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The anti-cancer activity of novel derivatives of dihydroartemisinin and tetraoxane dimers

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THE ANTI-CANCER ACTIVITY OF NOVEL DERIVATIVES OF DIHYDROARTEMISININ AND TETRAOXANE DIMERS

By

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ABSTRACT

Background:

Cancer is the leading cause of death in the developed countries and is also believed to be the second leading cause of death in the developing countries. Unfortunately many of the current chemotherapeutic agents are beset with limited efficacy, adverse side effects including unfavourable pharmacokinetics with some treatments known to induce secondary neoplasm and the agents are also expensive, all of which call for the discovery and development of new chemotherapeutic agents with improved profiles over existing ones. To this end some of the current research is focused on harnessing the chemotherapeutic potential of natural products. The antimalarial artemisinins have shown promising in vitro and in vivo activity against various types of cancer cells. In the current study we investigated the cytotoxicity and the underlying mechanism of action of novel derivatives of dihydroartemisinin JC3-39 and LLP271 and some tetraoxane dimers (RKA138, RKA149, RKA150, RKA151, RKA152, RKA155, RKA158 and RKA160) against HT29-AK (colon cancer) and HL60 (leukaemia) cell lines.

Methods used in the study:

The cytotoxicity of the test compounds against the cells were evaluated by MTT assay; ELISA assay was performed to determine the cellular levels of sTfR, survivin, IL-6 and TNF-α upon different drug treatment following the manufacture's

(www.rndsystems.com) protocol. Flow cytometric analysis was used to measure the expression of catalytically active cleaved caspase-3 and P-AKT upon drug treatment. The effect of test agents on DNA fragmentation was investigated using agarose gel electrophoresis and a model of wound healing (i.e. the scratch assay, backed by trypan blue dye exclusion assay) was used to determine the effects of the test agents on cell migration.

Key findings:

All of the agents were cytotoxic against both the cancer cell lines. The novel agents JC3-39 (IC_{50} of 14.20±0.22μM) and LLP271 (IC_{50} of 17.60±0.21μM) were more potent than DHA (IC_{50} of 61.40±0.45μM) against HT29-AK cells. Of the tetraoxane dimmers RKA150 was the most potent as compared to DHA (IC_{50} of 3.75±0.37μM vs. 61.40±0.45μM) against HT29-AK cell line. Unfortunately all the test agents showed less cytotoxicity against HL60 cells as compared to DHA (IC_{50} of 0.39±0.11μM). Artesunate was cytotoxic as well against HT29-AK and HL60 cell lines but the cytotoxicity was less than DHA. There were detectable levels of sTfR, survivin, IL-6 and TNF-α in HT29-AK cells, but the agents had variable effects on the cells. However there was increased activity of cleaved caspase-3 and P-AKT upon drug treatments in HT29-AK cell line and drug treatments also caused DNA fragmentation as evidenced by DNA smearing/laddering. All of the test agents caused a concentration-dependent inhibition of wound healing with a corresponding increase in dead cell number, with artesunate having the greatest effects on the inability of the wound to close.

Conclusion and implications:

The test agents were cytotoxic against both HT29-AK and HL60 cell lines. There is evidence that the agents mediated their cytotoxicity by apoptosis which stems from the observation that they cause an increase in catalytically active cleaved caspase-3 and P-AKT, decreased cellular survivin levels and caused DNA fragmentation. The drugs also showed potent inhibitory activity against the capacity of the wound to heal. Repeat studies are warranted to decipher the other cellular effects of the agents against the cells.

INTRODUCTION

Cancer is a large heterogeneous class of diseases in which a group of cells display unregulated growth ("What Is Cancer?"National Cancer Institute Retrieved 2009-08-17). Three properties of cancer cells are uncontrolled growth, local invasion and the ability to metastasize in regional lymph nodes or distant body sites and these properties differentiate malignant tumours from benign tumours. The tissue of origin gives the distinguishing characteristics of cancers and over 100 types of cancers have been identified including breast, liver etc. (World Health Organization, February 2006, Retrieved 2011-01-05). Cancers derived from epithelial cells (prostate, lung, colon etc.) are called carcinoma, cancers derived from connective tissues (bone, cartilage etc.) are called sarcoma; lymphoma and leukemia are two types of cancers that arise from hematopoietic (blood-forming) cells. Cancers derived from pluripotent cells (refers to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm, mesoderm and ectoderm) are called germ cell tumour.

Colon cancer and blood cancer:

Our present study is focused on two types of cancer cells; HT29-AK (colon cancer) and HL60 (leukaemia) cells. Colon cancer is the third most commonly diagnosed cancer in the world (Jemal *et al.*, 2007). It starts in the lining of the bowel frequently as a result of mutations (L Ricci-Vitiani *et al.*, 2006). The most commonly mutated gene in all colorectal cancer is the Adenomatous Polyposis Coli/ APC gene which produce the APC protein (M Tsujii *et al.*, 1997). APC is classified as tumour suppressor gene (CA O'Brien *et al.*, 2006). The APC protein acts as a brake on the accumulation of β-catenin protein. In the absence of APC, β-catenin accumulates to high levels and translocates into the nucleus. There it binds to DNA, and activates the transcription of genes that are normally important for stem cell renewal and differentiation (H Tazawa *et al.*, 2007). The inappropriate expression of APC to high levels is a predisposing factor to cancer (H Tazawa *et al.*, 2007). Leukaemia is a type of cancer of the blood or bone marrow which is characterized by an abnormal increase of immature white blood cells called "blasts" (RH Grimm *et al.*, 1995). Leukaemia is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases affecting the blood, bone marrow, and lymphoid system which are all known as hematological neoplasms (S Bonassi *et al.*, 2006).

Figure 1: The lining of bowel with a malignant tumour and a cancerous blood cell respectively.

Cancer epidemiology:

Cancer is the leading cause of death in the developed countries and is also believed to be the second leading cause of death in the developing countries (Ferlay *et al.,* 2010). It accounts for approximately 13% of all deaths each year worldwide with the most common being lung cancer (1.3 million deaths), stomach cancer (803000 deaths), colorectal cancer (639000 deaths), liver cancer (610000 deaths) and breast cancer (519000 deaths). In 2008 approximately 12.7 million cancers were diagnosed and 7.6 million people died of cancer worldwide. Deaths from cancer worldwide are projected to continue to rise to over 11 million by 2030 (Globocon 2010, I.A.R.C, 2010). In 2000, approximately 256,000 children and adults around the world developed some form of leukemia, and 209,000 died from it. About 90% of all leukemia is diagnosed in adults.

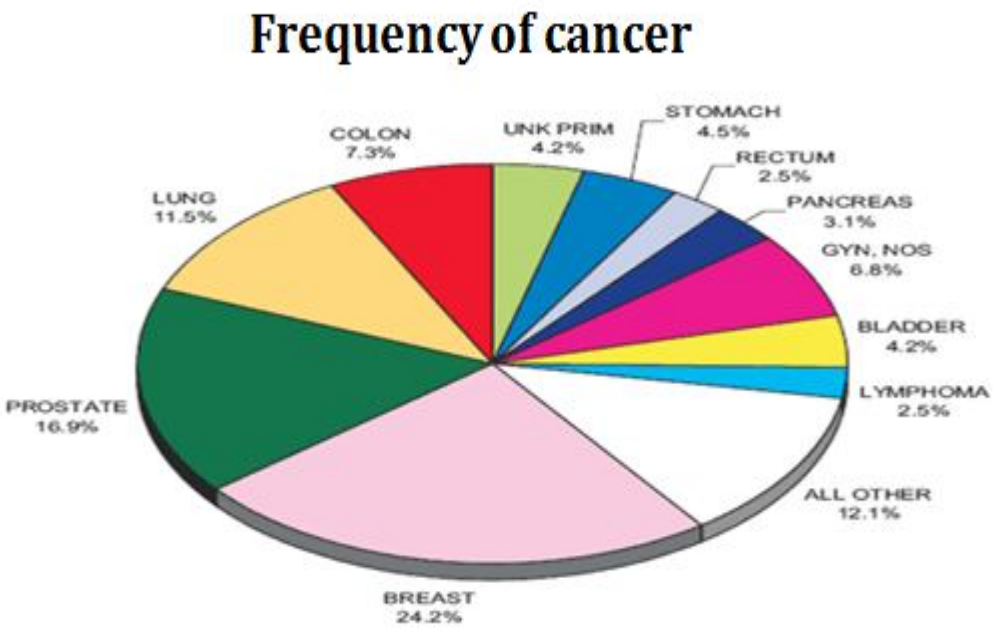


Figure 2: The 2010 distribution of primary diagnosis of various types of cancer.

Cancer rates in middle-aged men and women in Great Britain have gone up by nearly 20% in a generation and this represents an increase of 17,000 cases a year. Cases of cancer in men have risen from almost 20,000 in 1979 to almost 24,000 in 2008, while in women cases have increased from more than 24,000 to more than 36,500. Unfortunately, current chemotherapeutic agents have adverse side effects including unfavourable pharmacokinetics (Efferth, 2006; Brown *et al*., 2007; Shahrokni *et al*., 2009; Raschi *et al*., 2010; Cheung *et al*., 2010; Lee *et al*., 2010; Liu *et al*., 2010) and are expensive with some treatments known to induce secondary neoplasm (Pawelec *et al*., 2010). Therefore, there is an urgent demand for developing novel anti-cancer agents with improved activity that circumvents the limitations of some of the current agents. In order to identify chemotherapeutic agents with improved profiles over existing ones, some of the current research is focused on harnessing the chemotherapeutic potential of natural products.

Natural products in cancer chemotherapy:

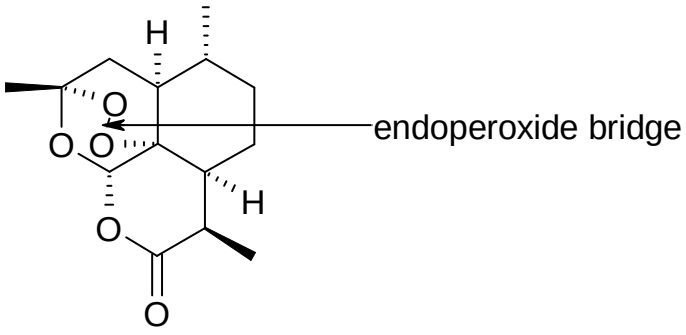
There is a large body of evidence on the potential utility of natural products as chemotherapeutic agents (Efferth, 2005; Cheung *et al*., 2010; Lee *et al*., 2010; Hussain *et al.,* 2011). Especially the traditional Chinese medicines has benefitted one fifth of the world's population in treating many diseases and its potentiality as a real healing option by the west is increasingly being recognized. Furthermore the rationale for combining conventional agents with modern biotechnological approaches to the delivery of traditional Chinese medicine is an avenue set to revolutionize the future treatment of cancer patients. For example, *Scutellaria baicalensis* commonly referred as '*Golden root'*, comprising of 12 herbs in various proportions have shown promising activities against

various cancer cell lines, such as PC-3 and DU145 (prostate cancer cell line), HL60 (leukaemia), KML562 (chronic myeloid leukaemia cell line), HeLa (cervical cencer cell line) and HO-8910 (ovarian cancer cell line) cell lines. All these herbs contain flavonoid-rich elements which are thought to impart anti-inflammatory, antibacterial, anti-neoplastic, pro-apoptotic, anti-proliferative and anti-angiogenic effects (Igney and Krammer, 2002; Po *et al.,* 2002;Nelson and Montgomery, 2003; Powell *et al.,* 2003; Parekh *et al.,* 2009; Gravett *et al.,* 2011; Soomro *et al.,* 2011).

Artemisinins as anti malarial and their mode of action:

Another such example is the Chinese medicinal herb qing-hao (*Artemisia annua L* or commonly referred to as sweet worm wood). It has been used in China for centuries for afflictions such as fevers, hemorrhoids and malaria (Parekh *et al*., 2009). In 1972, artemisinin was discovered to be the active ingredient responsible for the anti-malarial action of qing-hao (Singh and Lai, 2001). Artemisinin (Figure 3) is a sesquiterpene lactone peroxide containing an endoperoxide moiety or 1, 2, 4 trioxane (-C-O-O-C-) (Efferth *et al.,* 2004; Mercer *et al.,* 2007; Nakase *et al., 2008*; O'Neill, Barton and Ward,2010; Alessandro *et al.,* 2011). Several semi-synthetic derivatives of artemisinin have been produced including dihydroartemisnin (DHA), artesunate, artemether etc (Efferth *et al.,* 2001; Efferth *et al.,* 2002; Chen *et al.,* 2004; Parekh *et al.,* 2009). These drugs show exquisite activity against drug-resistant and sensitive forms of *Plasmodium falciparum*, the causative agent of malaria. The drugs mediate their anti-malarial action upon activation by intraparasitic haem, leading to the formation of carbon-centred free radicals (Singh and Lai, 2001). These carbon-centred radicals then cause cell death by interacting with cellular macromolecules such as proteins and membrane lipids (Efferth *et al*., 2004; Mercer *et al*.,2007; Hommel, 2008*;* Nakase *et al*.,2008; Jones *et*

al.,2009; Raghavamenon *et al*., 2011). Interestingly, due to their rapid rate of division most cancer cells take up a large amount of iron as it plays a vital role in cell physiology and growth (Kwok and Richardson, 2002). Given that artemisinins require iron for activation, it has been shown some 18 years ago that the artemisinins have cytotoxicity effect both in vitro and in vivo against various types of cancer cells (Moore *et al.,* 1995; Posner *et al.,* 1997; Beekman *et al.,* 1997; Beekman *et al*., 1998; Efferth *et al.,* 2001; Jeyadevan *et al.,* 2004; Mercer *et al*., 2007; Parekh *et al.,* 2009).



Artemisinin

Figure 3: Chemical structure of artemisinin with the endoperoxide bridge.

Artemisinins as antitumour agents and their mode of action:

Since the early 1990s subsequent studies have observed artemisinin and its analogues showing antitumor activity towards many cancer cell lines; the highest activity being reported against leukaemia and colon cancer cell lines (Disbrow *et al*., 2005). Recent studies using rationally designed and synthesized lead compounds, based on the DHA backbone, have shown cytotoxic activity against a range of tumour cell lines including colorectal (HT29-AK, HCT-116 and SW-480), leukaemia (HL-60), hepatoma (HEP-G2 and HUH-7), lung (SPC-A1), prostate (C4-2 and LNCaP), breast (MDA-MB-231 and MCF-7), endothelial (HMEC-1), osteosarcoma and pancreatic

(BxPc3-RFP) cancer cells (Li and Zhou, 2007; Mercer *et al*., 2007; Mu *et al*., 2007; HOU *et al*., 2008; Shao *et al*., 2008; Zhou *et al*., 2008; Jones *et al*., 2009; Riganti *et al*., 2009; Lu *et al*., 2008 and 2010; Morrissey *et al*., 2010; Aung *et al*., 2011; Noori and Hassan, 2011; Alessandro *et al*., 2011; Ji *et al*., 2011).

