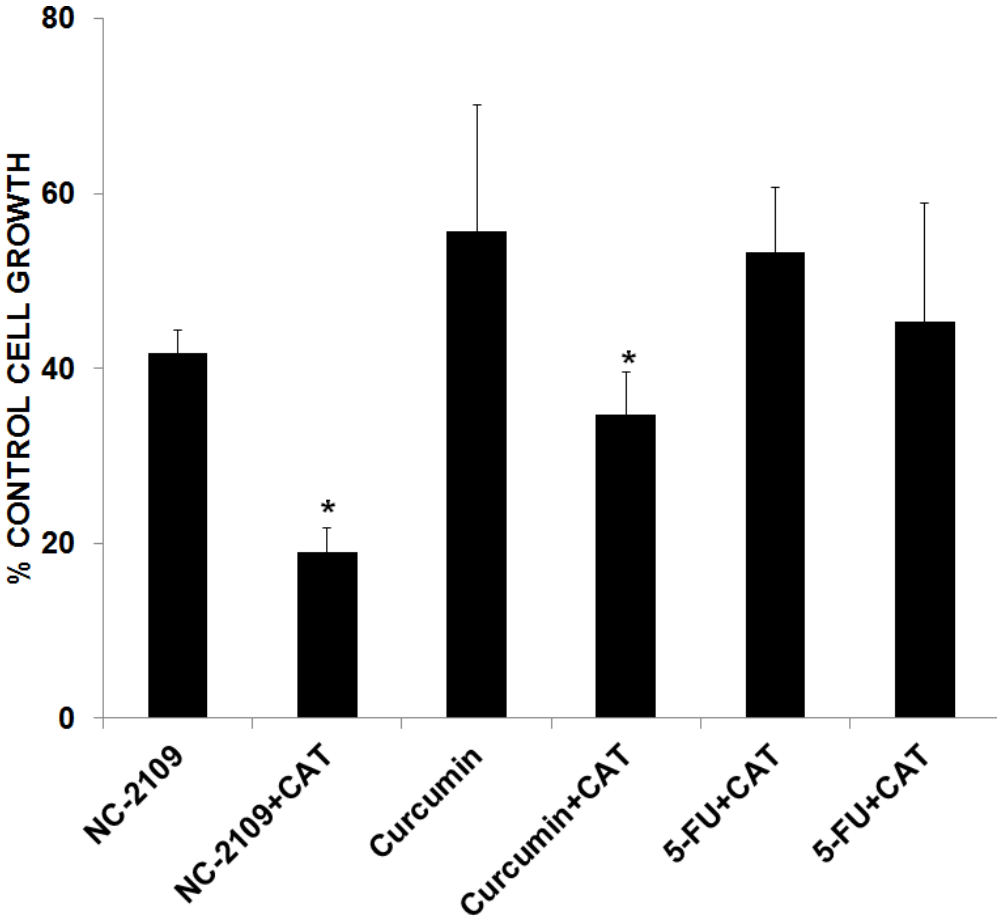


Fig 16: ***Effects of drug treatment in combination with carboxyatractyloside on cell growth:*** HCT-116 colon cancer cells were treated in the presence and in the absence of carboxyatractyloside (CAT) upon being treated simultaneously with the test agents for 8 h incubation time. *Statistically significant ($p < 0.05$) from drug treatment in the absence of carboxyatractyloside based on two-way ANOVA with Tukey's post-hoc analysis. Bars represent means ± SD of an experiment carried out in triplicate.

6.0 DISCUSSION

Curcumin, a component of turmeric shows both cytotoxic and anticancer properties (Aggarwal. et al., 2003, Anand et al., 2008). Also, there is an epidemiological connection between the intake of turmeric and the lower incidence of colon cancer (Ravindran, et al., 2009). However major drawbacks in the development of curcumin and its analogues as anticancer agents are its poor solubility and extremely low bioavailability. It may also be possible that the dimethylaminomethyl groups can acquire a positive charge which could facilitate uptake by mitochondria (especially tumor mitochondria).Two portions of the curcumin molecule which are able to react with cellular thiols are indicated as **(A)** and **(B)** in **Figure 17**. These groups have been primarily incorporated into the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore which has led to the discovery of quite a few candidate anticancer agents (Das et al., 2009a, Das et al., 2007, Das et al., 2009b). A major drawback with acyclic dienones is that these molecules can assume a variety of different conformations which creates a difficulty in understanding which of them contribute to its bioactivity. Hence, the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has been mounted on heterocyclic and cycloaliphatic scaffolds as shown by the general structure **(C)** in **Figure 17**.

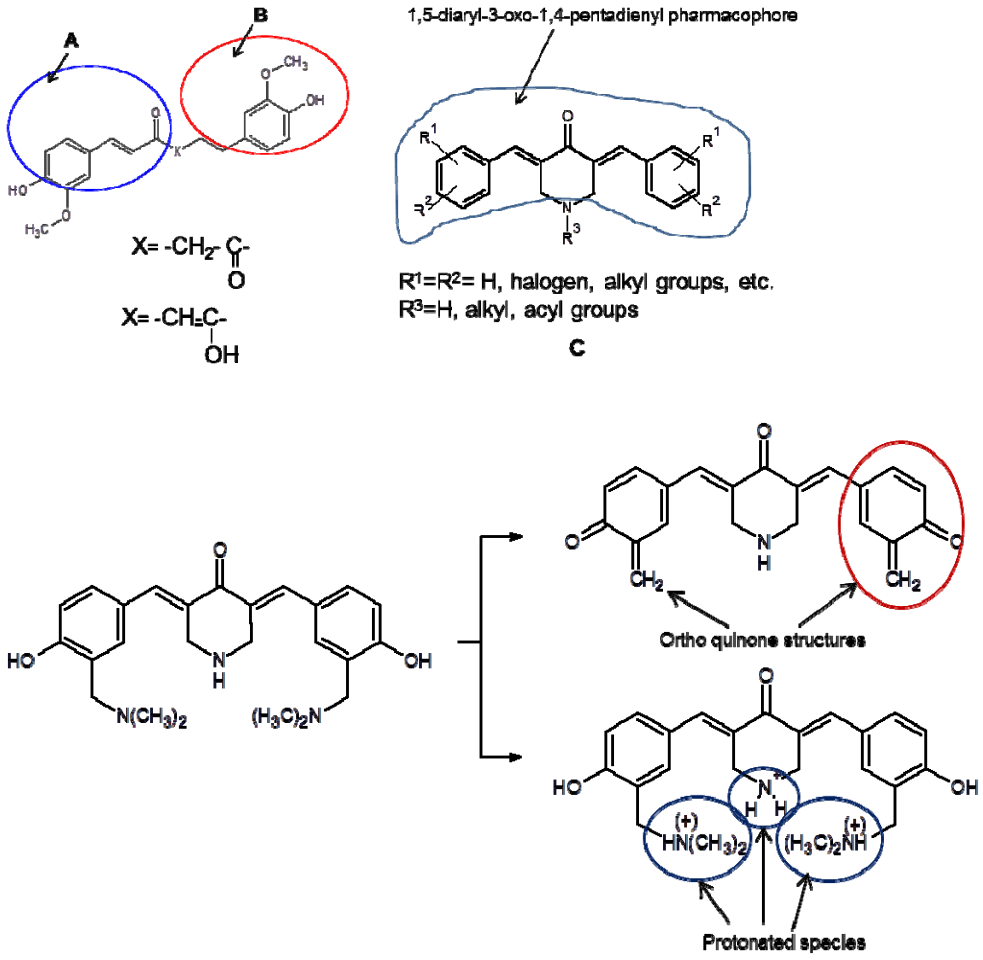


Fig 17: Structures related to various unsaturated ketones

A problem encountered usually with series (**C**) is often their lipophilicity. Many of these synthetic derivatives exhibit logP values in the region of 5-8 (Das et al., 2008a). Hence, recently a new programme was initiated by undertaking two molecular modifications in order to address this issue. First, hydroxyl groups were incorporated into the aryl rings since this functional group is hydrophilic having a Hansch π value of -0.67 (Hansch and Leo, 1979). Second, dimethylaminomethyl groups were placed in the aryl rings since when protonated, the ionic bonds formed would potentiate their solubility in aqueous solvents. Furthermore, there is the possibility that the dimethylaminomethyl groups undergo deamination to produce orthoquinone methides which are highly chemically reactive. In addition, the

thiol alkylating capacity of dienones is influenced by the magnitude of the charge on the olefinic carbon atoms. While the Hammett σ value of the dimethylaminomethyl group is 0.00, when protonated [$CH_2 N^+H(CH_3)_2$] the σ value rises to 0.40 (Perrin et al., 1981), which decreases the electron density on the olefinic carbon atoms. This will determine the rate and extent to which thiol alkylation would occur. Since the pH of a number of malignant tumors is lower than is found in normal cells, (Wike-Hooley et al., 1985), the chemical reactivity of the dienones and hence toxicity may be greater in tumors than normal cells. Also when they are charged derivatives, the molecules will likely have an affinity for mitochondria in general and tumor mitochondria in particular due to the high mitochondrial membrane potential of many tumors than is found in nonmalignant cells (Modica-Napolitana and Aprille, 2001).

In this thesis, I hypothesized that these compounds will be able to bring about the formation of reactive oxygen species upon lowering the reduced glutathione levels thus resulting in mitochondrion- directed apoptosis. I also hypothesized that these compounds would selectively target the cancer cells by causing the alkylation or oxidation of some of the important mitochondrial thiol proteins such as the ANT2 isoform that is upregulated in cancer states. Furthermore, I asked whether a combination approach using ANT2 siRNA would further improve the outcome, and whether the ANT2 inhibitor CAT would potentiate the apoptotic process in cancer cells.

The IC_{50} values of these synthetic compounds were previously determined in leukemia cell lines (Molt4/C8, CEM and L1210), showing NC-2346 to be the most potent, followed by NC-2109 and NC-2352 in these cell lines (**Table 1**). In this thesis I compared the cytotoxicities and selectivities of these compounds in HCT-116 colon cancer cells compared to curcumin and 5-fluorouracil (a standard colon cancer treatment drug), and investigated the mechanisms involved.

6.1. Cytotoxicity of curcumin and the synthetic analogues in colon cancer (HCT-116) and normal colon epithelial (CRL-1790) cells

In the colon cancer cell line, HCT-116, NC-2109 and NC-2346 showed very good, submicromolar GI_{50}'s. However NC-2352, which has four dimethylaminomethyl moieties in comparison to NC-2346 (which has two dimethylaminomethyl functional groups) was less active as observed through its higher GI_{50} value towards HCT-116 cells. One possible explanation for the lesser cytotoxicity of NC-2352 may be steric impedence of the olefinic carbon atoms by the extra dimethylaminomethyl groups thus diminishing the interactions with cellular constituents.

On the other hand, NC-2109, in spite of being devoid of the dimethylaminomethyl groups, showed potent cytotoxicity towards the HCT-116 colon cancer cells (GI_{50} value – 0.87±0.38 μM). The absence of dimethylaminomethyl functional groups could make it less bulky and may possibly help this molecule to interact at binding sites.

All of the analogs as well as curcumin have two phenolic hydroxy groups. The presence of hydroxy groups could generate a reactive intermediate phenoxy radical (Youssef and El-Sherbeny, 2005). However, it is important to have a strong oxidant to generate such a byproduct. If the cellular environment has high levels of hydrogen peroxide or other ROS, such a cytotoxic intermediate may be produced which would increase the oxidative stress. In addition, the proton of the phenolic group can form hydrogen bonds with different cellular constituents. For example, the hydroxy hydrogen atom could bond with the nitrogen, oxygen and sulfur atoms of proteins and also with a carboxylate anion [COO^-].

The relative toxicity of synthetic analogs versus the natural agent curcumin towards a neoplastic cell line has not been determined previously by our group. The cytotoxic properties of NC-2346 and NC-2109 towards HCT-116 cells were greater (~4 times more potent) than displayed by curcumin. This

observation may be due to the dienones having rigid structures which are preferable to the acyclic curcumin. In addition, if curcumin exists predominantly in the enolic form, it has only one 3-aryl-2-propenoyl group in contrast to NC-2346 and NC-2109 which have two such structures. Both of these compounds, as well as curcumin, were more potent than 5-fluorouracil.

The GI_{50} of 5-fluorouracil is 5.47 μM towards HCT-116 cells and is similar to the value reported by the National Cancer Institute of 4.55 μM (http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp). Of particular interest are the observations that curcumin can sensitize colorectal cancer cells to various antineoplastic agents. Thus curcumin has enhanced the cytotoxicity of both 5-fluorouracil (Shakibaei et al., 2013) and folfox (Yu et al., 2009). Hence in developing NC-2346, NC-2109 and related compounds, the ability of these unsaturated ketones to chemosensitize neoplasias to established anticancer drugs should be investigated.

An issue of major importance in evaluating candidate anticancer agents is the differential in toxicity between neoplasms and non-malignant cells. Consequently NC-2346, NC-2109 and NC-2352 as well as curcumin and 5-fluorouracil were evaluated against human CRL-1790 colon cells and the data are presented in **Table 2**. All of the compounds had lower cytotoxicity (higher GI_{50} values), in CRL-1790 colon cells compared to HCT-116 cells, and had much greater selectivity than 5-fluorouracil. This finding suggests that these agents may have fewer cytoxic side effects than the standard colon cancer drug 5-fluorouracil.

A recent review has outlined several studies which revealed that curcumin was more toxic to neoplasms than non-malignant cell lines (Lopez, 2008). For example, the EC_{50} value of curcumin towards B-CLL chronic lymphocytic leukemic cells is 5.5 μM but 21.8 μM towards human mononuclear cells, creating a SI value of 3.96 (Everett et al., 2007). The GI_{50} value of curcumin in HCT-116 colon cancer cells in my study gave 3.5 μM. On the other hand, the GI_{50} value in CRL-1790

normal colon epithelial cells was found to be 58.64 μM, thus generating an SI value of 16.75.

There were substantial differences in the toxicity of these compounds in CRL-1790 cells. The differences in the toxicity between NC-2346, NC-2109 and NC-2352 may be explained by similar arguments used in interpreting the GI_{50} results against HCT-116 cells, namely the accessibility of the hydroxy groups and olefinic carbon atoms to interact with cellular constituents as well as the atomic charges on the olefinic carbon atoms. Thus steric crowding of the phenolic hydroxyl groups in NC-2352 may contribute to its low toxicity. While NC-2109 has aryl hydroxyl groups available for reacting with cellular substituents, under acidic conditions it has no dimethylmethylene ammonium group (-$CH_2N^+H(CH_3)_2$) to increase electrophilic attack of thiols and hence it is less toxic than NC-2346.