The well accepted antitumor mechanism of action is similar to the anti-malarial mechanism. Rapid proliferation is the basic feature of malignant cells, where high intracellular iron content is needed as a cofactor of DNA metabolism and continued cell proliferation (Li and Zhou, 2007). Many examples of increased requirement and dependency on iron by cancer cells to proliferate can be found in the research literatures. For example, breast cancer cells have 5-15 times more transferrin receptor (transferrins are iron-binding blood plasma glycoproteins that control the level of free iron in biological fluids) than normal breast cells (Beguin, 1992; Harford, 1994). Transferrin receptors are over expressed only on the cell surface of breast carcinoma cells but not on benign breast tumor cells (Singh and Lai, 2001). Artemisinin has been shown to be selectively toxic to human breast cancer cells (Singh and Lai, 2001). In another study artemisinin was tested on two breast cell lines, HTB 125, a normal human breast cell line and HTB 27, a radiation-resistant human breast cancer cell line (Hsieh *et al*., 1996). It has been found that only 2% of breast cancer cells were still alive after a 16 hour treatment with both DHA and holotransferrin (iron bounded transferrin) On the other hand, treatment with DHA alone or DHA+holotransferrin had little effect on normal human breast cells. These data indicates that artemisinin is selectively toxic to the radiation resistant human breast cancer cell line but not to normal breast cells (Hsieh *et al*., 1996). Furthermore it was found that holotransferrin significantly enhanced the cytotoxicity of artemisinin on breast cancer cells (Mercer *et al.*, 2007; Oh

et al., 2009*;* Zhang *et al*., 2010*;* O'Neill, Barton and Ward, 2010). In chronic myelogenous leukemia (CML) cells also express more transferrin receptors on their cell surface than normal cells. In addition, the dependence of CML cells on iron intake via the transferrin mechanism is suggested by the finding that antibody to transferrin receptors retards the growth or kills CML cells (Calzolaria *et al*., 2004). Leukemia cells have been shown to be the most sensitive to artesunate, an artemisinin analogue, among various other cancer cell lines such as breast cancer cell line and prostate cancer cell lines (Kawabata *et al*., 2001). Interestingly, elevated iron levels allow the selective toxicity of artemisinins towards the cancer cells (Lazarus *et al*., 1995). Given that iron is central to the activity of artemisinin; various manipulators have been attempted with the view to enhance the cytotoxic effects of the drugs. For example, conjugation of artemisinin or DHA to transferrin has been shown to increase their targeted delivery and cytotoxic activity against tumouric cells (Lai *et al*., 2005; Nakase *et al*., 2008; Oh *et al*., 2009; Xie *et al*., 2010). The selective toxicity of the artemisinins have also been demonstrated by studies showing that they are approximately 100 times more potent in killing human leukemia cells than normal lymphocytes, with the selectivity stemming from the differential levels of iron in the two cell types (Singh and Lai, 2006). In vitro studies have verified the potent activity of artemisinin-transferrin conjugate against Molt-4 leukaemia cell line, while DHA-transferrin conjugate showing even up to 172 times cytotoxic activity in MCF-7 human breast neoplastic cell line (Oh *et al*., 2009; Xie *et al*., 2010). Furthermore, a recent study found that the oral co-administration of artesunate and ferrous sulphate induced apoptosis in cancer cells as compared to artesunate alone (Fafowora *et al*., 2011).

The primary mechanism of 1, 2, 4-trioxanes by which they exert their anti-tumour activities has been hypothesized to be through the induction of caspase-dependent apoptotic cell death (Efferth *et al*., 2004; Singh and Lai, 2006). The caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family (NA Thornberry *et al*., 1997). Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (GS Salvesen *et al*., 1999).

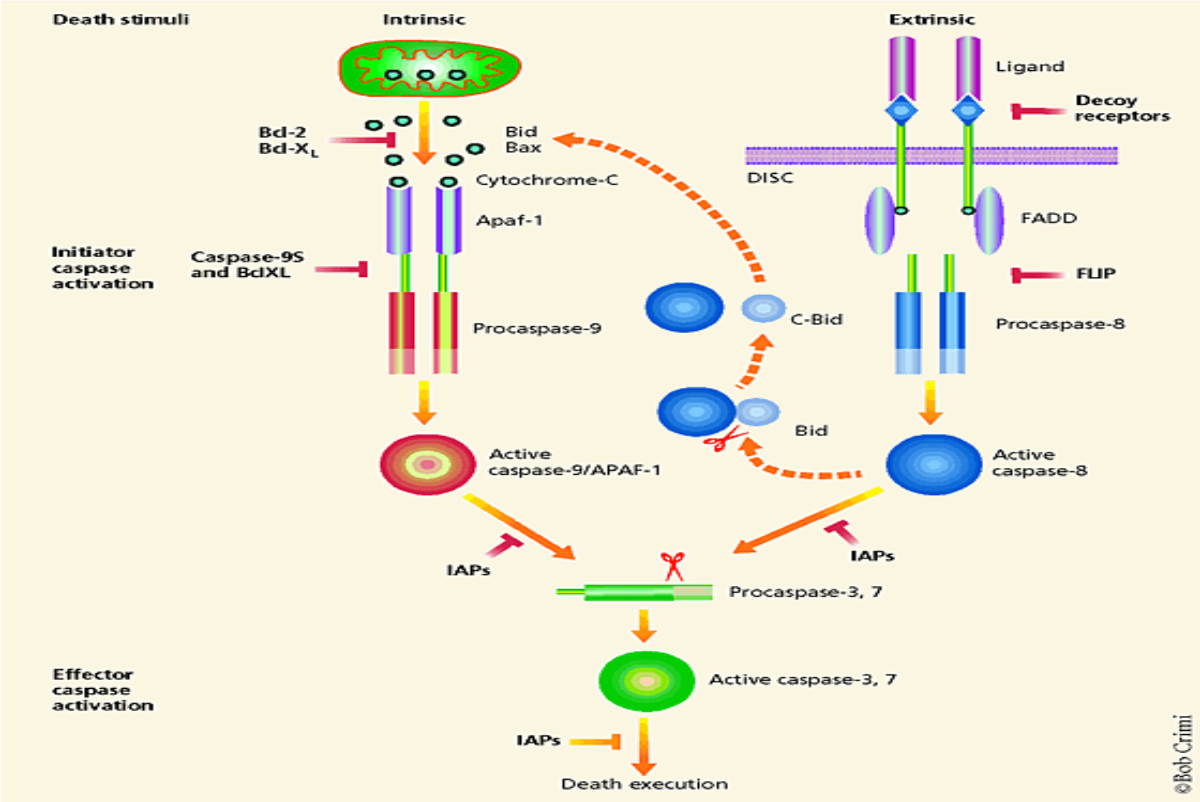


Figure 4: Both the intrinsic and extrinsic pathways lead to the activation of caspases in a stepwise fashion.

It has been accompanied with mitochondrial endoplasmic reticulum stress, release of cytochrome c, activation of effectors caspases-3 and-7, induction of cell cycle arrest at go/g1 phase (Parekh *et al*., 2009). Moreover, studies in human leukaemia, colon and prostate tumour cells demonstrated that the compounds-induced apoptosis is highly correlated with loss or significant decrease of the anti-apoptotic protein survivin, down-

regulation of anti-apoptotic Bcl-2 and over-expression of pro-apoptotic Bax proteins (Jiao *et al.,* 2007; Zhou, Wang and Li, 2008; Aung *et al.,* 2011). B-cell lymphoma 2 (Bcl-2) is an apoptosis regulator protein encoded by the BCL2 gene whereas Bcl-2–associated X protein, or Bax is a protein of the Bcl-2 gene family. It promotes apoptosis by competing with Bcl-2 (Ji *et al.,* 2011; Liang *et al.,* 2011).

Additionally, it has been reported that 1, 2, 4-trioxanes are known to block the signaling pathway NF-κb (nuclear factor kappa beta) and may therefore induce apoptosis (Efferth, 2005, Gao *et al*., 2011). Indeed, it has been demonstrated that artesunate in human rheumatoid arthritis inhibits tumour necrosis factor (TNF)-α-induced production of pro-inflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Interleukin-8 (IL-8) through suppression of signaling pathway NF-κb (Alessandro *et al*., 2011). Therefore, it can be hypothesized that 1, 2, 4-trioxanes may exert similar anti-inflammatory activities in malignancy cells.

Moreover, extensive studies has been done aiming at pharmacoenhancing the activity of 1, 2, 4-trioxanes especially the main active compound DHA against cancer cells (Jones *et al*., 2009). Studies aimed at pharmacoenhancing the antitumour effects of artemisinin have generated artemisinin-based hybrids with the view to enhancing the DNA-targeting property of artemisinin (Jones *et al*., 2009). These derivatives are referred to as dihydroartemisinin-acridine hybrids. Acridine is a potent DNA-intercalating agent and there is also some evidence that the carbon-centred radicals of artemisinins cause DNA damage. The rationally designed artemisinin-acridine hybrids displayed promising anti-tumour activity in a variety of cancer cells types including leukemia, colon and breast cancer cells and they induced cell death by apoptosis (Li and Zhou, 2007, Jones *et al*., 2009, Noori and Hassan, 2011).

Effects of artemisinins on apoptosis:

There are evidences that artemisinin induces apoptosis in the presence of iron in vitro and is lethal towards human leukemia and breast cancer cells (Efferth *et al*., 2004; Singh and Lai, 2006). Clearly, apoptosis is guided by a range of complex multi-step, multi pathway programmes that consequently lead to the breakdown of cellular DNA leading to cell death (Parekh *et al*., 2009). One of these multi-steps includes the induction of caspase-dependent apoptotic cell death (Rathmell and Thompson, 1999; Mercer *et al*., 2007; Parekh *et al*., 2009; Ji *et al*., 2011; Raghavamenon *et al*., 2011). It is postulated that the activation of the endoperoxide bridge leads to apoptosis via caspase-dependent pathways, in which 'initiator' caspases (caspases 8, 9 and 10) have the primary role of processing and activation of both pro-enzymes (procaspases-8,-9 and-10) and 'executioner' caspases (caspases-3,-6 and -7), which ultimately cause cell death (Rathmell and Thompson, 1999; Mercer *et al*., 2007; Parekh *et al*., 2009; Ji *et al*., 2011; Raghavamenon *et al*., 2011). Some of the remaining apoptotic programs include induction of cell cycle arrest at g_o/g_1 phase, induction of mitochondrial endoplasmic reticulum stress, release of cytochrome-c and subsequent DNA fragmentation and the artemisinins have been shown to affect all these important processes required for cell survival (Parekh *et al*., 2009). DHA also induce apoptosis in vitro and in vivo through a process that involves inactivation of MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated protein kinase), Mcl-1 (myeloid cell leukaemia protein) down-regulation, culminating in cytochrome-c release and caspase activation (Gao *et al*., 2011). DHA is well documented to cause apoptosis in micro

vascular endothelial cells (HMEC-1) via caspase 3 and 7 activation, phosphatidylserine exposure and G_2 cell cycle arrest with high doses of drug in combination of oxygen (Alessandro *et al* 2011).

DHA	Induction of apoptosis	1. By inactivation of MEK/ERK. 2. By the down-regulation of Mcl-1 in leukemia cells (Gao *et al*., 2011). 3. Via caspase 3 and 7 activation in micro vascular endothelial cells (HMEC-1) (Alessandro *et al* 2011).

There are a series of key receptors, genes and enzymes whose activation and suppression lead to a cascade of intracellular events which ultimately result in cell death. For example, the mitochondrion plays a central role in cell survival and many of the triggers of apoptosis are known to act in a mitochondria-dependent manner. DHA has been shown to cause apoptosis via mitochondrial pathway, by reducing cellular iron influx, which results as a consequence of decreased expression of transferrin receptors. these effects are believed to be mediated via the down regulation of proliferating cell nuclear antigen (PCNA), a key regulator of DNA synthesis and repair; anti-apoptotic protein Bcl-2, VEGF (vascular endothelial growth factor) and up-regulating pro-apoptotic protein Bax in pancreatic cancer cells under normoxic but not hypoxic conditions (Ji *et al*., 2011). It has been reported that the effects of the artemisinins on these important molecules ultimately cause apoptosis following the downstream activation of caspase-3 (Green and Evan, 2002).

Recently, it has been reported that DHA up-regulates the expression of Bax, FAS and cyclin D1 by activation of caspase-3,-8,-9; down-regulated the expression of Bcl2, Cdc25b and cyclin B1, consequently leading to apoptosis. DHA has also been shown to decrease the level of splenic $CD4^+$, $CD25^+$ and $FOXP3^+$ regulatory T cells in vivo, which was demonstrated by the reduction of tumour size and a decrease in cytokine levels (Jiao *et al.,* 2007, Liung *et al.,* 2011).

Clearly, all cells have to control the level of apoptosis that is occurring as such many pathways involving inhibitors of apoptosis, such as survivin which promote cell survival. Survivin is also known as Baculoviral IAP/ inhibitor of apoptosis protein repeat-containing protein 5 (BIRC5), and it is an apoptosis inhibitor 4 (API4) and a member of the inhibitor of apoptosis protein (IAP) gene family. It plays a vital role in cell division and suppression of apoptosis by suppressing cell death, initiated by both intrinsic and extrinsic apoptotic pathways (Ambrosini, Adida and Altieri, 1997; Salvesen and Duckett, 2002; Schimmer, 2004). There is also evidence that it plays a role in tumour formation, tumour cell resistance to anti-cancer agents and may act as a marker and prognostic indicator for certain cancers including pancreatic and colorectal cancers (Ambrosini *et al.,* 1997;Muchmore *et al.,* 2000; Altieri, 2003; Wheatley *et al.,* 2005). Survivin is highly expressed in human malignancies including colorectal cancer and leukaemia, but rarely in healthy tissues (Tamm *et al*., 1998; Dohi *et al.,* 2004; McNeish *et al.,* 2005; Lu, Luo and Tao, 2007; Ryan, O'Donovan and Duffy, 2009). The anti-apoptotic effect of survivin stems from its inhibitory action on caspase 3 and 7 (Zaffaroni *et al.,* 2005). Survivin has been reported to interact with a number of other proteins. It binds and inhibits the activity of the pro-apoptotic mitochondrial protein SMAC (second mitochondria-derived activator of caspases) /Diablo (direct IAP binding protein with

low pI), and stabilizing XIAP (X-linked inhibitor of apoptosis protein) by preventing ubiquitination and subsequent proteasomal degradation of the proteins. DHA has been shown to down-regulate protein expression of survivin in the lung cancer cell lines SPC-A-1, due to down-regulation of its mRNA (Tamm *et al.,* 1998).

Recent in vitro studies in human prostate malignant cells have shown that DHA-mediated apoptosis is also correlated with inhibition of the PI3-kinase/AKT (phosphoinosine 3-kinase/serine-threonine protein kinase) and extracellular signal regulated kinase (ERK) survival pathway (He *et al.,* 2010). AKT, also known as Protein Kinase B (PKB), is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as cell proliferation and apoptosis (M Pap and GM Cooper, 1998). Since it can block apoptosis, and thereby promote cell survival, AKT has been implicated as a major factor in many types of cancer (O'Gorman *et al.,* 2000; Testa and Tsichlis, 2005; De Souza, Russell and Kearsley, 2009).