The excellent tolerability of NC-2352 is noteworthy. Hence in developing this series of compounds, the placement of atoms and groups having different physicochemical properties in the 3 and 5 positions of the 4-hydroxyphenyl rings should be undertaken in an effort to find compounds with low toxicity to normal cells but improved potency towards human cancer cells. As indicated in **Table 2**, NC-2352 has a very high selectivity index of 59.01. However, its potency against HCT-116 cells was relatively low, which might limit its therapeutic usefulness.

The current cytotoxicity studies carried out in the colon cancer cells (HCT-116 cells) and colon epithelial normal cells (CRL-1790) show that NC-2109, NC-2352 and curcumin, and to a moderate extent NC2346 are selective towards cancer cells over the normal colon epithelial cells. This is an important finding as it implies that normal cells will be less affected by the molecules, yet they would be potent towards the cancer cells. Based on the results obtained thus far, the next stage would be to evaluate both the natural molecule, curcumin and its synthetic analogues, NC-2346 and NC-2109 using *in vivo* studies.

6.2 Mechanisms of action

The mechanisms of cytotoxicity were explored using isolated rat liver mitochondria, in order to determine the capabilities to influence mitochondrial respiration and cause mitochondrial swelling (MPT), in HCT-116 cells.

6.2.1 Effects on rat liver mitochondria

6.2.1.1 Oxygen consumption

The oxygen consumption experiments were primarily carried out to evaluate if curcumin and its analogs are able to serve as electron transport chain inhibitors. Such inhibition could induce cell death through energetic catastrophe (eg. Zhang et al., 2010) or increased ROS generation, or inhibit proliferation through the prevention of necessary oxidative functions (such as in pyrimidine biosynthesis (eg. Desler et al, 2010)). Both curcumin and curcumin analogues were unable to inhibit mitochondrial respiration which suggests that cytotoxicity was not mediated through inhibition of the mitochondrial respiratory chain.

However, the standard mild uncoupling agent dinitrophenol, NC-2109 and 5-fluorouracil were able to behave as uncouplers at concentrations as low as 0.1 μM. The increase in respiration occurred almost immediately after the addition to the mitochondrial suspension, which was before significant mitochondrial swelling occurs (which could produce uncoupling and stimulate respiration). This observation suggests chemical uncoupling as a protonophore has occurred. The phenolic hydroxy group is a weakly acidic group which may have been involved in the overall uncoupling phenomena.

A few previous studies have investigated the effects of curcumin on mitochondrial respiration. In the observations provided by Tapia et al., (2012) curcumin was unable to induce significant changes with respect to mitochondrial respiration in renal mitochondria. Ligaret et al. (2004a) studied two fluoro substituted based curcumin derivatives. These compounds were able to depolarize the mitochondrial

membrane potential, brought about intensification of mitochondrial respiration, and decreased ROS formation upon treatment with a recoupling agent, 6-ketocholestanol, but not the MPT pore inhibitor cyclosporine A, that these effects were reversed. This result strongly implies that these compounds may serve as uncoupling agents. Moreover, they were able to induce MPT opening by promoting the oxidation of thiol groups and the release of cytochrome c, making these two molecules candidates for the induction of apoptosis (Ligaret, et al., 2004a).

6.2.1.2 Mitochondrial swelling

It was important to understand whether curcumin and the curcumin analogs are able to bring about MPT pore formation (mitochondrial swelling). Based on the mitochondrial swelling results in the rat liver mitochondria, all the compounds (except 5-FU) both natural as well as synthetic showed the potential at low concentrations to induce mitochondrial swelling.

Swelling is an expansion of the inner mitochondrial membrane which involves opening of the MPT. Thus curcumin and curcumin analogues were able to successfully cause important changes with respect to mitochondrial function and, chiefly to stimulate the opening of the MPT in rat liver mitochondria. NC-2109 and NC-2346 were able to cause swelling in rat liver mitochondria at a concentration as low as 0.1 μM. NC-2352 did not cause swelling at this particular concentration. On the other hand, curcumin was able to cause observable swelling at 0.1 μM, but a higher concentration (i.e., 2.5 μM) was needed to produce effects of similar magnitude to NC-2346 and NC-2109. NC-2346, NC-2109, and NC-2352 showed GI_{50} values of 0.90 μM, 0.87 μM and 9.07 μM respectively in HCT-116 colon cancer cells. With respect to their potencies in rat liver mitochondria, NC-2346, NC-2109, NC-2352 were found to be potent at 0.1 μM, 0.1 μM and 10 μM concentrations respectively. This observation shows that the relative potencies of these compounds in the two models are very similar.

The effect of curcumin on mitochondrial swelling is a paradoxical outcome for a natural molecule which is also known as an antioxidant and chemopreventive agent in diverse models (Hagar et al., 1997, Shoskes, 1998). The antioxidant activities of curcumin are generally seen in the presence of some other oxidative stress. Morin et al., have shown that curcumin induces MPT along with causing oxidation of protein thiols (Morin et al., 2001). Antioxidant activities also could be due to induction of antioxidant enzymes via Keap1 thiol oxidation/alkylation resulting in Nrf2 activation and induction of antioxidant response element (ARE) enzymes (Albena et al., 2002).

This seems to be in agreement with the studies carried out by Jaruga and coworkers (Jaruga et al., 1998b). In their study, they have shown that curcumin does not cause glutathione loss in dexamethasone-treated thymocytes. This observation is probably because curcumin increased glutathione biosynthesis through Nrf2 activation (which induced glutathione synthase). So this increase could most likely be in response to an electrophilic/prooxidant effect of curcumin.

One mechanism that may explain such paradoxical effects is a direct reaction of curcumin with protein thiols. Thiols are present on mitochondrial membrane proteins which plausibly control the MPT opening (Kowaltowski and Vercesi, 1999). This mechanism is supported by observations that the hydrophobic thiol-alkylating reagent N-ethylmaleimide prohibited the opening of the MPT pore by the oxidant tert-butylhydroperoxide or the thiol cross-linking agent 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (Kowaltowski et al., 1997). On the other hand, hydrophilic reagents, such as monobromobimane and mersalyl, showed negligible effects. These results demonstrate that thiol groups must be deeply embedded in the membrane or limited to its hydrophobic pocket. One other point that needs to be addressed is how the curcumin molecule and its analogues interact with thiol groups, which may allow cross-linking. Flynn and coworkers (1991) have succinctly explained how curcumin is able to react with thiols giving rise to additive products. Such an interaction might be

possible with two thiols adjacent to each other and may denote a probable structure for the thiol–curcumin interaction.

Protein thiols may have the 'switch on' or 'switch off' effects depending on the cellular calcium levels. The cellular/endogenous calcium is regulated by calcium transport systems, which are highly sensitive to redox environments (Fulvio et al, 2009). Some of the experimental findings show that ROS can stimulate Ca^{2+} increases by its release from internal stores and damage of the Ca^{2+} clearance systems. There is clear correlation of Ca^{2+} increases with diseased states and associated ROS levels (Camello-Almaraz et al., 2006).

It is believed that Ca^{2+} is essential for both curcumin-stimulated oxidation and mitochondrial swelling thus endorsing the part it plays in MPT induction. The observation that the calcium antagonist ruthenium red inhibits mitochondrial swelling without affecting thiol oxidation (Kowaltowski, et al., 1997) shows that intra-mitochondrial Ca^{2+} is not needed for the stimulation of thiol group oxidation but is required to induce mitochondrial swelling. The oxidation of protein thiols could relate to the binding of Ca^{2+} to key locations that regulate the mitochondrial pore opening (Bernardi et al., 1993). The regulation of thiol groups by Ca^{2+} may stimulate their reactivity with a prooxidant (Kowaltowski, et al., 1997).