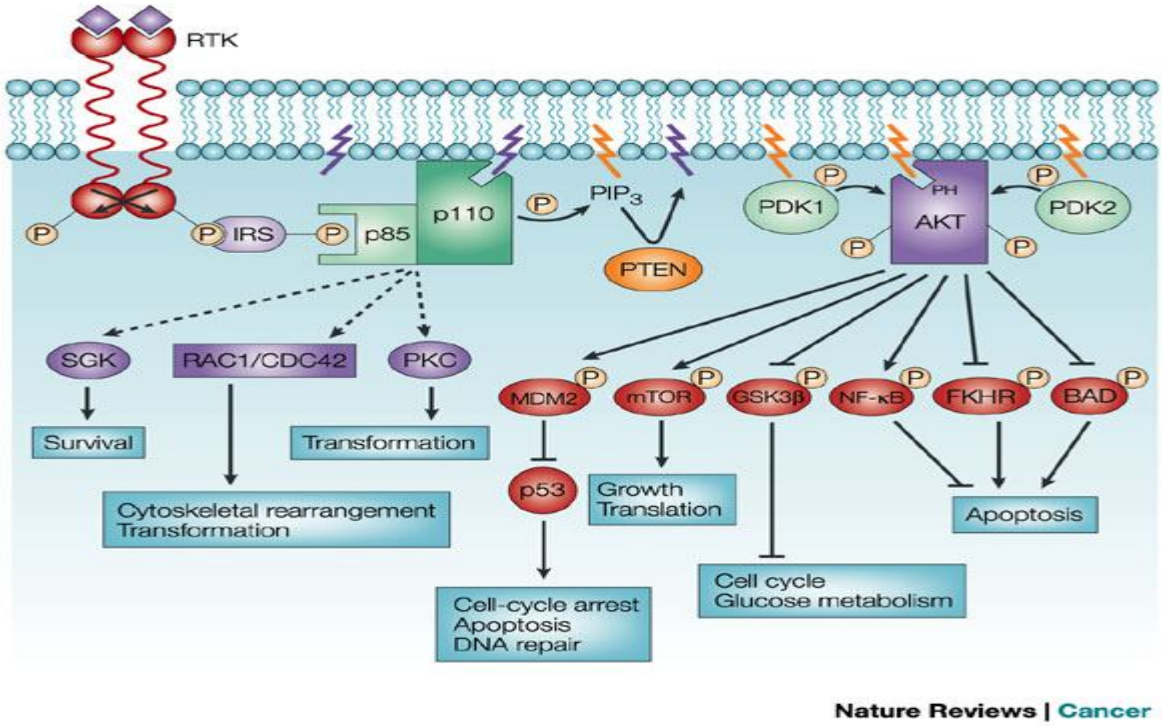


Figure 5: The phosphatidylinositol 3-Kinase–AKT pathway in human cancer.

Effects of artemisinins on cytokine secretion:

Cytokines such as Interleukin-6 (IL-6) and TNF-α are pleiotropic cytokines which play a major role in a number of diseases (Jones *et al*., 2005; Hodge *et al*., 2005; Tzanavari, Giannogonas And Karalis, 2010). IL-6 plays a major role in malignancy and has shown to be an inhibitor of apoptosis and angiogenesis in cancer cells. Normal human circulating IL-6 is in the 1pg/ml range, with modest elevations in certain cancers (Kozłowski *et al*., 2003; Hong, Angelo and Kurzrock, 2007; Lukaszewicz, Mroczko and Szmitkowski, 2007). It has also been reported that IL-6 promotes inflammation-associated carcinogenesis, such as colitis-associated cancer (Mouawad *et al*., 1996; Naugler and Karin, 2008).

TNF-α, also known as cachectin, plays an important role in inflammation, immune system development, apoptosis and lipid metabolism (Idriss and Naismith, 2000;

Hehlgans and Pfeffer, 2005; Chen *et al.,* 2009; Salek and Croft, 2010; Van *et al.,* 2010). TNF-α is produced by tumour cells and assembled intracellularly to form non-covalently linked homotrimer which is expressed on the cell surface (Perez *et al.,* 1990; Tang, Hung and Klostergaard, 1996; Watts *et al.,* 1999).

Interestingly, artesunate have been reported to cause the suppression of TNF-α and production of IL-6, as well as activation of PI3K-AKT pathway. Additionally, it has been reported that 1, 2, 4-trioxanes are known to induce apoptosis by blocking the signalling pathway NF-kβ. There is evidence that artesunate leads to the suppression of signalling pathway NF-kβ signalling and consequently inhibits TNF-α-induced production of the pro-inflammatory cytokines, such as IL-1, IL-6 and IL-8, in human rheumatoid arthritis (Lukaszewicz, Mroczko and Szmitkowski, 2007; Xu *et al.,* 2007). Recently, it was reported that DHA inhibits angiogenesis in human pancreatic cancer by inhibition of NF-kβ DNA-binding activity and also decreased the expression of NF-kβ-targeted pro-angiogenic gene products, VEGF (Vascular endothelial growth factor), IL-8, COX-2 (Cyclooxygenase-2) and MMP-9 (Matrix metalloproteinases-9)in vitro and in vivo (Efferth, 2005). In-vivo experiments using DHA also demonstrated increased levels of IFN-γ but decreased the levels of IL-4. In HCT116 cells it has been reported that the apoptotic genes, such as TNF-α, TRAILR, CASP, GADD45 were expressed at much higher levels, while some survival genes, such as, Bcl2, AKT, BAD were expressed at lower levels, inducing apoptosis upon DHA treatment (Wang *et al*., 2011; Ji *et al*., 2011).

Effects of artemisinins on DNA fragmentation:

Apoptotic cells also have a major distinctive characteristic of DNA fragmentation, which occurs by endogenous endonucleases activation, producing oligonucleosomes (Noori

and Hassan, 2011; Farsam *et al*., 2011). It is postulated that the carbon-centred free radicals generated by artemisinin and its derivatives upon reductive cleavage by iron alkylates into DNA which may cause DNA fragmentation (Noori and Hassan, 2011; Farsam *et al*., 2011).

Effects of artemisinins on cell migration/metastasis:

Metastasis and invasion are fundamental uncontrolled properties of malignant cancer cells, which is a leading cause of morbidity and mortality. The invasion of lymph and blood vessels are important steps in metastasis which requires increased expression of matrix metalloproteinases (MMPs), cell-extracellular matrix (ECM) interactions and disconnection of intercellular adhesion and degradation of ECM. MMPs have been implicated in malignancy and members of its family are involved in degradation of ECM. The activity of MMPs is kept in check by TIMPs (tissue inhibitor of matrix metalloproteinases) (Hwang *et al.,* 2010). It has been reported that inhibition of the expression of MPPs or enzyme activity can be used as early targets for preventing cancer metastasis (Liotta, Steeg and Stetler-Stevenson, 1991; Deryugina *et al.,* 1997; Stamenkovic, 2000; Hwang *et al.,* 2010)).

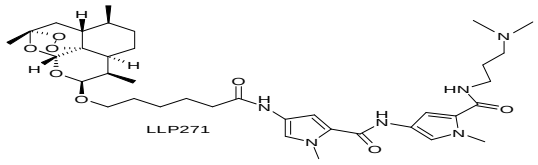
Artemisinin has been reported to have inhibitory effect on invasion and metastasis of human hepatocellular carcinoma (HCC) cell lines (HEPG2 and SMMC-7721). The drugs inhibited, in a concentration-dependent manner, the invasion and migration of the cells by reducing the levels of MMP2 and inducing cellular levels of TIMP2 (Liabakk *et al*., 1996; Hwang *et al.,* 2010; Wang *et al.,* 2011). They have been also demonstrated to activate Cdc42, which enhance E-cadherin activity, resulting in greater cell-cell adhesion

and consequently reduced metastasis (Du *et al.,* 2009; Weifeng *et al.,* 2011). DHA is also an effective anti-metastatic agent that functions by down-regulating MMP-9 gene expression, through inhibition of PKCa/Raf/ERK and JNK phosphorylation in human fibrosarcoma HT-1080 cells (Du *et al.,* 2009; Weifeng *et al.,* 2011).

Aim of our research:

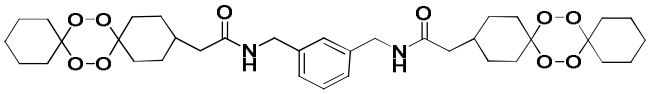
Cancer treatment continues to pose a significant economic burden to health care systems. Artemisinin derivates have shown great promise as anticancer agents and may have potential clinical utility. Unfortunately, currently many of the agents are effective against tumour cells within the micromolar range as opposed to the nanomolar range seen against plasmodium parasites, but it is hoped that via rational drug design this range can be reduced. In the current study we have access to a number of novel derivatives of dihydroartemisinin minor groove binding conjugates like JC339 and LLP271 through a collaborative research partnership with the chemistry department of the University of Liverpool. Our aim was to evaluate the cytotoxicity of those derivatives against human colon cancer cell line (HT29-AK) and leukemia cell line (HL60) and to compare the observed effects with the effects of the parent compound (DHA). Our experiments also aimed to determine the mechanism of action of JC339 and LLP271 by using a range of techniques such as MTT assay, Flow cytometry, ELISA and wound healing assay etc. We have also evaluated the cytotoxicity of some of the novel tetraoxane RKA compounds against human colon cancer cell line (HT29-AK) and leukemia cell line (HL60). The figure below shows the chemical structures of all the test agents used in this study,

(A)

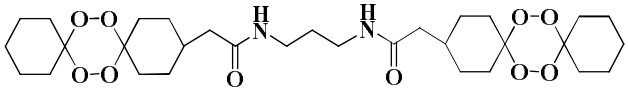


(B) JC3-39, Structure unknown.

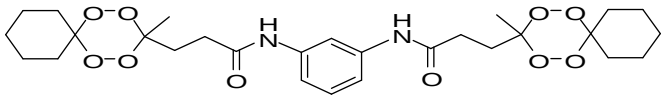
(C)



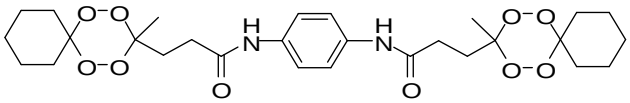
(D)



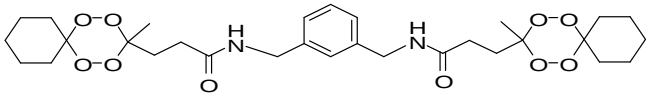
(E)



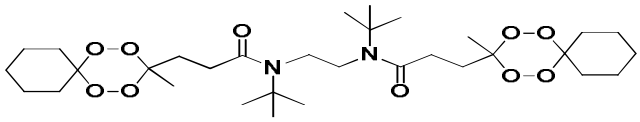
(F)



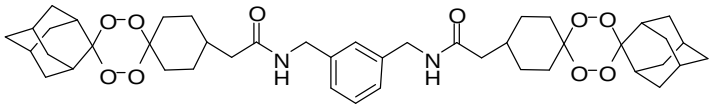
(G)



(H)



(I)



(J)

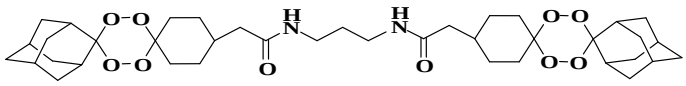


Figure 6: The chemical structures of the test agents. (A) is the chemical structure of the dihydroartemisinin hybrid LLP271 (B) is JC3-39 whose structure is unknown.

The structures of the tetraoxane dimmers RKA138 (C), RKA149 (D), RKA152 (E), RKA150 (F), RKA151 (G), RKA158 (H), RKA155 (I) and RKA160 (J).

MATERIALS AND METHODS

Reagents and chemicals:

The semi-synthetic derivative of artemisinin (dihydroartemisinin/DHA) was purchased from Dafra-Pharma (Belgium). Derivatives of DHA (JC3-39 and LLP271) and the novel tetraoxane RKA compounds (RKA138, RKA149, RKA150, RKA151, RKA152, RKA155, RKA158 and RKA160) were kindly donated by the University of Liverpool (Liverpool, U.K). All the ELISA kits which include the QUANTIKINE Human total survivin immunoassay, Human TNF-alpha, Human IL-6 and Human sTfR were purchased from R&D Systems (Minneapolis, U.S.A). The antibodies (AKT and catalytically active cleaved caspase-3) for flow cytometric analysis were purchased from Cell-Signaling Technology (New England Biolabs, Herts; U.K). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich (Poole, U.K). All media were supplied by Biosera and Invitrogen and chemicals, unless mentioned otherwise, were supplied by Fisher Scientific, Leicestershire (UK). All the drugs were dissolved in dimethylsulfoxide (DMSO) with the final concentration in the incubation media being less than 1%.

Cell culture:

Human colonic adenocarcinoma cells (HT29-AK) and Human promyelocytic leukemia cells (HL60) were obtained from the European Collection of Cell Cultures (ECACC). The cells were maintained in the log phase of growth. They were cultured in EMEM and

RPMI-1640 medium respectively supplemented with 10% fetal bovine serum (FBS). The EMEM media was further supplemented with 1% L-glutamine (Lonza, Verviers, Belgium). Cells were sub cultured twice a week and maintained at 37°C humidified incubator with 5% CO_2. Cell counting involved the use of an electronic counter (NucleoCounter ®, Chemometec, Allerod, Denmark).

Trypsinization (for the adherent cells):

Trypsinization process was used to get the adherent cells (HT29-AK) in suspension in the initiation and termination step of each experiment and for sub-culturing. First the medium from culture flasks was aspirated. Then the attached cells were washed thrice with 5mL of PBS. All the PBS was removed before 1mL of trypsin (Lonza, Verviers, Belgium) was added to each flask and incubated at 37°C for about 3-5 minutes. Then 10-15mL of EMEM medium supplemented with 10% FBS and 1% L-glutamine was added to each flask to inactivate the trypsin. The cell suspension was then counted and used for subsequent assays.

Measurement of cytotoxicity:

MTT (3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay for measuring the cytotoxicity of drugs.

HT29-AK and HL60 cells (1×10^4 cells/well) were seeded in flat bottom 96-well plates. The cells were then exposed to varying concentrations (0-100µM) of the drugs (DHA, RKA138, RKA149, RKA150, RKA151, RKA152, RKA155, RKA158, RKA160, JC339 and LLP271) and incubated for 24 hours (at 37^0C and 5% CO_2). Following incubation, cell viability measurements were carried out by the addition of 20µl of 5mg/ml of MTT solution into each well and incubating the plates for 2 hours (at 37^0C and 5% CO_2).

Thereafter, 100µl of lysis solution (15% sodium dodecylsulphate in 50% N, N-dimethyl formamide) was added into each well to dissolve the formazan crystals. The plates were further incubated overnight. The absorbance of the samples were measured at 492nm using a micro-plate reader (Stingray software; Anthos-2001, Anthos laboratory instruments, UK).

DATA ANALYSIS TO DETERMINE THE IC_{50} VALUE OF THE DRUGS: The IC_{50} value (inhibitory concentration of a drug at which 50% of cells are killed) was determined by calculating the percentage of growth from the optical density readings obtained from the plates by the following equation:

$$\% \text{ of cell growth} = \frac{\text{Optical density of drug treated cells} \times 100}{\text{Optical density of control cells}}$$

The mean percentage of cell growth was plotted against the concentration of the drugs and the IC_{50} was analyzed with the 4 parameter logistic analysis using Grafit (Erithacus Software, UK).

Effect of drug treatment on cleaved caspase-3 and P-AKT activity:

AKT, also known as Protein Kinase B (PKB), is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as apoptosis and cell migration. Caspase-3 protein is a member of the cysteine-aspartic acid protease (caspase) family.

Sequential activation of caspases plays a central role in apoptosis. We used flow cytometric analyses to determine if the drugs mediate their cytotoxicity via targeting these important molecules.

In order to determine the effects of drug treatment on cleaved caspase-3 activity, the cells (HT29-AK and HL60 at 5×10^6 cells/flask) were incubated in the absence or presence of 3 fixed concentrations of the drugs representing their ¼IC50, ½IC50 and IC50 for 24hrs (37°C, 5% CO_2). HT29-AK cells were incubated without or with DHA (15.3μM, 30.7μM and 61.4μM), JC339 (3.5μM, 7.1μM and 14.2μM) LLP271 (4.4μM, 8.8μM and 17.6μM) RKA138 (9.3μM, 18.6μM and 37.2μM), RKA149 (10.4μM, 20.9μM and 41.8μM), RKA150 (0.9μM, 1.8μM and 3.7μM), RKA151 (8.2μM, 16.4μM and 32.8μM), RKA152 (3.4μM, 6.8μM and 13.6μM) and RKA158 (11.6μM, 23.1μM and 46.3μM) for 24hrs at 37°C in the presence of 5% CO_2.

In another experiment, HL60 cells were similarly incubated without or with DHA (0.1μM, 0.2μM and 0.4μM), RKA138 (1.7μM, 3.3μM and 6.7μM), RKA149 (1.7μM, 3.3μM and 6.6μM), RKA150 (3μM, 6.1μM and 12.2μM), RKA151 (8.6μM, 17.1μM and 34.3μM), RKA152 (1.8μM, 3.6μM and 7.1μM), RKA152M (7.8μM, 15.6μM and 31.2μM), RKA155 (4μM, 8μM and 16.1μM) and RKA158 (3.9μM, 7.9μM and 15.7μM) for 24hrs at 37°C in the presence of 5% CO_2.

The assay was terminated by centrifugation (at 1200 rpm for 2 minutes) and the cell pellets were then re-suspended in 250μl of PBS. Samples were then fixed in 250μl of 6% formaldehyde at 37^0C for 10 minutes. Thereafter the samples were centrifuged (at 1200 rpm for 2 minutes) after chilling on ice for 1 minute. The samples were then permeabilized by re-suspending them in ice-cold 90% methanol. The samples were then centrifuged (at 1200 rpm for 2 minutes) followed by 2 wash steps in 200μl of

incubation buffer (0.5% BSA in PBS stored at 4^0C). The samples were re-suspended in 100µl incubation buffer and incubated at room temperature for 10 minutes. Thereafter samples were incubated for 1 hour at room temperature with 100µl of primary antibody (pro-apoptotic marker caspase-3). A further wash step was done followed by resuspending the cell pellet in 500µl of PBS. Finally, the cell samples were being analysed on the BD FACS Calibur® flow cytometer on the FL-1 channel. The fluorescence of the cells was plotted against the number of events and the data were registered on a logarithmic scale prior to calculation of the mean relative fluorescence units.

In a separate experiment, we investigated the effect of drug treatment on apoptosis using AKT antibody. For this only HT29-AK cells were incubated without or with DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM). The cells were collected by trypsinisation and then centrifuged (at 1200 rpm for 2 minutes) and the cell pellets were re-suspended in 250µl of PBS. The samples were then fixed in 250µl of 6% formaldehyde at 37^0C for 10 minutes. Thereafter the samples were centrifuged (at 1200 rpm for 2 minutes) after chilling on ice for 1 minute. The samples were then permeabilized by re-suspending them in ice-cold 90% methanol. The samples were then centrifuged (at 1200 rpm for 2 minutes) followed by 2 wash steps in 200µl of incubation buffer (0.5% BSA in PBS stored at 4^0C). The samples were re-suspended in 100µl incubation buffer and incubated at room temperature for 10 minutes. Thereafter samples were incubated for 1 hour at room temperature with 100µl of primary antibody (pro or anti-apoptotic marker AKT). A further wash step was done followed by re-suspending the cell pellet in 500µl of PBS. Finally, the samples were being analysed on the BD FACS Calibur® flow cytometer on

the FL-1 channel. The fluorescence of the cells was plotted against the number of events and the data were registered on a logarithmic scale prior to calculation of the mean relative fluorescence units.

Effect of drug treatment on DNA damage by gel electrophoresis:

An aliquot of HT29-AK cells (5×10^6 cells/flask) and media were incubated without or with DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM) for 24 hours (at 37^0C and 5% CO_2). The cells were collected by trypsinisation and centrifuged at 1200 rpm for 2 minutes. The pellets were re-suspended in 200µl of EMEM media. DNA was purified following the manufacturer's (QIAGEN, Maryland, USA) protocol. Measurement of DNA concentrations was then carried out with Nano-Drop (N.D) Spectrophotometer (ND-1000 software, Labtech International, UK) at 260nm.

An equal concentration and volume of DNA (10ng/µl DNA and 25µl volume, the volume was adjusted using sterile distilled water) for all the samples was loaded into each well of a 1% agarose gel stained with 10µl of ethidium bromide. One of the control samples was further heat treated at 95^0C for 25 minutes. 60mA current for each gel slab was used to run DNA. The samples were then visualized and photographed under UV-light.

Effect of drug treatment on cellular survivin level:

Survivin is a protein which functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death (35). It is expressed highly in most human tumours.

To investigate the effects of drug treatment on survivin levels, HT29-AK cells (2×10^6 cells/well) were incubated in 6 well flat bottomed plates in the absence or presence of DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM) for 24 hours (at 37^0C and 5% CO2). The cells were collected by trypsinisation and centrifuged as described previously. The cell pellets were washed in PBS by centrifugation (at 1200 rpm for 2 minutes) and solubilized in Lysis Buffer 6 as described by the manufacturer (www.rndsystems.com).

The lysates were centrifuged (at 1200rpm for 5 minutes) and 60µl of the supernatant was mixed with 60µl of the assay diluents. Aliquots of cell lysate and media were retained for analysis. Then 100µl of each standard and sample was added in each well of the ELISA plate. The plates were then incubated for 2 hours at room temperature, covered with the provided adhesive strip. The wells were washed 3 times with wash buffer and then 100µl detection antibody was added. The plates were again incubated for 2 hours at room temperature. After incubation the wells were washed as described previously. Furthermore, 100µl of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature. The wells were washed three times with wash buffer. Then 100µl Substrate solution was added and incubated for 20 minutes at room temperature. Thereafter 50µl of stop solution was added to each well. The optical density was measured at 450nm within 30 minutes and the data was further analysed by plotting standard graph for known survivin concentrations. The unknown survivin concentrations of samples were calculated by plotting the absorbance values in standard graph.

Effect of drug treatment on wound healing:

HT29-AK cells (2×10^6 cells/well) were cultured (at 37^0C, 5% CO_2) in 6 well flat bottomed plates until confluency was reached (3-7 days). Once the cells were confluent, a uniform wound was created using a sterile P10 pipette tip. Then non-adherent cells were washed twice using EMEM media supplemented with 10% FBS and 20mM L-glutamine. A picture of wells with the confluent cells and the wound-created cells were taken. Thereafter the cells were incubated without or with DHA (6.1μM, 15.3μM, 30.7μM and 61.4μM), JC339 (1.4μM, 3.5μM, 7.1μM and 14.2μM) and LLP271 (1.8μM, 4.4μM, 8.8μM and 17.6μM) for 3 days at 37^0C and 5% CO_2. The assays were terminated by taking pictures of the wells again. The effects of drug treatment on wound healing were assessed by comparing the control pictures (confluent non-treated wells; wells with wound, but without any drug treatment) with the pictures taken of drug treated wells (confluent treated wells; wells with wound and drug-treated).

Trypan blue test to evaluate cell death in the wound healing assay:

Here, an aliquot of the culture media was collected from each well (untreated and drug-treated) of the wound healing assay in order to check the viability of the cells and to compare it with the viability of the control sample. Equal volumes (20μl each) of the collected samples and trypan blue dye were mixed before being loaded on the glass-slide and viewed under light microscope at 10x magnification and pictures were taken. Along with this, the numbers of dead cells were counted using a haemocytometer following the equation mentioned below:

Cell count = average number of cells in large corner square X $1x10^4$ cells/ml X2

Effect of drug treatment on the cellular level of TNF-α:

TNF-α (Tumor Necrosis Factor-alpha) is a cytokine. It is produced chiefly by activated macrophages. TNF-α is able to induce apoptotic cell death and to inhibit tumourigenesis.

To determine the effect of drug treatment on the cellular level of TNF-α, we incubated HT29-AK cells (5×10^6 cells/flask) without or with DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM) for 24 hours at 37^0C and 5% CO_2. Thereafter 200µl of the incubation was taken and stored at -20^0C for batch analysis of TNF-alpha levels as described by the manufacturer (www.rndsystems.com).

Effect of drug treatment on the cellular level of IL-6:

Interleukin 6 (IL-6), also known as interferon-beta 2, is an α-helical cytokine that plays important role in cancer progression (36).

To determine the effect of drug treatment on the cellular level of IL-6, we incubated HT29-AK cells (5×10^6 cells/flask) without or with DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM) in small culture flasks for 24 hours at 37^0C and 5% CO_2. Thereafter 100µl of the incubation medium was taken and stored at -20^0C for batch analysis of IL-6 levels as described by the manufacturer (www.rndsystems.com).

Effect of drug treatment on the cellular level of sTfR:

Soluble transferrin receptor (TfR) is a trans-membrane, disulfide-linked dimmer that arises from the proteolysis of TfR (37). It delivers iron to the cell cytosol which acts as a

cofactor for DNA metabolism. As we have hypothesized that the specificity of our drugs to cancer cells is based on the cellular level of iron, measuring the levels of sTfR may give us an indirect measure of the relative levels of iron within the cells in the absence or presence of drug.

This assay is based on the micro plate sandwich enzyme immunoassay technique using two different monoclonal antibodies specific for sTfR. Briefly, the HT29-AK cells (5×10^6 cells/well) were incubated without or with DHA (15.3µM, 30.7µM and 61.4µM), JC339 (3.5µM, 7.1µM and 14.2µM) and LLP271 (4.4µM, 8.8µM and 17.6µM) in a 6-well plate for 24 hours (at 37^0C and 5% CO_2). The cells were collected by trypsinisation and centrifuged at 1200rpm for 5 minutes. The cell pellets were solubilized in Lysis Buffer 6 as described in the survivin assay. Thereafter 50µl of the cell-lysate was taken and stored at -20^0C for batch analysis of sTfR levels as described by the manufacturer (www.rndsystems.com).

First, 100µl of sTfR assay diluent was added into each well. Then 20µl of each standard and sample was added to the wells and incubated for 1 hour at room temperature. The wells were washed three times with wash buffer. Thereafter 100µl of sTfR conjugate was added and incubated again for 1 hour at room temperature. The wells were washed again as described previously. Then 100µl of substrate solution was added and incubated for 30 minutes at room temperature. The assay was terminated by adding 100µl of stop solution. The optical density was measured at 450nm within 30 minutes and the data was further analysed by plotting standard graph for known sTfR concentrations. The unknown sTfR concentrations of samples were calculated by plotting the absorbance values in standard graph.

Statistical analysis:

Data are expressed as mean ± SD and was assumed to be non-normal; the Shapiro-Wilk test was used to assess the distribution of the data. Statistically significant differences between controls and test samples were then assessed either by one-way ANOVA followed by Mann-Whitney U test using Stats-Direct statistical software, version 2.6.3, 2007 (Cheshire, UK).

RESULTS

The cytotoxicity of the compounds against HL60 and HT29-AK cells:

At the start of these investigations, the cytotoxicity of artesunate (a semi-synthetic derivative of artemisinin) against HL60 cells and HT29-AK cells were evaluated. In the case of HT29-AK cells, the cells were treated with artesunate at time 0 (i.e. cells and drug added at the same time), at proliferating and at confluence and the results are represented in Table 1. We observed that artesunate was more cytotoxic against HL60 cells than HT29-AK cells ($1.45\pm0.08\mu M$ vs. $160.44\pm1.62\mu M$). The rank order of potency of artesunate against HT29-AK cells at time 0, at proliferating and at confluence was confluence proliferating time 0. The IC_{50} value of artesunate against confluent HT29-AK cells was $51.75\pm2.94\mu M$, which was significantly ($P\leq0.05$) more potent than DHA.

Table 1 The cytotoxicity results for artesunate against HL60 and HT29-AK tumour cell lines over different times of confluency. The IC_{50} values are means±S.D of three independent experiments with six replicates in each. HL60 cells were incubated with varying concentrations (0-100μM) of artesunate for 24 hours before the cytotoxicity was measured using MTT assay. Whether in case of HT29-AK cells, for time 0, cells were incubated with artesunate (0-750μM) as soon as the cells are seeded in the 96-well microtitre plates; at proliferating, cells were allowed to grow for 2-3 days before being treated and at confluence, cells were treated at least 3-4 days post seeding respectively. The cytotoxicity was measured as described previously. *P<0.05 as tested by Mann-Whitney U test.

Cells	**IC_{50} (μM)** **Mean±SD**
HL60	1.45±0.08
HT29-AK	160.44±1.62[a]
	125.54±3.30[b]
	51.75±2.94[c]*

a,b and c represent the data obtained against HT29-AK cells at time zero, proliferating (2-3 days post seeding) and upon reaching confluency (3-4 days post seeding), respectively.

In the subsequent studies, the cytotoxicity of the test agents (JC3-39, LLP271 and the tetraoxane dimers, the RKA series) was evaluated in comparison to DHA using the MTT assay and the results can be seen in Table 2. Interestingly, all the test agents were cytotoxic against both HL60 and HT29-AK cell lines, with HL60 cells being more susceptible compared to HT29-AK cells. DHA was more potent than all the other

compounds tested in case of HL60 cells. However, all of the novel agents were significantly ($P \leq 0.05$) more potent than DHA against HT29-AK cell line. The rank order of activity of the tetraoxane dimers against HL60 cells was as follows: RKA149≈ RKA138≈ RKA152≈ RKA150≈ RKA158 ≈ RKA155≈ RKA152M≈ RKA151. Against HT29-AK cells the rank order of potency of the agents was as follows: RKA150≈ RKA152≈ RKA151≈ RKA138≈ RKA149 ≈ RKA158. The IC_{50} values of JC3-39 and LLP271 against HT29-AK cells were 14.20±0.22μM and 17.60±0.21μM respectively, both of which were significantly ($P<0.001$) more potent in comparison to DHA. Overall, the data showed that HL60 cells were more susceptible to DHA as only 0.39±0.11μM was needed to kill 50% cells, whereas HT29-AK cells were more susceptible towards the novel agents (Table 2).

Table 2: Cytotoxicity results for the different compounds in comparison to DHA against HL60 and HT29-AK tumour cell lines. The IC_{50} values are mean of three independent experiments with six replicates in each ± standard deviation. The cytotoxicity of RKA152M and RKA155 against HT29-AK cells and the cytotoxicity of JC3-39 and LLP271 against HL60 cells were not determined (N.D). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ as tested by Mann-Whitney U test.