Curcumin is able to bring about oxidation of key mitochondrial thiol membrane proteins causing MPT pore formation in the presence of very low levels of Ca^{2+} (Morin et al, 2001). The paradox of curcumin's anti- and pro-apoptotic activities may depend on a balance between its antioxidant defensive effect and its ability to cause MPT pore opening, thus influencing the cell to select between life or death. It is thought that MPT would be influenced by the cellular environment and thus could clarify the contrary effect of curcumin observed with apoptosis (Kuo et al., 1996, Khar et al., 1999, Ozaki et al., 2000, and Jaruga et al.,1998a). A difference in cellular environment may be one of the

mechanisms by which curcumin selectively stimulates cell death in the tumor cells.

6.2.2 Mechanisms in HCT-116 cells

6.2.2.1 Effects on mitochondrial membrane potential

Having established that curcumin and its analogues are able to strongly induce ROS, the next step was to evaluate whether this natural molecule and its synthetic counterpart, NC-2109 were able to cause depolarization of the mitochondrial membrane. This significant phenomenon in mitochondrion occurs as a result of ROS production and oxidation (or perhaps alkylation) of critical thiols which open the MPT pore (Kowaltowski and Vercesi, 1999). Quite a few publications point to curcumin's role in depolarizing the mitochondrial membrane potential in cancer cells such as in HepG2, and acute myeloid leukemic (AML) cells lines such as *Kasumi-1*, *KG-1a,* and *U937* (Cao et al. 2006, Rao et al., 2011). The investigations in AK-5 histiocytoma cells carried out by Bhaumik and coworkers (1999) confirm that mitochondrial membrane potential collapse and apoptosis induced by curcumin was preceded by the generation of ROS.

In the current studies curcumin was able to lower the mitochondrial membrane potential in HCT-116 cells, consistent with published results (Rao et al., 2011, Cai et al., 2012). NC-2109, a synthetic analog was also able to moderately depolarize the membrane potential. Based on these results we can infer that both curcumin and NC-2109 at their GI_{50} concentrations can bring about mitochondrial membrane depolarization. Furthermore, due to the presence of strong electrophilic centers in NC-2109 it is able to selectively target protein thiols in cancer cells. Cancer cells also have a higher mitochondrial membrane potential over normal cells. Lowering or a collapse of this potential would typically result in the MPT formation eventually leading to cell death.

6.2.2.2 ROS Induction by curcumin and curcumin analogues

For the hypothesis regarding ROS-induced ROS release (RIRR), the next step was to ascertain whether curcumin and analogs were able to serve as ROS inducers. Therefore, a time course of ROS was carried out at different time points in HCT- 116 colon cancer cells. We proposed that these compounds act by inducing ROS which provokes an ephemeral burst of mitochondrial ROS generation. A result of such ROS-induced ROS release is the opening of the mitochondrial permeability transition pore (MPT), which plays an important part in bringing about apoptosis (Liu et al, 2008, D'Autreaux, and Toledano, 2007). As hypothesized through our model, these curcumin and curcumin analogues have been designed to either act by direct oxidation/alkylation of protein thiols and/or indirectly by ROS induction.

Based on time course experiments, NC-2109, curcumin, and 5-fluorouracil followed a characteristic biphasic profile. They brought about an early onset of cellular ROS release which triggered a second surge in ROS consistent with RIRR. Thus, the pattern that these compounds follow is very similar to the positive control (H_2O_2). A spike in cellular ROS would therefore result in an increase in mitochondrial superoxide levels. Such an observation has also been reported by Xiaoxiao and coworkers, who have shown that methyl 3-(4-nitrophenyl)propionate belongs to a new class of P450-mediated ROS inducers that is able to selectively target a tumor cell and bring about apoptosis (Xiaoxiao et al., 2013). These curcumin derivatives which are α,β-unsaturated keto compounds have electrophilic centers susceptible for a nucleophile attack by a GSH peptide or glutathione-S-transferase (GST) enzyme (Shiraki et al., 2005). One mechanism by which these compounds may act therefore is via the depletion of GSH resulting in an increase in the cellular ROS levels. Once the endogenous levels of ROS increase, it would prime a cell to produce more ROS in the cell.

All of the compounds brought about ROS formation, with NC-2109 and 5-FU giving the largest initial and secondary increase. The initial ROS generation is an important step for the formation of MPT. As

indicated by our swelling results, curcumin and the curcumin analogues were able to cause mitochondrial swelling in rat liver hepatocytes. It is suggested that initial thiol oxidation due to an early ROS onset or a direct alkylation of the protein thiols by these curcumin analogues would have a 'domino effect' or a sequential steps leading to or resulting in the formation of MPT. A subsequent release of cytochrome *c* due to pore formation will bring about a further increase in ROS production.

5-FU was capable of bringing about intracellular ROS generation and this feature has not been previously reported. However, 5-FU did not cause mitochondrial swelling at submicromolar concentrations in rat liver mitochondria. 5-FU has a relatively high GI_{50} value when compared to the submicromolar GI_{50} values of NC-2346 and NC-2109 in HCT-116 colon cancer cell cells. Therefore it is possible that 5-FU could not cause mitochondrial swelling at a concentration of 0.1 μM, but at its GI_{50} concentration of 5.4 μM it was able to induce ROS release.

A few previous studies have suggested that 5-FU acts through mechanisms other than the well documented inhibition of thymidylate synthase. (Choi et al., 2005; Borralho et al., 2007, Aresvik et al., 2010, ; Arisan et al., 2011, Xavier et al., 2011). A study by Lamberti et al. (2012) showed that 5-FU may be indirectly responsible in 'turning on'the mitochondria-mediated apoptosis program through bringing about an increase in the intracellular ROS. In another study, Stevenson and coworkers (Stevenson et al., 2011) have shown that following the treatment of colorectal cancer cells with 5-FU in the presence of a calcium binding protein (CALB2) the mitochondria-induced apoptosis pathway is activated.

All of the curcumin analogues tested, NC-2109, NC-2346, NC-2352, curcumin and 5-FU showed a significant increase in mitochondrial superoxide levels at early time points in HCT-116 cells. These experimental results substantiate our hypothesis of RIRR. An early onset of ROS is seen in all of the curcumin and curcumin analogues (except NC-2346). Such a priming due to ROS inception could

necessarily trigger the MPT formation and cytochrome c release that subsequently brought about another burst of ROS (as shown by the DCF-DA results showing a biphasic pattern of ROS release).

Previous research on curcumin has also shown that it can act as a prooxidant (Sharma et al, 2005, Bouayed and Bohn, 2010). It was observed that curcumin in a time - and dose - dependent manner was able to bring about ROS formation and DNA damage in HepG2 hepatoma cells (Mersch- Sundermann et al., 2004). This damage to the DNA was not restricted to nuclear DNA but occurred to the mitochondrial DNA too. This is not unusual as mitochondria are a main site for ROS generation (Jastroch, et al., 2010), and mitochondrial DNA is not protected with histones; hence it is highly susceptible to oxidative damage (Rothfuss et al., 2010).