Test agents	IC_{50} against HL60 Mean ±S.D (μM)	IC_{50} against HT29-AK Mean ±S.D (μM)
DHA	0.39±0.11	61.40±0.45
RKA138	6.99±0.12	37.16±0.16*
RKA149	6.56±0.16	41.79±0.24*
RKA150	12.24±0.68	3.75±0.37***
RKA151	34.29±0.63	32.77±0.31**
RKA152	7.14±0.15	13.59±0.11***
RKA158	16.44±0.54	46.29±0.31*
RKA152M	31.21±1.81	N.D
RKA155	18.81±2.30	N.D
JC3-39	N.D	14.20±0.22***
LLP271	N.D	17.60±0.21***

Effect of drug treatments on the cellular concentration of sTfR in HT29-AK cells:

Iron is central to the cytotoxicity of artemisinins as it mediates the reductive conversion of the drugs to reactive species which ultimately kill the cancer cells. Here the cellular sTfR levels were measured and also the effects of drug treatments on cellular sTfR level

in the absence and presence of the test agents was evaluated. This gave us a very crude measure of cellular transferrin level and the effect of drug treatment on receptor expression. There were measurable levels of sTfR in the control cells ranging from 4.3±0.5nmol/L to 5.9±0.6nmol/L (Figure 7). There was a significant ($P<0.05$) decrease in cellular sTfR concentration when cells were treated with 15.3μM of DHA as compared to control (Figure 1A). However, surprisingy, none of the other concentrations (30.7μM and 61.4μM) tested of DHA significantly altered the level of sTfR (Figure 7A). Upon treatment with 3.5μM and 7.1μM of JC3-39, there was a significant ($P\leq0.05$) increase in cellular sTfR level as compared to the control. The cellular sTfR concentration in the control samples was 4.3±0.5nmol/L; 5.2±0.3nmol/L and 5.9±0.5nmol/L was measured in samples treated with 3.5μM and 7.1μM of JC3-39 respectively. There was no significant alteration in the cellular level of sTfR when the cells were treated with 14.2μM of JC3-39 as compared to control (Figure 7B). When HT29-AK cells were treated with 4.4μM of LLP271, the cellular sTfR concentration was significantly ($P<0.001$) decreased as compared to control (3.9±0.3nmol/L vs. 5.6±0.4nmol/L). None of the other concentrations tested of LLP271 (8.8μM and 17.6μM) significantly altered the levels of sTfR compared to control (Figure 7C).

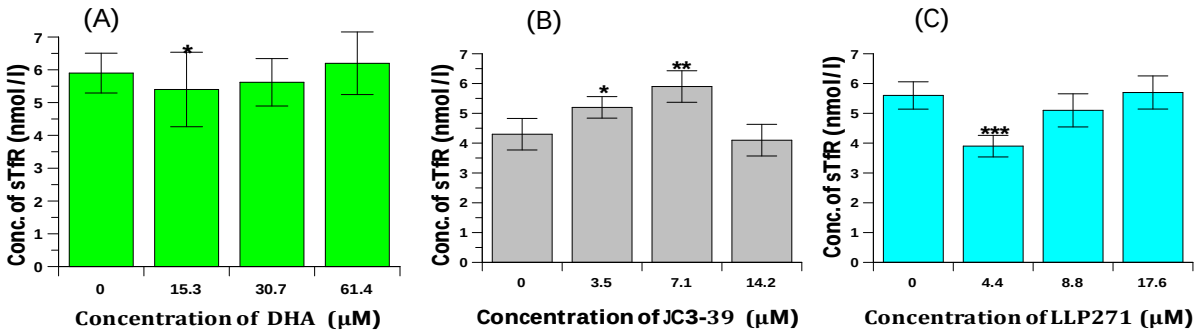


Figure 7 The effects of different test agents on the cellular concentration of sTfR in HT29-AK cells. Cells were treated without or with various concentrations of DHA, JC3-39 and LLP271 for 24 hours before sTfR levels were measured by ELISA. Results are means ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test.

Effect of drug treatments on cellular P-AKT levels in HT29-AK cells:

Given that the agents are cytotoxic, we were interested to understand if drug treatments can alter the P-AKT levels as it plays a critical role in controlling cell survival and apoptosis (F Pene, YE Claessens, O Muller, F Viguie and P Mayeu - Oncogene, 2002 - ukpmc.ac.uk). As can be seen in Figure 8, we found that all the drugs tested markedly increased the levels of P-AKT in HT29-AK cells in a concentration dependent manner as compared to control. The concentration of P-AKT in the control samples ranged from

47.9±0.8 RFU to 62.6±3.4 RFU. Cells treated with 15.3µM DHA showed a significant ($p<0.001$) increase in P-AKT activity as compared to control (139.14±8.45 RFU vs. 53.4±3.22 RFU). Similarly, the HT29-AK cells treated with 30.7µM and 61.4µM DHA also showed a significant ($p\leq0.05$) increase in P-AKT activity as compared to control (Figure 8A). When cells were treated with 3.5µM of JC3-39, a P-AKT activity of 88.25±10.52 RFU was measured which was significantly ($p<0.05$) greater than control (62.59±3.47 RFU). The P-AKT activity was increased by ~1.5 and ~1.4 folds as compared to control when cells were treated with 7.1µM and 14.2µM of JC3-39 respectively (Figure 8B). When cells were treated with 4.4µM, 8.8µM and 17.6µM of LLP271, the relative fluorescence of P-AKT measured were 110.4±10.63, 132.84±1.8 and 155.46±2.2 RFU respectively which were significantly ($P\leq0.05$) greater than control (Figure 8C).

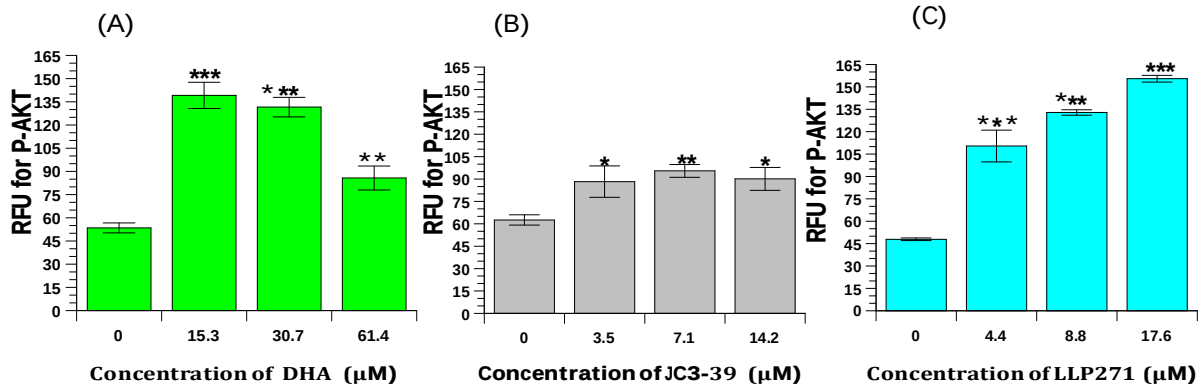


Figure 8 Graphs showing the effects of the test agents on P-AKT activity in HT29-AK cells. Cells were first treated without or with different concentrations of DHA, JC3-39 and LLP246 for 24 hours as mentioned in the methods section before P-AKT activity

was measured by flow cytometric analysis. Results are expressed as the mean ±SD values obtained from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test.

Effect of drug treatments on cleaved caspase-3 activity in HT29-AK cells:

To further investigate whether the dimers have the potential to increase apoptosis, the effects of the agents on caspase 3 activity was evaluated against HT29–AK cells. The results obtained (Figure 9) showed that all the compounds tested increased apoptosis by increasing catalytically active caspase-3 activity. The levels of cleaved caspase-3 in the control cells ranged from 46.5±11.9 RFU to 62.3±3.86 RFU. The catalytically active cleaved caspase-3 activity was significantly ($p \leq 0.05$) increased when cells were treated with 15.3μM DHA as compared to control (145.6±8.32 RFU vs. 62.3±3.86 RFU). Similarly, in 30.7μM and 61.4μM of DHA treated samples we have observed ~1.9 and ~1.7-folds increase in fluorescence respectively as compared to control (Figure 9A). The catalytically active cleaved caspase-3 activity was significantly ($p \leq 0.05$) increased upon treating the cells with 3.5μM of JC3-39 as compared to control (77.8±2.2 RFU vs. 46.6±11.9 RFU). The activity of cleaved caspase-3 was increased by ~2.08-fold when HT29-AK cells were treated with 7.1μM of JC3-39 as compared to control. A significantly greater (P<0.01) relative fluorescence of 100.5±5.3 RFU was measured when cells were treated with 14.2μM of JC3-39 as compared to control (Figure 9B). A concentration dependent increase in catalytically active cleaved caspase-3 activity was observed in HT29-AK cells treated with LLP271. Cleaved caspase-3 activity was increased by ~2.25-

fold when the cells were treated with 4.4μM of LLP271. Similarly, it was increased by ~2.7 and ~3.4 folds as compared to control when the cells were treated with 8.8μM and 17.6μM of LLP271 respectively (Figure 9C).

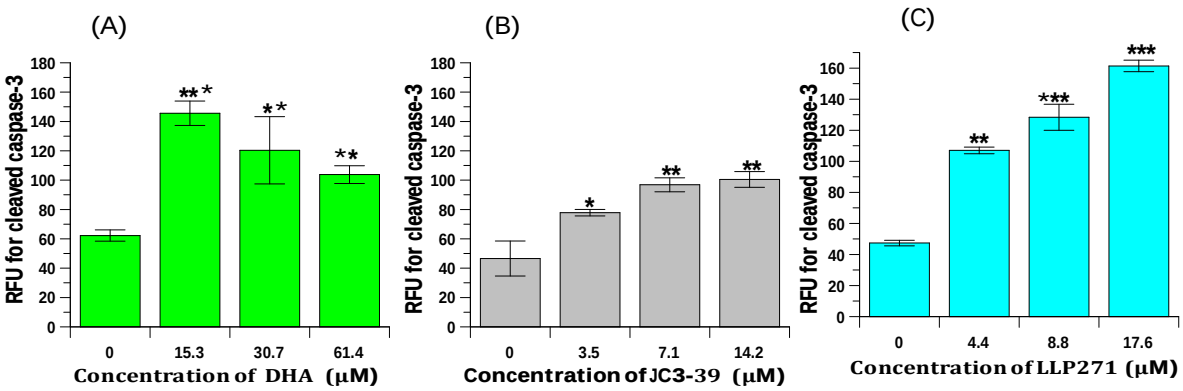


Figure 9 The effects of different drug treatments upon catalytically active cleaved caspase-3 activity in HT29-AK cells. Cells were first treated without or with different concentrations of DHA (15.3μM, 30.7μM and 67μM), JC3-39 (3.5μM, 7.1μM and 14.2μM) and LLP271 (4.4μM, 8.8μM and 17.6μM) for 24 hours as described in the methods section before catalytically active cleaved caspase-3 activity were measured by flow cytometric analysis. Results are the mean ±SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test.

Drug treatments induced DNA fragmentation of HT29-AK and HL60 cells:

Given that the drugs increased the levels of catalytically active caspase-3, which is an important signal that the drugs caused apoptosis, the next experiments investigated the effects of drug treatments on DNA fragmentation. Induction of HT29-AK DNA fragmentation upon drug treatments was analyzed using agarose gel electrophoresis after incubating the cells without or with the drugs for 24hours. As can be seen in Figure 10, all the agents (DHA, JC3-39 and LLP271) caused some DNA fragmentation which is illustrated in the lanes by smearing or laddering. There were two types of control (without any drugs) samples, one was non-heat-treated control (lane 2) and another one was heat-treated (at 95^0C for 20 minutes) control (lane 3). The heat-treated control was included to enable the interpretation of data acquired from the drug-treated samples. The effect of different concentrations of DHA on DNA damage can be seen in lanes 4, 5 and 6. In lane 4 the cells were treated with 15.3μM DHA. Similarly in lane 5 and 6 the cells were treated with 30.7μM and 67μM DHA respectively; with all treatments showing DNA smearing compared to control. The effect of 3.5μM, 7.1μM and 14.2μM of JC3-39 on the cells showed some DNA damage in lanes 7, 8 and 9 respectively. LLP271 treatment also caused DNA damage in lanes 10, 11 and 12. In lane 10 the cells were treated with 4.4μM of LLP271, whereas in lanes 11 and 12 the cells were exposed to 8.8μM and 17.6μM LLP271 respectively. The characteristics DNA smear pattern in all the above mentioned lanes at different concentrations of the test agents represents degradation of genomic DNA into smaller, low molecular weight fragments being definitive signs of the induction of apoptosis. The difference in band sizes in all of the lanes is unfortunately a result of operator error making it impossible

to make an accurate comparison of the effects of the agents on DNA damage. As a consequence of which different amounts of DNA was loaded into the wells. However, there was some background DNA damage in the control samples (Figure 10, non-drug-treated, lane 2), but the heat-treated samples appear to display greater DNA damage compared to their non-heat-treated, non-drug treated counterparts (Figure 10, lane 3). But despite this error it can be observed that compared to normal control (lane 2) and heat treated control (lane 3) drug treatment of the cells caused some DNA fragmentation.

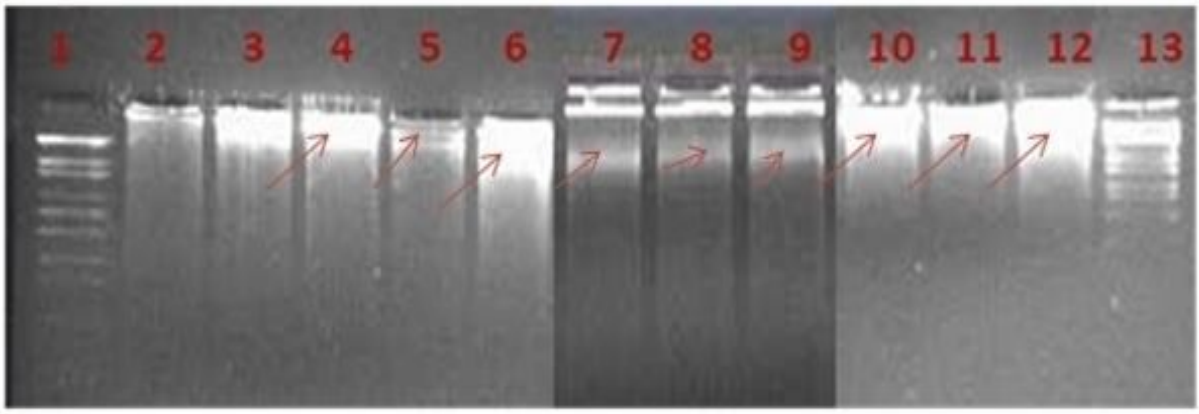


Figure 10 A representative gel image for the effects of drug treatments on DNA fragmentation using agarose gel electrophoresis. HT29-AK cells were treated without or with DHA (lane 4:15.3μM, lane 5:30.7μM and lane 6:61.4μM); JC3-39 (lane 7:3.5μM, lane 8:7.1μM and lane 9:14.2μM) and LLP271 (lane 10:4.4μM, lane 11:8.8μM and lane 12:17.6μM) for 24hrs. The gel was then visualised under U.V light after running for 30 minutes under 60mA current. *E. coli.* molecular marker ladder (Bioline, UK) was used to determine DNA bands from 421 kDa to 19329 kDa, as shown in lanes 1 and 13. The red arrow-heads in the figure indicate the DNA smearing.

We also investigated the effects of the tetraoxane dimers (the RKA compounds) on DNA damage against HL60 cells and the results can be seen in Figure 11. As in figure 10,

unfortunately, variable concentrations of DNA were loaded in each well due to operator error. RKA138 (at 3.4µM, 6.9µM and 13.8µM) induced DNA damage in HL60 cells as can be seen in lanes 4, 5 and 6 respectively. Similarly, lanes 7, 8 and 9 indicate the DNA damaging effects of RKA149 on DNA damage in HL60 cells treated with 3.3µM, 6.6µM and 13.2µM RKA149 respectively. Lanes 10, 11 and 12 represent the effects of 6.1µM, 12.2µM and 25.4µM RKA150 on DNA damage respectively. The effects of RKA152 on DNA damage was observed in lanes 13, 14 and 15 that were treated with 3.5µM, 7.1µM and 14.2µM RKA152 respectively. The effects of 8.2µM, 16.4µM and 32.8µM of RKA158 on DNA damage can be seen in lanes 16, 17 and 18 respectively. We observed DNA smearing in all the different concentrations of respective drugs.

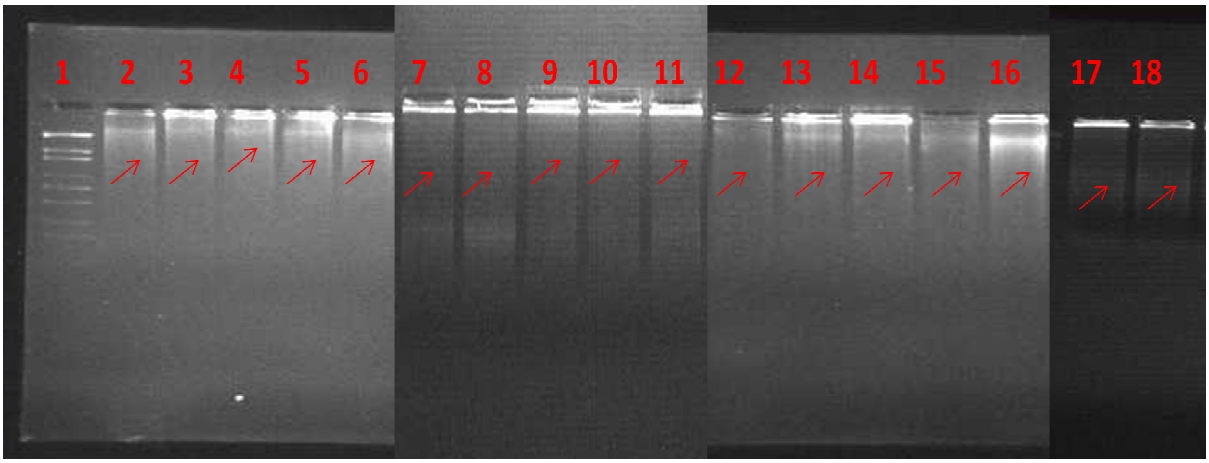


Figure 11 The effects of drug treatments on DNA fragmentation using agarose gel electrophoresis. HL60 cells were treated without or with RKA138 (lane 4:3.4µM, lane 5:6.9µM and lane 6:13.8µM); RKA149 (lane 7:3.3µM, lane 8:6.6µM and lane 9:13.2µM); RKA150 (lane 10:6.1µM, lane 11:12.2µM and lane 12:25.4µM); RKA152 (lane 13:3.5µM, lane 14:7.1µM and lane 15:14.2µM) and RKA158 (lane 16:8.2µM, lane 17:16.4µM and lane 18:32.8µM) for 24hrs. There were 2 controls: non-drug treated (lane 2) and non-drug and heat-treated (at 95^0C for 20 minutes, lane 3) controls. DNA was visualised under U.V light after running for 30 minutes under 60mA current. *E. coli*. Molecular

marker ladder (Bioline, UK) was used to determine DNA bands from 421 kDa to 19329 kDa, as shown in lane1. The red arrow-heads in the figure indicate the DNA smearing.

Drug treatments caused variable changes in cellular survivin level in HT29-AK cells:

The cellular survivin levels upon treatment with different concentrations of the agents were measured and the results are illustrated in Figure 12. We found no significant alterations in survivin level when cells were treated with 15.3μM and 30.7μM of DHA respectively as compared to control, but a higher concentration of DHA (61.4μM) caused a ~5.7-fold increase in cellular survivin concentration as compared to control (Figure 12A). There was no significant alterarion of survivin concentration when HT29-AK cells were treated with 3.5μM and 7.1μM JC3-39 as compared to control. However cells treated with 14.2μM of JC3-39 showed a significant ($P<0.01$) decrease in survivin concentration as compared to control (Figure 12B). Similarly cells treated with 4.4μM and 8.8μM of LLP271 did not alter the cellular survivin concentration but we observed a significant ($P<0.05$) decrease in the cellular survivin concentration in samples treated with 17.6μM LLP271 (Figure 12C).

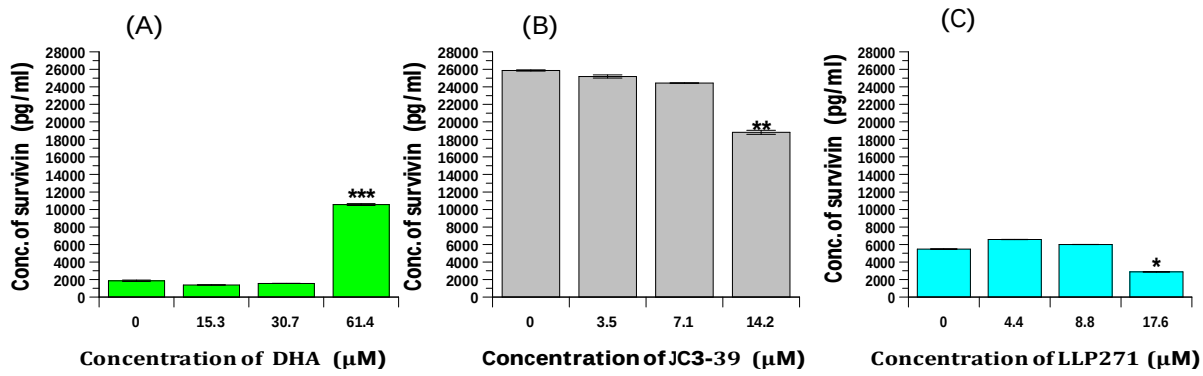
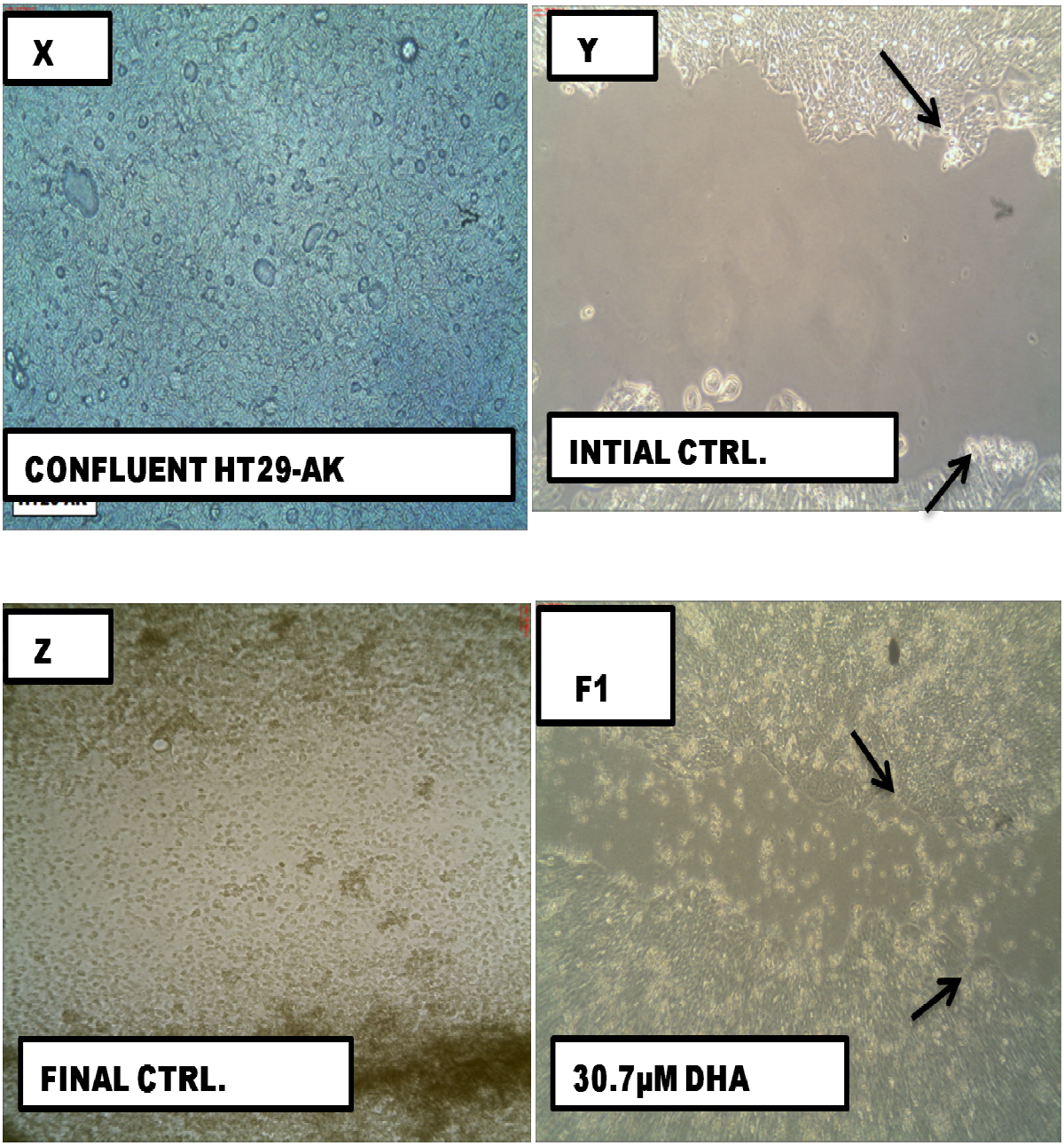


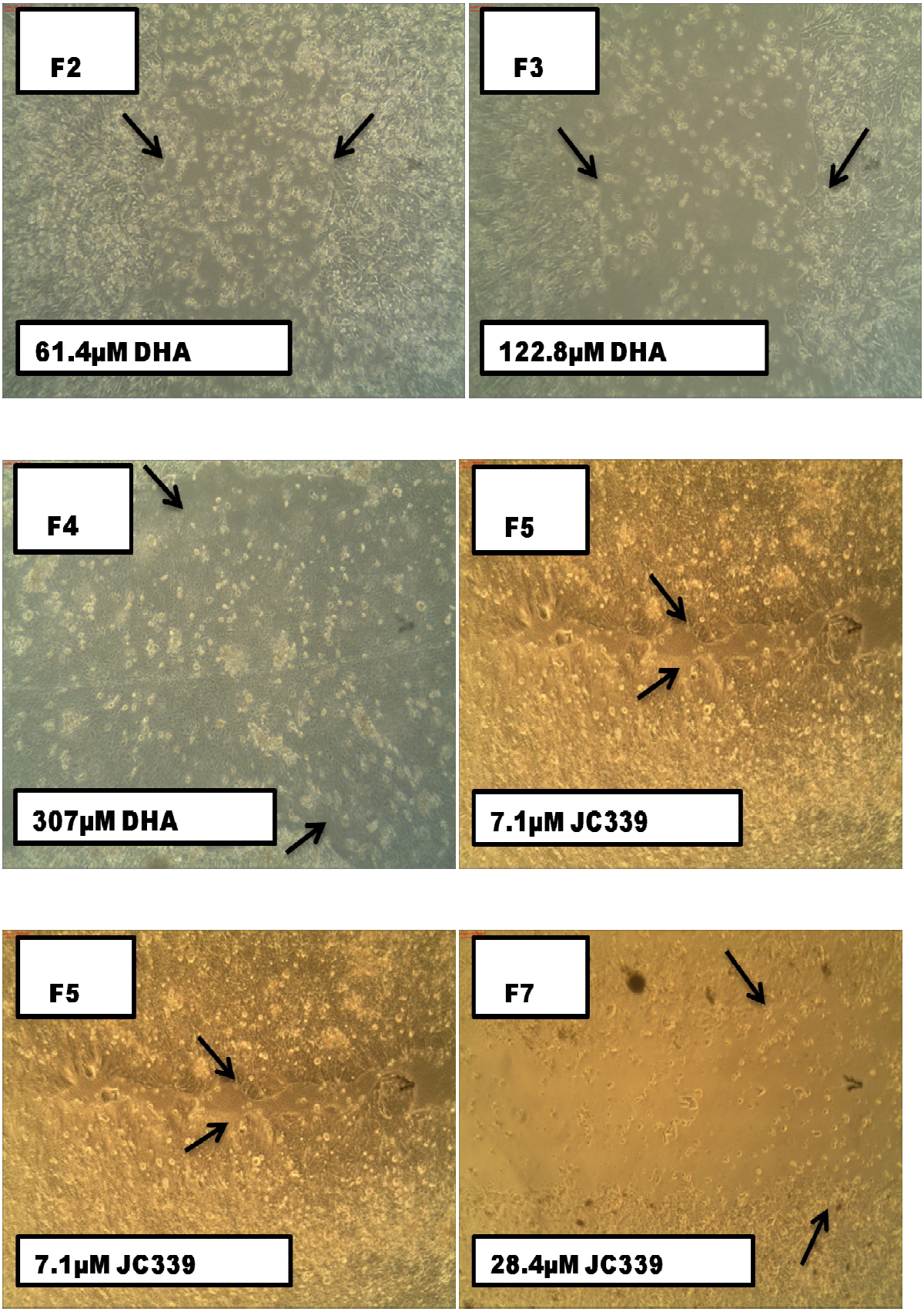
Figure 12 The effects of different drug treatments on cellular survivin concentrations in HT29-AK cells determined by ELISA. Results are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test. The differences in control samples is perhaps due to freeze thawing.