Kunwar et al. (2009) in a similar kind of study showed increased levels of ROS at 2 h and 18 h after the addition of 1-25 μM of curcumin to mouse spleen lymphocytes and EL4 lymphoma cell lines. It was however observed, that there was a decrease in ROS levels after an initial increase at 2 h. These changes were also reflected in the levels of non-protein thiols which dwindled at the 2 h time point after the addition of curcumin, while at 18 h the levels of non-protein thiols increased significantly with increasing concentration (1-25 μM) of curcumin (Kunwar et al., 2009). Consistent with these observations, in my experiments using colon carcinoma cells (HCT-116) I found that curcumin and the curcumin analog NC-2109 were able to initially increase ROS levels, but the levels declined by 8 h to those of the control or lower.

There have been other studies that point to a similar prooxidative role of curcumin while taking into consideration its capacity to promote oxidative stress in transformed cells under *in vitro* conditions. The effects positively correlate with the acceleration of ROS generation, altering the redox balance/homeostasis such as depleting the GSH levels and depolarizing the mitochondrial membrane potential (Sandur et al., 2007a, Sandur et al., 2007b, Syng-Ai, et al., 2004, Fang et al., 2005). The

mechanism by which curcumin regulates its prooxidant effects is not yet fully understood. The final possibility of curcumin's role as a prooxidant could be through a GSH depletion mechanism. In this mechanism it may form a Michael adduct through the electrophilic carbon and a nucleophile such as a cysteine residue on key mitochondrial proteins or GSH peptide (Das et al., 2007).

Other research on the mechanism of oxidative stress points to curcumin binding to mitochondrial thioredoxin reductase in an irreversible fashion and inhibiting its activity by alkylating the cysteine residue present in the catalytic pocket of the enzyme's NADPH oxidase site (Singh & Misra, 2009) This alkylation step is reportedly due to the α,β-unsaturated carbonyl group of curcumin, and results in ROS generation (Anand et al., 2008).

Another reason for the role of curcumin as a pro-oxidant is understood to be due to the formation of phenoxyl radicals of curcumin by the heme peroxidase-H_2O_2 system (Galati et al, 2002). These phenoxyl radicals could possibly be reduced by endogenous GSH or NADH which results in GS radical formation and may allow the GS or GSSG radical to reduce O_2 to O_2^-.and in turn cause elevations of ROS.

Kunwar et al. (2009) point out that so far research suggests that curcumin has the capacity to switch from an antioxidant to a prooxidant which is conditional on the cell type, redox atmosphere and its dosage. In addition, curcumin often acts as an antioxidant in normal cells while exhibits prooxidant characteristics in tumor cells. It is these different features of the natural molecule that make it a useful antitumor agent. Based on the above features, the synthetic analogues of curcumin (excepting NC-2346) may follow a pattern similar to that of the curcumin molecule.

As already pointed out in the literature review section, curcumin may also serve as a free radical scavenger due to its phenolic functional groups (Patro et al., 2002, Wei et al., 2006). Based on the biochemical calculation conducted by Wright (2002), the most important region to serve as a free

radical scavenger is the OH group.

Taking together, the curcumin molecule can serve both as a prooxidant and as an antioxidant. At the concentrations used in my *in vitro* experiments, as determined by ROS assays, this molecule was adopting the role more of a prooxidant than an antioxidant. Our results strongly point out that the synthetic analogs of curcumin (NC-2346, NC-2109 and NC-2352) all serve as strong prooxidants and are able to increase the cellular ROS levels which can be a good method to target cancer cells selectively.

6.2.2.3 GSSG/GSH levels in HCT-116 cells treated with curcumin and curcumin analogues

One therapeutic approach and possible mechanism for the action of curcumin analogues has focussed on depleting cellular GSH to remove its cytoprotective effects. This approach could reduce the established resistance of tumors to the cytotoxicity effects of chemotherapeutic agents. The literature strongly points to cancer cells having a very strong antioxidant defense system (Calvert et al., 1998, Estrela, et al., 2006). Different experimental trials involving the lowering of the concentrations of cellular thiols have revealed a problem of achieving greater toxicity to neoplasms than non-malignant cells. A subsequent administration of an anticancer drug may result in causing greater harm to tumors than normal cells (Wu and Batist, 2013).

In the current experiments in HCT-116 colon cancer cells, curcumin produced a decrease in GSH, measured after 48 h. The other curcumin analogs, NC-2109 and NC-2346 also decreased the GSH levels with concomitant increases in the cellular ROS as indicated by their GSSG/GSH ratios.

Syng-Ai et al. (2004) have previously reported the depletion of GSH by treatment with curcumin in tumor cells lines (MCF-7, MDAM, HepG2) and normal cells (rat hepatocytes). In addition, it was also seen that GSH depletion with l-buthionine-(S, R)-sulfoximine (BSO), a strong inhibitor of γ-glutamyl cysteine synthetase in the GSH synthesis pathway, was able to sensitize the cells to the effects of

curcumin and this was possibly due to the prooxidant effects of curcumin. It was however, observed in HepG2 cells that the content of GSH only lowered by 21%. This may be due to presence of high levels of endogenous GSH in HepG2 cells that may be responsible for the chemoresistance in these cells (Syng-Ai et al., 2004).

GSH depletion has been shown to be effective in both *in vivo* and *in vitro* experiments, and causes chemosensitization in different tumor cell types to various chemotherapeutic approaches and anti-hormonal therapies (Dorai and Aggarwal. 2004). In addition curcumin's effects on GSH depletion were observed in on both normal and tumor cells (Sandor et al., 1995, Lewis-Wambi et al., 2009). However, curcumin's experimental use was limited due to its very short half-life with the consequential necessity for prolonged periods of infusions (Caroll et al., 2011). Although, every effort was made to synthesize analogs which were tumor selective, they met with little success (Sun et al., 2009). This is an important observation as curcumin is more selective towards cancer cells as opposed to normal cells (results herein, and Syng-Ai et al., 2004). The same premise could therefore, apply on its synthetic counterpart, NC-2109, which showed greater potency and selectivity than curcumin.

Another important point that needs to be considered is that a cancer treated with these test agents could be initially chemosensitized. This phenomena is due to the presence of the 1,5-diaryl-3-oxo-1,4-pentadienyl group in the dienones. Thus an initial thiol alkylation at one of the olefinic carbon atoms would lower the concentrations of cellular thiols. Hence the cells are now chemosensitized to a further chemical insult caused by thiol alkylation at the remaining olefinic carbon atom.

6.3 Combination approach in cancer therapy

In order to study my hypothesis that curcumin and curcumin analogues are able to enhance/potentiate mitochondria-induced apoptosis through interactions with ANT, a combination study was carried out with agents affecting the ANT proteins. The improvement of combination approach based therapies for cancers has an extensive and renowned past (Frie, 1972, DeVita and Schein, 1973, DeVita et al., 1975). For example, the simultaneous use of cytotoxic agents each with therapeutic potential as a single agent for a specific type of cancer, which have dissimilar recognized mechanisms of action and that do not overlap in terms of toxicities, proved extremely significant for the long-term control of leukemia. However, a problem of selecting cytotoxic agents is their tendency to result in one or more forms of cell death in proliferating cells. Thus the progress of molecularly targeted anticancer agents as therapeutic agents promises extensively greater biochemical selectivity in tumour killing versus toxicity to normal host tissues and has, for the most part, supplanted the development of relatively nonspecific cytotoxic agents (Murgo et al., 2008). There are continuing challenges for the improvement of combination-targeted therapeutics for cancer. The main challenge is to evaluate the connection of targeted agent development with the thorough understanding of the biology of the target molecule, both in terms of the applicable disease and in the extensive array of their prospective drug interactions with different downstream effector molecules (Hait and Hambley, 2009). The extent of our understanding of the mechanism(s) of action of new molecularly targeted anticancer agents may be insufficient, even at the time of regulatory approval. Thus, the mitochondrial protein ANT was chosen in order to carry out combination studies mainly to see if both the natural molecule and the synthetic molecule are able to show improved activities in terms of mitochondrial membrane potential collapse and a decrease in cell growth.