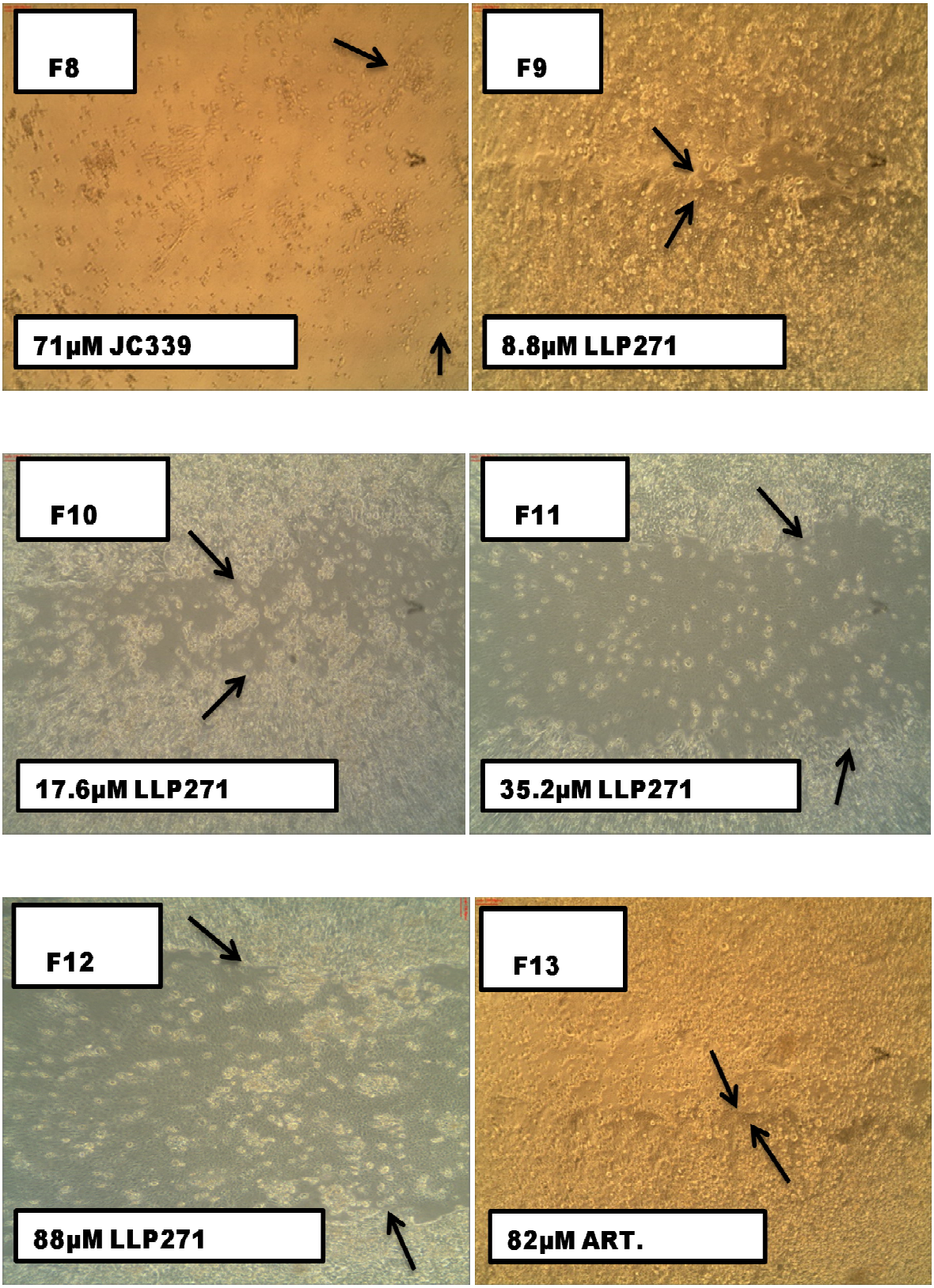
Inhibition of the capacity of the wound to heal in HT29-AK cells upon drug treatments:

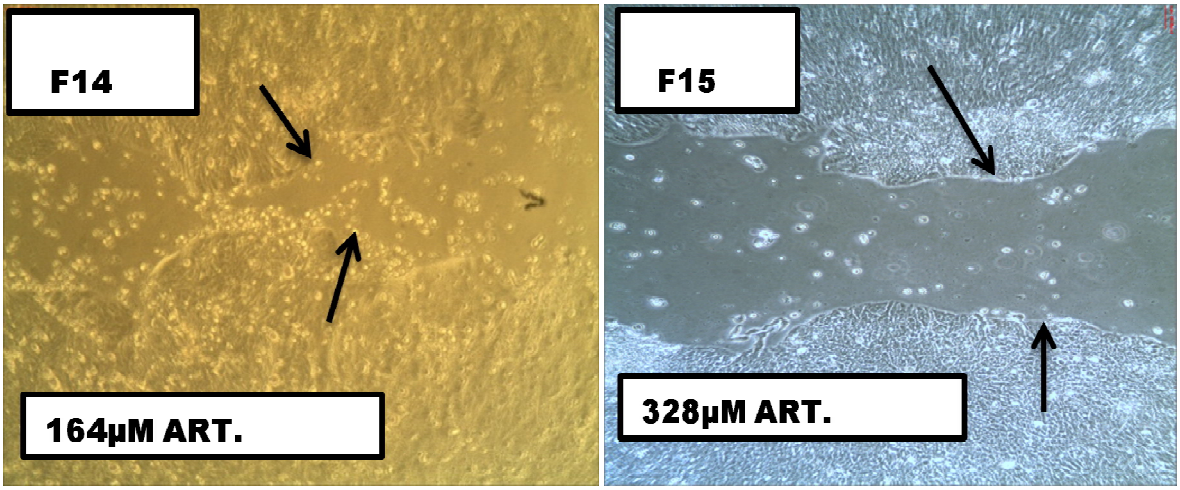
Tumour cell invasion and migration play a major role in metastasis which is a major cause of morbidity and mortality (K Nabeshima, T Inoue, and Y Shima - Pathology, 2002 - Wiley Online Library). Matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases play a crucial role in the metastatic and invasion processes. To evaluate the effects of drug treatment on HT29-AK cell migration/invasion, we investigated the effects of drug treatment on wound healing (Figure 13) as a scratch assay. Cells were first allowed to reach confluency (Figure 13X) and then a uniform wound was created (Figure 13Y) using a sterile P10 pipette tip and the cells were

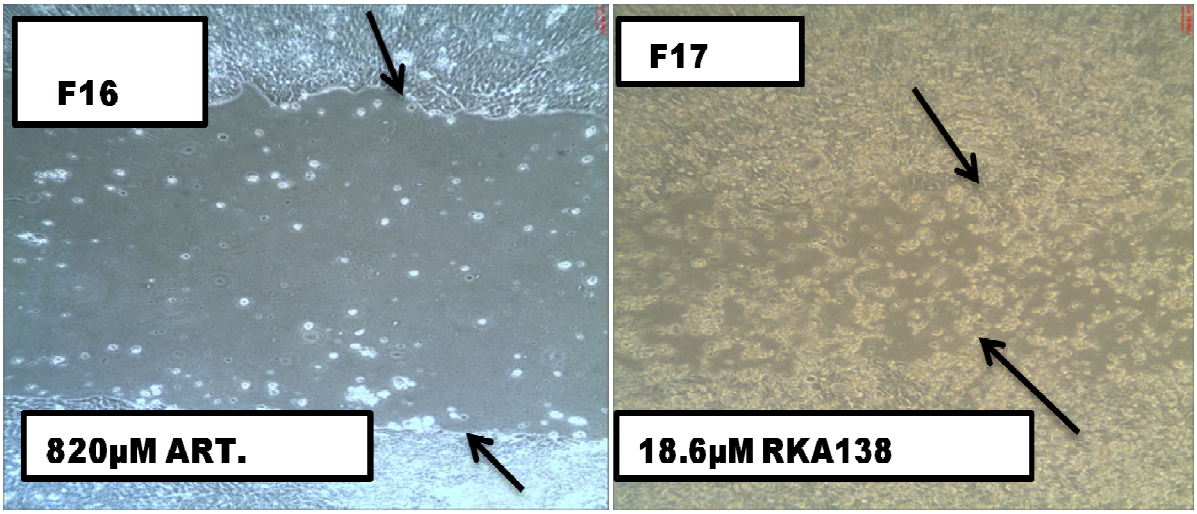
treated without or with four different concentrations of the drugs as shown in F1-F36 following several washings to remove the non-adherent cells. Our observation demonstrates that the initial wound created (see Figure 13Y) was completely closed after 7 days of culture in the media containing no drug (see Figure 13Z). There was a concentration dependent inhibition of the ability of the wound to heal/close in case of all the drugs used for this experiment. At the start of the experiment, the size of the wound was 72.5μm (initial control, Figure 13), but this wound was completely closed upon 7 days of culture without drug treatment (final control, Figure 13). Compared to the final control, the ability of the wound to heal was markedly reduced by artesunate, DHA and other novel compounds (JC3-39, LLP271) in a concentration dependent fashion. Similarly all the tetraoxane dimers (the RKA compounds) caused a concentrations dependent decrease in the ability of the wounds to close (Figure 13). At concentrations corresponding to their IC_{50}s, RKA152 had the weakest effect on the ability of the wounds to close; the following rank order of effectiveness at preventing wound healing was observed: artesunate> RKA138> RKA149> RKA150> RKA158> LLP271> DHA> JC3-39> RKA152 (Figure 13). Overall the data showed that the dimers were more potent at reducing the capacity of the wounds to heal compared to DHA.

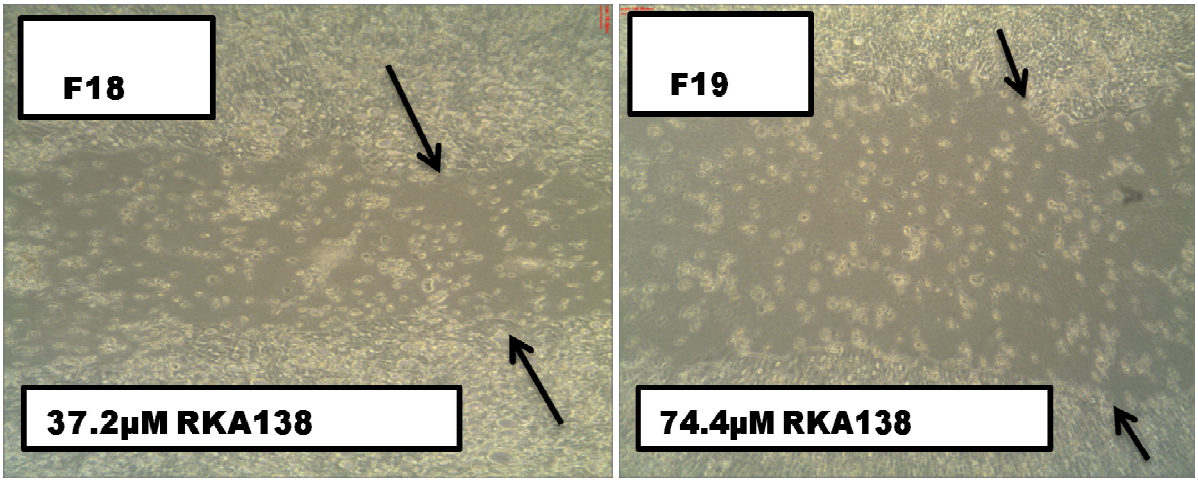

X
CONFLUENT HT29-AK
Y
INTIAL CTRL.
Z
FINAL CTRL.
F1
30.7µM DHA

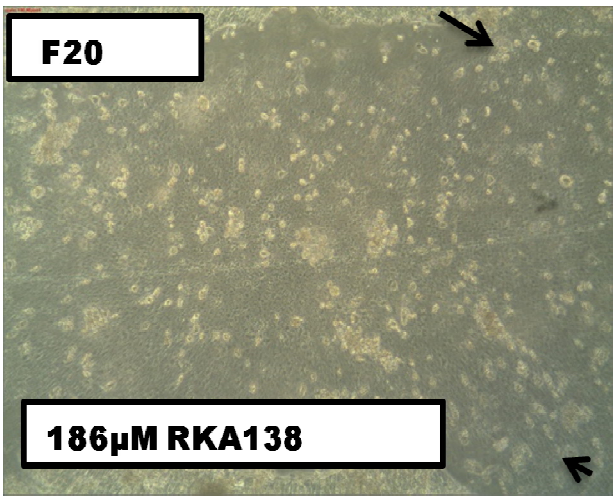

F2
61.4μM DHA
F3
122.8μM DHA
F4
307μM DHA
F5
7.1μM JC339
F5
7.1μM JC339
F7
28.4μM JC339


F8
71µM JC339
F9
8.8µM LLP271
F10
17.6µM LLP271
F11
35.2µM LLP271
F12
88µM LLP271
F13
82µM ART.


F14
164μM ART.
F15
328μM ART.