6.3.1 Combination approach using curcumin and NC-2109 in the presence of ANT2siRNA knockdown

ANT2 levels are high in cancer states, which may decrease the sensitivity to apoptosis. Therefore, ANT2 is a potential mitochondrial target for anticancer therapy (Chevrollier et al., 2011, Sharaf et al., 2011). In this research I used an ANT2siRNA knockdown approach in order to see if MPT pore formation (involving other ANT isoforms) is augmented and increases apoptosis in the presence of curcumin and curcumin analogs.

I thus conducted a pilot experiment transfecting HCT-116 cells with ANT2siRNA and observed the effect on mitochondrial membrane potential in the absence and presence of curcumin and NC-2109 (results shown in **Appendix 1**). In this experiment, ANT2siRNA alone showed no effect on the mitochondrial membrane potential. It was observed that even in the absence of ANT2siRNA, curcumin, NC-2109 and 5-fluorouracil treatments for 48 h were able to decrease the mitochondrial membrane potential by >50%, and no additional benefit of ANT2siRNA was evident. Because of this (and the expense involved) the experiments with ANT2siRNA were not pursued further. However subsequent analysis of the cell numbers in the bright field images (only one per condition) from this experiment suggests that ANT2siRNA may have substantially increased the growth inhibition provided by curcumin, NC-2109 and 5-fluorouracil (**Appendix 1**).

Previous studies by Jang et al. (2008, 2011) have examined the anti-cancer effects of ANT2siRNA or ANT2shRNA in breast cancer cells. ANT2siRNA in one of the studies in the breast cancer cell line MDA-MB-231 suppressed cell growth and cell proliferation, through cell cycle arrest, ATP depletion and apoptosis that included marked disruption of the mitochondrial membrane potential (Jang et al., 2008). In another study with breast cancer stem-like cells, ANT2shRNA additionally increased the chemosensitivity to doxorubicin (Jang et al., 2011).

6.3.2 Combination approach using curcumin and NC-2109 in the presence of carboxyatractyloside

The main idea of using a combination approach in my research was to see if there is synergism by which the programmed cell death is accelerated in colon cancer cells. In this approach the presence of the ANT inhibitor, carboxyatractyloside (CAT), which stabilizes the 'c' (cytoplasmic-side open) conformation, was investigated for its ability to potentiate cell death in HCT-116 colon epithelial cancer cells treated with curcumin or NC-2109.

Initial experiments on mitochondrial membrane potential using a microscope (the plate reader wasn't working) suggested that CAT increased the effect of curcumin at the 8 h timepoint. The brightfield images also suggested a further decrease in cell numbers by curcumin in the presence of CAT.

Subsequent experiments where the mitochondrial membrane potential was measured using the microplate reader showed that the presence of CAT significantly increased the ability of curcumin to bring about mitochondrial membrane depolarization at both 8 and 16 h of treatment. There was a suggestion that CAT potentiated the effect of NC-2109 at 8 h, but it was not found to be significant.

Considering the results of mitochondrial membrane potential measurements and the bright field images, sulforhodamine B cell viability studies in the presence and absence of CAT were conducted. These experiments showed that CAT significantly increased the ability of curcumin and NC-2109 to inhibit cell growth. Thus, in the combination approach using a thiol alkylator such as NC-2109 and curcumin, the presence of CAT was able to show synergism. Through this combination approach it is clear that ANT has an important role in the mitochondria-mediated cell death caused by these compounds. The results also suggest that the utility of these compounds in cancer treatment could be facilitated by combination therapy with CAT.

7.0 CONCLUSIONS

Curcumin and its synthetic analogues, NC-2346, NC-2109 and NC-2352 possessing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore inhibited the proliferation of the colon cancer HCT-116 cells. However they exercised less toxicity towards normal colon epithelial cells (CRL1790). The GI_{50} values for these agents ranged from 0.9-9.1 μM and 4.2-535 μM for cancer and normal colon cells, respectively. These agents showed better potency and selectivity than 5-fluorouracil, and therefore may have improved efficacy and decreased cytotoxic side effects in the treatment of colon cancers.

The actions of curcumin and the analogs tested are possibly by direct and indirect mechanisms. The direct mechanism includes oxidation of key mitochondrial proteins such as ANT, resulting in MPT pore opening and apoptosis. The results of potentiation by the ANT inhibitor CAT support this mechanism. The indirect mechanism involves depletion of reduced GSH levels, which the results also supported. This brings an overall increase in the cellular ROS levels which triggers the phenomena called ROS-induced ROS release leading to the formation of the MPT pore and resulting in apoptosis.

The observed increase in ROS levels caused by these compounds were consistent with ROS-induced ROS release, and subsequent depolarization of the membrane potential across the mitochondrial inner membrane. These mechanisms were supported experimentally and this mechanism seems to be the most likely pathway for the increased ROS generation. These results were substantiated with the swelling results indicative of the formation of MPT pore.

Another way by which these test agents could act, and was tested for, is by blocking the electron transport chain (inhibition of oxygen consumption) leading to a decrease in ATP and an increase in mitochondrial ROS that eventually results in apoptosis. Our results however, did not show any

inhibition of respiration in isolated rat liver mitochondria.

Based on the combination studies, there was a clear indication that the presence of ANT inhibitor CAT was able to potentiate mitochondria-directed cell death based on the mitochondrial membrane potential results and also the cell viability tests. This result supports an involvement of ANT in the mechanism of cytotoxicity to colon cancer cells.The other alternate combination approach using a ANT2 directed siRNA suggested that curcumin in the presence of ANT2 siRNA was able to increase cytotoxicity, but these studies have to be validated further using different incubation times.

8.0 FUTURE DIRECTIONS

The first step in expanding the project is to design and then synthesize further analogs of NC-2346, NC-2109 and NC-2352. Some suggestions have been made previously which are recorded in this thesis. In the case of NC-2346 and NC-2109, the formation of the corresponding quarternary ammonium compounds should be undertaken since charged molecular species are mitochondriotropic. In addition, the rate and extent of ractions of quarternary ammonium salts with tumor mitochondria may well be greater than with mitochondria of nonmalignant cells due to the higher mitochondrial membrane potential in tumor mitochondria. The nitrogen atoms in the piperidine ring and for the dimethylaminomethyl groups can be quarternized. In addition, structure-activity relationships should be developed such as exploring the size and nature of the dialkylaminomethyl groups which may affect the alignment at binding sites. Furthermore, the formation of analogs of NC-2346 and NC-2109 in which the two bis(arylidene) groups are mounted on cycloalkane scaffolds should be attempted in order to evaluate the importance of the piperidine ring to cytotoxic properties and selective toxicity. These new compounds should be subjected to the same biochemical evaluations as reported herein. The principal aim is to find correlations between structures and the results generated in the bioassays namely the effects of compounds on HCT-116 and CRL-1790 cells, mitochondrial respiration, the MMP, extent of ROS formation and lowering of GSH concentrations in neoplastic cells. When such correlations are found, then predictions can be made as to which further analogs should be prepared with a view to increasing potencies and selectivity indices.

A second cluster of experiments to be implemented is the evaluation of NC-2346, NC-2109, NC-2352 and the new analogs against a wide range of neoplastic and non-malignant cells. The reasons for these studies are as follows:

(1) The aim is to identify those groups of tumors which are particularly sensitive to these compounds. Furthermore if selectivity is observed, e.g., greater toxicity to colon and breast cancers than to renal and

leukemic tumors, then this selectivity may also indicate a capacity for greater toxicity to neoplasms than normal cells. Furthermore, if a group of neoplasms are particularly sensitive to these dienones, the screening of both a dienone and an established drug for the treatment of these tumors should be undertaken using the GI_{50} concentrations of the compound and the drug. The purpose of this experiment is to find if synergy was noted and if this is the case, reduced doses can be given of both compounds and toxicity is lowered.

(2) Promising compounds should also be evaluated against various nonmalignant cells, e.g. human Hs27 foreskin cells and murine NIH-3T3 fibroblasts with the aim of identifying compounds with high selectivity indices. Those compounds having favourable properties will be evaluated for adequate membrane permeability using Caco-2 cells which is a useful prerequisite before *in vivo* experimentation is conducted. When these *in vitro* experiments have been completed, various *in silico* determinations should be made such as obtaining the logP values of the compounds. In this way, for example, the lipophilicity of the molecules may be shown to correlate with cytotoxic potencies which in turn can lead to the rational design of further analogs.

(3) In the future the preparation of GSH adducts of one or more of the dienones should be prepared and examined for their cytotoxic properties and stabilities. Is potency of the adduct decreased or increased compared to the dienone? If potency is decreased, then alkylation of cellular thiols is likely to be the mode of action of the unsaturated ketones. On the other hand, if the adduct is more potent than the parent dienone, it is conceivable that the adduct penetrates the cells better than the dienone where it either remains intact or releases GSH and the cytotoxin. In other words, the adduct is a prodrug.

(4) The results strongly point towards mitochondria-mediated cell death. Other key mitochondrial proteins such as ANT, VDAC could play a role in the mitochondrial cell death. One of our approaches was to knockdown an isoform of ANT, which did not give us conclusive results. It would be good to

improve the knockdown experiments with ANT2 by reducing the incubation time in order to have less than maximal effects of the test agent alone.

(5) To better understand and validate the CAT findings, it would be useful to use an ANT ligand such as bongkrekic acid, which maintains ANT in its 'm' rather than 'c' conformation, in the presence of curcumin and curcumin analogues. If these test agents are able to induce MPT in spite of the presence of this inhibitor, it would mean that these test agents act in an ANT-independent fashion.

(6) Potentially clinically effective combinations should be tested in murine xenograft models. This would help in finding out if the curcumin and curcumin analogues when administered together with known anticancer drugs such as 5-FU or cisplatin, act synergistically to increase efficacy. This would also help understand their therapeutic indices and toxicity levels (LD_{50}) when administered singly or when given as a combination therapy *in vivo*. Xenograft experiments could also be useful to confirm the possible utility of CAT to increase the potency of curcumin and curcumin derivatives.

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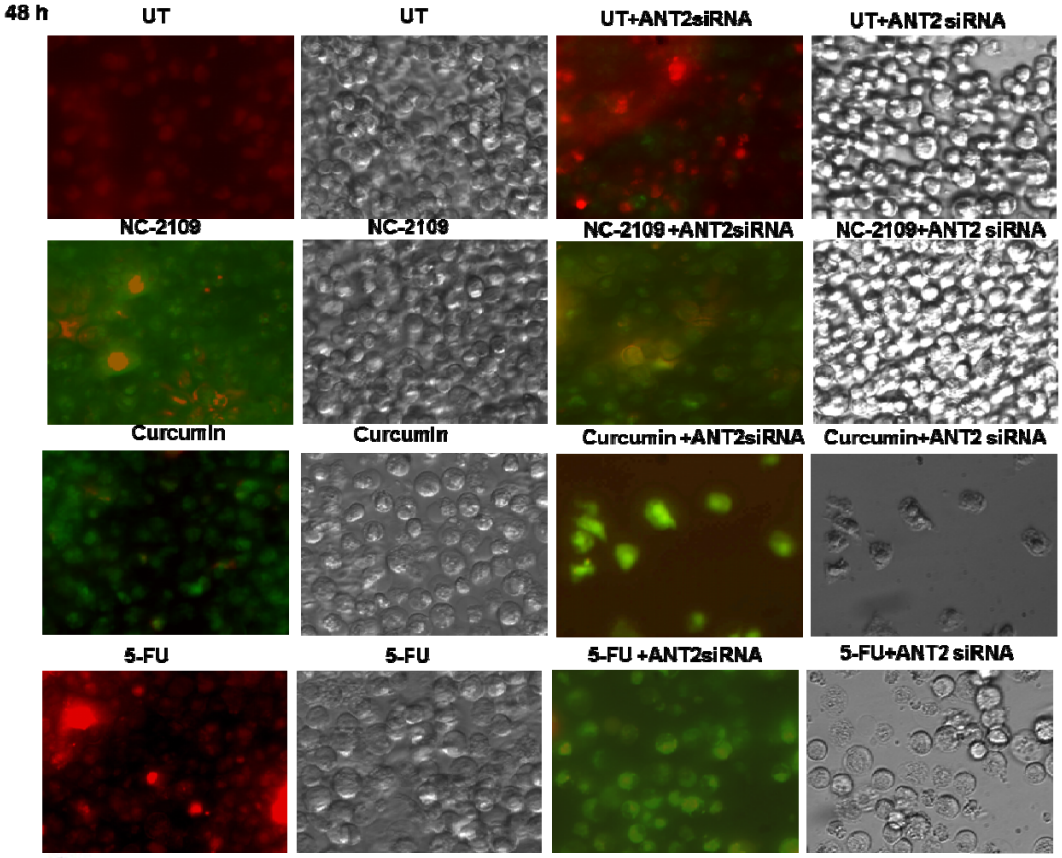
APPENDIX A

COMBINATION APPROACH USING ANT2siRNA

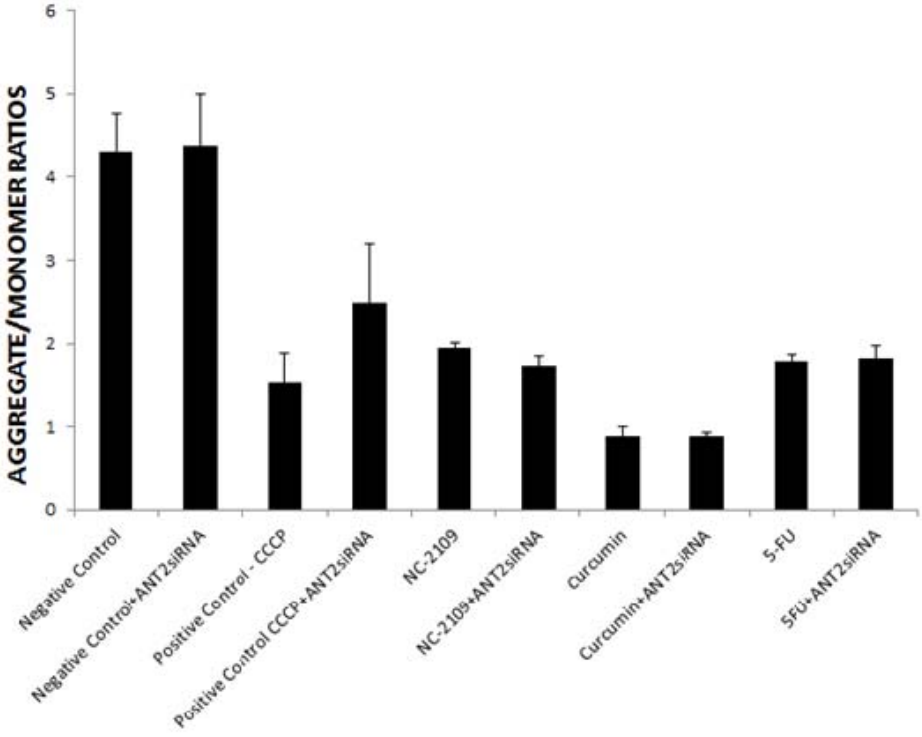
Effects in the presence/absence of ANT2 directed siRNA in HCT-116 cells:

As cancer cells overexpress ANT2, an ANT2 knockdown approach in the presence of curcumin and its synthetic analogues could help in augmenting the process of mitochondria-directed apoptosis. For this reason I did a pilot experiment using ANT2siRNA. **Figure 18 Panel A** shows the fluorescence and bright field images after treatment for 48 h, and **Figure 18 Panels B and C** show quantitation of the effects on the mitochondrial membrane potential and cell growth. The presence of ANT2siRNA alone had no effect on the mitochondrial membrane potential. It is seen that even in the absence of ANT2siRNA, curcumin and NC-2109 treatments for 48 h were able to lower the mitochondrial membrane potential to very low levels, and no additional benefit of ANT2siRNA was evident. With 5-fluorouracil however, it had no effect alone on membrane potential, but the mitochondrial membrane potential was strongly decreased in the combined presence of ANT2siRNA.

A.


48 h
UT
UT
UT+ANT2siRNA
UT+ANT2 siRNA
NC-2109
NC-2109
NC-2109 +ANT2siRNA
NC-2109+ANT2 siRNA
Curcumin
Curcumin
Curcumin +ANT2siRNA
Curcumin+ANT2 siRNA
5-FU
5-FU
5-FU +ANT2siRNA
5-FU+ANT2 siRNA

B.


AGGREGATE/MONOMER RATIOS
0
1
2
3
4
5
6
Negative Control
Negative Control+ANT2siRNA
Positive Control - CCCP
Positive Control CCCP+ANT2siRNA
NC-2109
NC-2109+ANT2siRNA
Curcumin
Curcumin+ANT2siRNA
5-FU
5FU+ANT2siRNA

C.

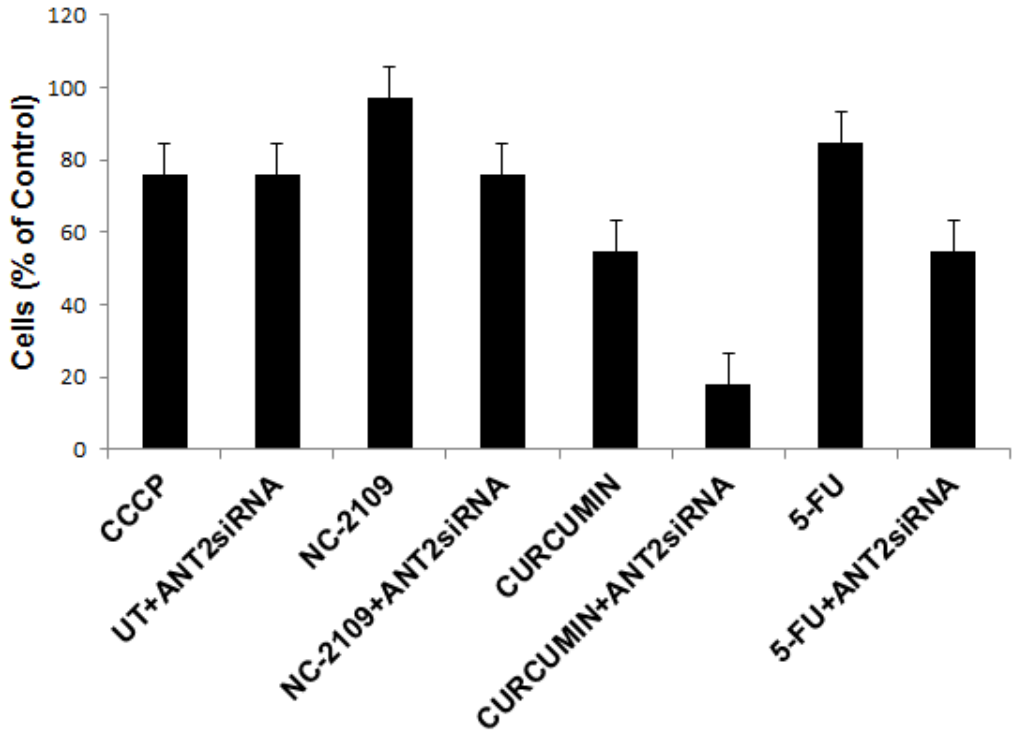


Fig 18: ***Combination approach where the HCT-116 cells were treated with ANT2 directed siRNA in the presence of the test agents incubated for 48 h***. **Panel A.** shows fluorescence and bright field images of UT-Untreated, NC-2109, curcumin and 5-fluorouracil in the presence and absence of ANT2 siRNA. . The fluorescence microscopic images were taken at 400x The image is one representative out of three wells. **Panel B.** shows the quantitated mitochondrial membrane potential in HCT-116 cells treated with ANT2 directed siRNA in the presence of the test agents. For half of the wells the cells were transfected with ANT2siRNA as described in Materials and Methods, prior to treating with the test agents. GI_{50} concentrations of the drugs were used to treat the cells. Following the 48 h incubation period the cells were processed for JC-1 staining. In this Panel, the bars for test agent alone were from the experiment shown in **Figure 7**, normalized to the current negative control. The bars represent means ± SD of the experiments carried out in triplicate. **Panel C.** shows the cell count (as % of Control) following the 48 h incubation period. The cells were counted under a microscope at 100x magnification. The bars represent means ±SD of one well –three different regions were taken.

From the same experiment, the effects on mitochondrial membrane potential were quantified by fluorescence measurements in a plate reader **(Figure 18 Panel B.)**. The results show that the potentials were already decreased by 60-80% by the drug treatments compared to the untreated control ($p < 0.05$), and treatment with ANT2siRNA did not show any effect compared to drug treatment alone. With 5-fluorouracil, the strong additional effect of ANT2siRNA shown in **Figure 18 (Panel A.)** was not seen in the plate reader results **(Panel B.)**, but this might be because the plate reader measurement of membrane potential for 5-FU alone was drawn from a different experiment and normalized to control.

These results in the presence of ANT2siRNA were further clarified in the same experiment, based on the bright field images as shown beside the fluorescence image in **Figure 18 Panel A.** and quantified in **Figure 18 Panel C**. In the case of curcumin, the number of cells in the presence of ANT2siRNA was much lower than with curcumin alone, showing decreased cell growth. The standard anticancer agent, 5-fluorouracil showed a moderate effect on cell growth, which also was significantly improved by the presence of ANT2siRNA. In this experiment the synthetic analogue, NC-2109 did not show any effect in the absence or presence of ANT-2 siRNA.