F16
820μM ART.
F17
18.6μM RKA138


F18
37.2μM RKA138
F19
74.4μM RKA138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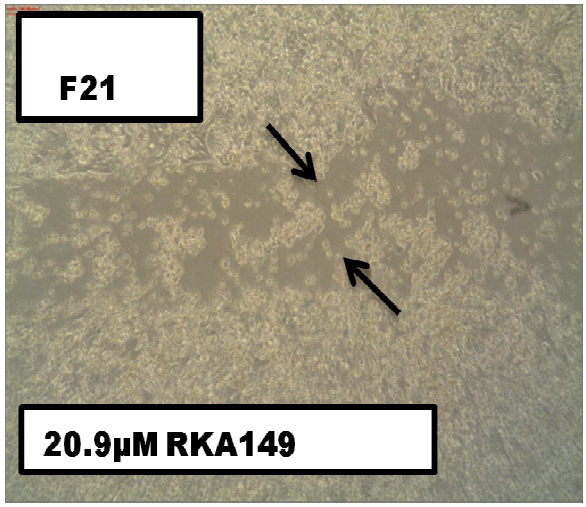
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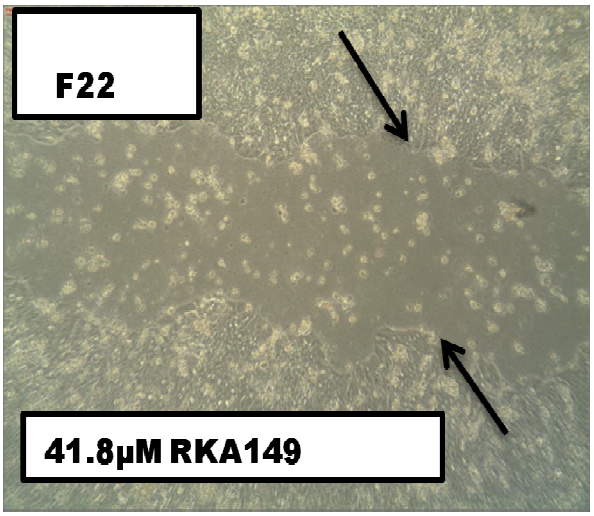
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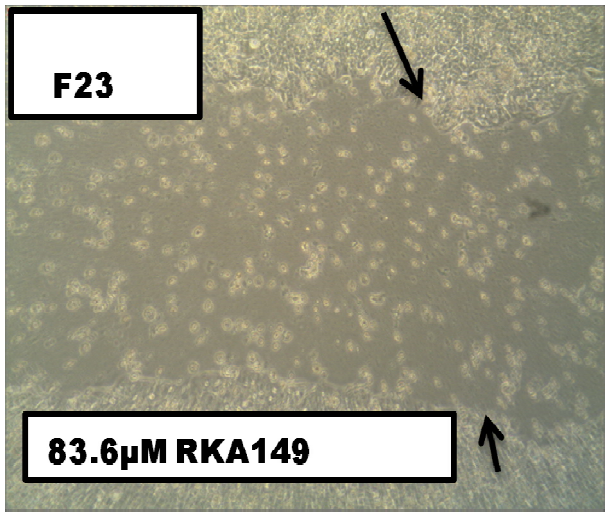
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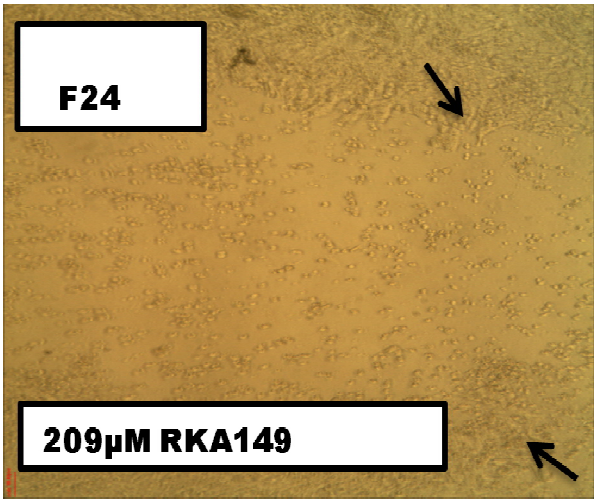
83.6µM RKA149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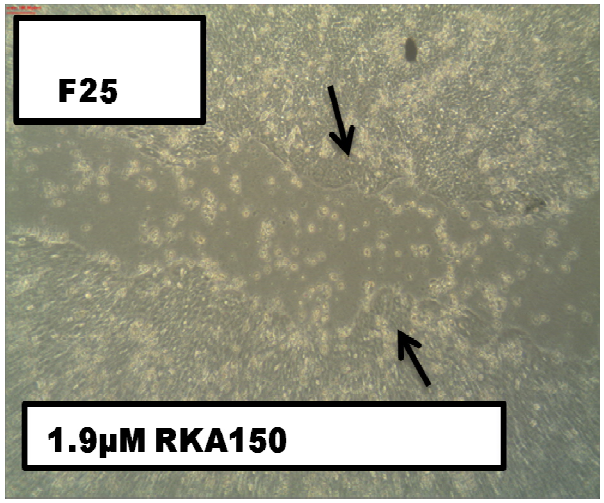
209µM RKA149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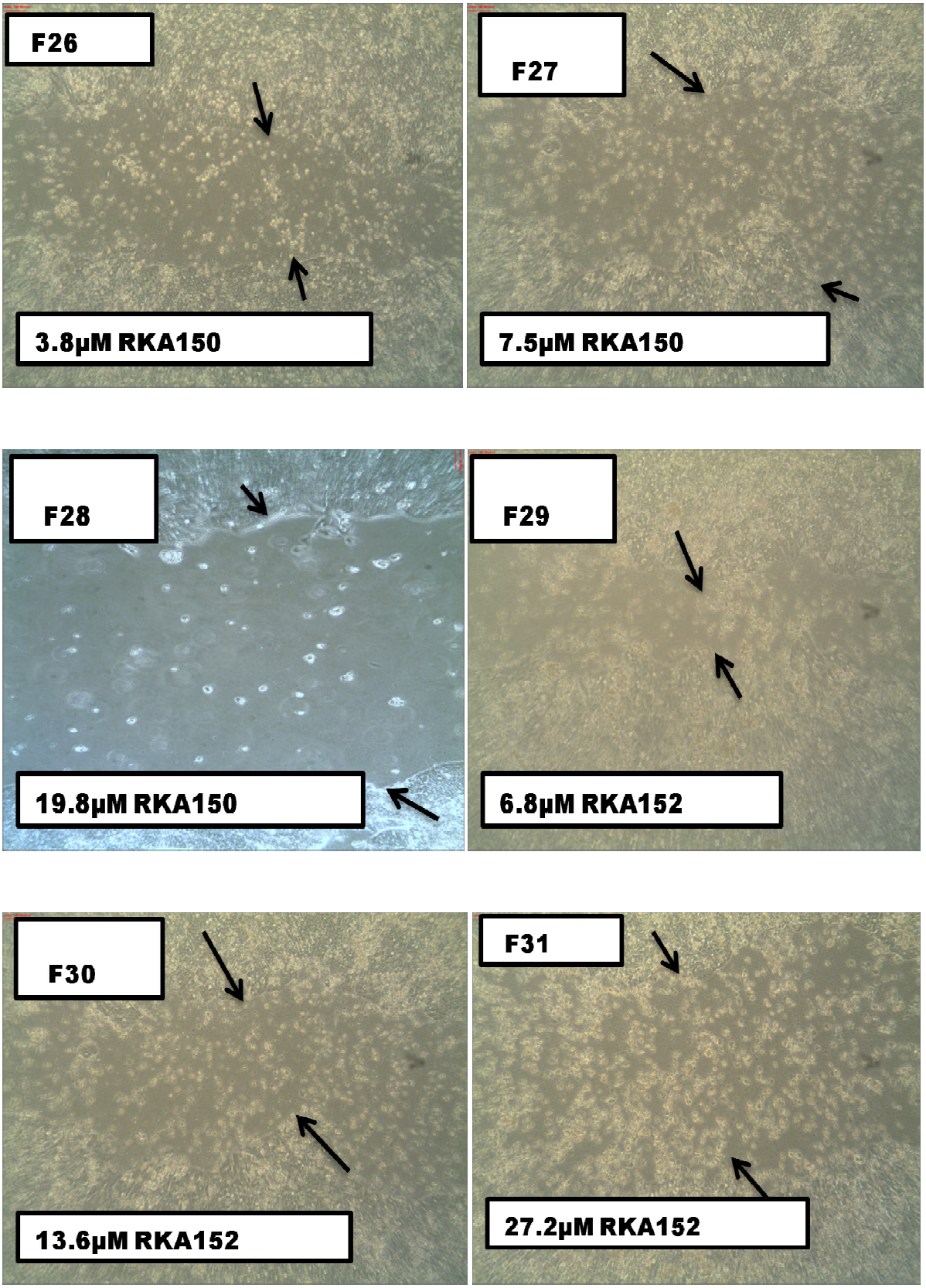
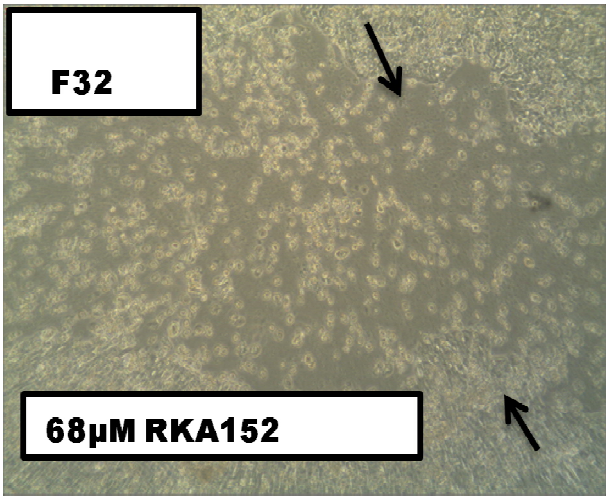
1.9µM RKA150


F26
3.8µM RKA150
F27
7.5µM RKA150
F28
19.8µM RKA150
F29
6.8µM RKA152
F30
13.6µM RKA152
F31
27.2µM RKA152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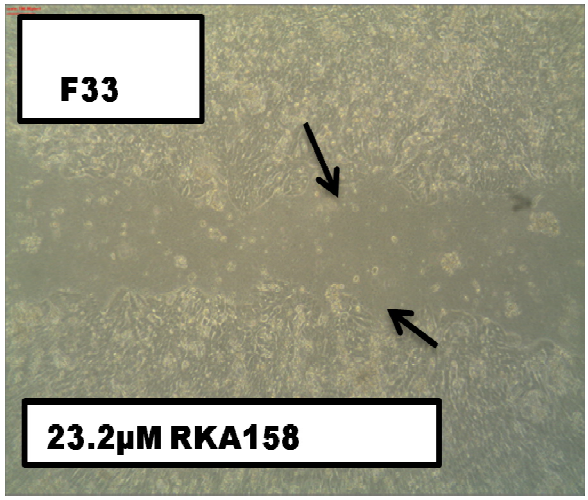
68µM RKA152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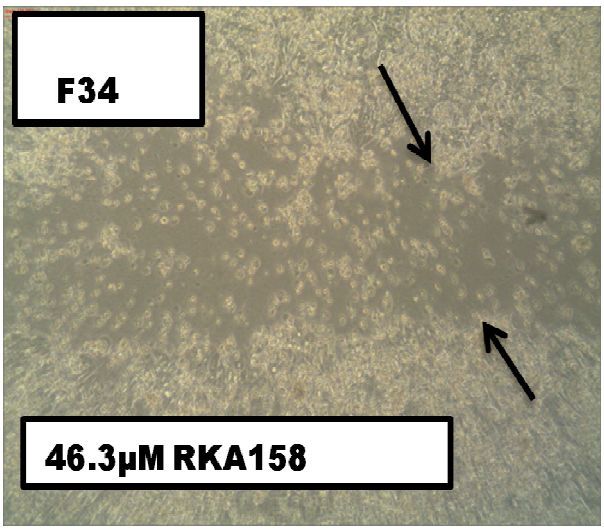
23.2µM RKA158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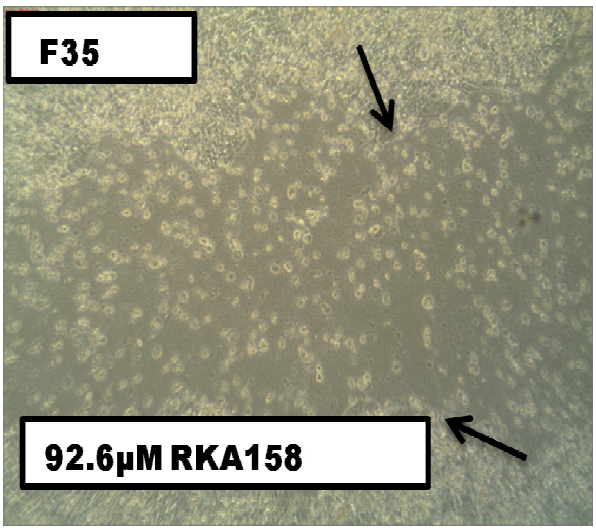
46.3µM RKA158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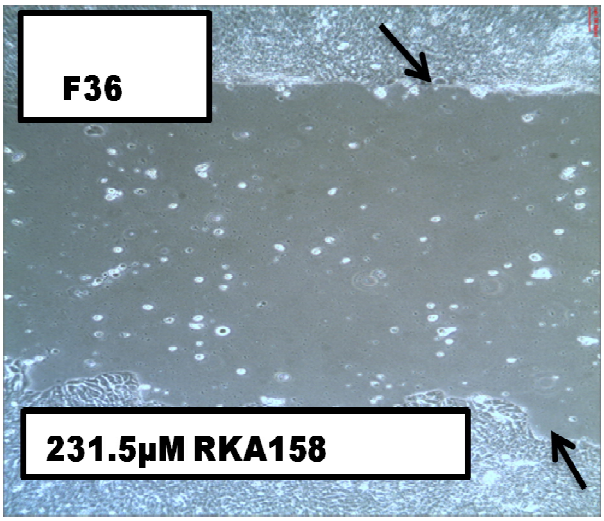

F36
231.5µM RKA158

Figure 13 The light-microscopic images of the concentration-dependent effects of the drugs (DHA, JC3-39, LLP271, artesunate, RKA138, RKA149, RKA150, RKA152 and RKA158) on wound healing in HT29-AK cells. All the pictures were taken using the same 10X magnification. Cells were cultured for 7 days (at 37^0C and 5% CO_2) to allow the establishment of confluency (Figure 13X) and following the creation of a wound using a sterile P10 pipette tip (Figure 13Y). Figure 13Z shows the closure of the wound after 7 days of culture without drug treatment. Effects of 30.7μM, 61.4μM, 122.8μM and 307μM DHA (F1-F4); 7.1μM, 14.2μM, 28.4μM and 71μM JC3-39 (F5-F8); 8.8μM, 17.6μM, 35.2μM and 88μM LLP271 (F9-F12); 80.2μM, 160.4μM 320.8 μM and 802μM artesunate (F13-F16); 18.6μM, 37.2μM, 74.4μM and 186μM RKA138 (F17-F20); 20.9μM, 41.4μM, 83.6μM and 209μM RKA149 (F21-F24); 1.9μM, 3.8μM, 7.5μM and 19.8μM RKA150 (F25-F28); 6.8μM, 13.6μM, 27.2μM and 68μM RKA152 (F29-F32) and 23.2μM, 46.3μM, 92.6μM and 231.5μM RKA158 (F33-F36) on wound healing. Arrows represent the wound area which can be seen to have closed or widened in some of the images.

We also measured the diameter of the wounds and the results are represented in Figure 14. It can be seen that as the concentrations of the test agents increased, the capacity of the wound to heal decreased i.e. the diameter of the wound increased. The initial wound in Figure 14Y was completely closed after 7 days of culture (Figure 14Z). The sizes of the wounds were 6.1μm, 17.5μm, 52.6μm and 76.4μm when cells were treated with 30.7μM, 61.4μM, 122.8μM and 307μM DHA respectively. At the highest concentration of JC3-39 (71μM) we measured a wound diameter of 63.6μm which gradually closed down to 5.7μm when cells were treated with 7.1μM of JC3-39. The wound size was 7.2μm when cells were treated with 8.8μM of LLP271. Similarly, the sizes of the wounds were 23.6μm, 58.8μm and 74.9μm when cells were treated with 17.6μM, 35.2μM and 88μM of

LLP271 respectively. We observed a wound size of 76.8µm when cells were treated with 802µM artesunate. As the concentration of artesunate decreases the wound size decreases as well with the lowest being 14.2µm in case of 80.2µM treatment. The sizes of the wounds were 10.6µm, 46.2µm, 67.4µm and 75.1µm when cells were treated with 18.6µM, 37.2µM, 74.4µM and 186µM RKA138 respectively. Diameters of the wounds were 9.7µm, 21.1µm, 59.2µm and 75.3µm when cells were treated with 20.9µM, 41.4µM, 83.6µM and 209µM RKA149 respectively. We observed a wound size of 73.6µm when cells were treated with 19.8µM RKA150. As the concentration of RKA150 decreases the wound size decreases as well with the lowest being 8.5µm in case of 1.9µM treatment. The measured diameters of wounds were 4.6µm, 27.3µm, 67.6µm and 71.8µm when cells were incubated with 6.8µM, 13.6µM, 27.2µM and 68µM RKA152 respectively. At the lowest concentration of RKA158 (23.2µM), we measured a wound of 7.9µm in diameter. The diameter gradually increased to 75.7µm when cells were treated with 231.5µM of RKA158.

We observed that the agents that are proved to be very much cytotoxic against HT29-AK cell line could not inhibit the wound healing as much as expected (Table 1 and 2 versus Figure 14). This could be because the diameters of the wound that we measured do not represent the average diameter of the wound or the total number of dead cells in the vicinity of the wound. Only two random areas of the wound were measured rather than measuring several regions of the wound. The inadequate mixing of the media while counting the number of dead cells also caused variation. We also observed a lot of cell clumping in samples that were treated with higher concentrations of the drugs. This cell clumping made it impossible to count the accurate cell number in it (for example figures

4D, 9A and 9D). However from the observations it is clear that all the drugs decreased the migration of the cells and caused cell death (Figure 14).

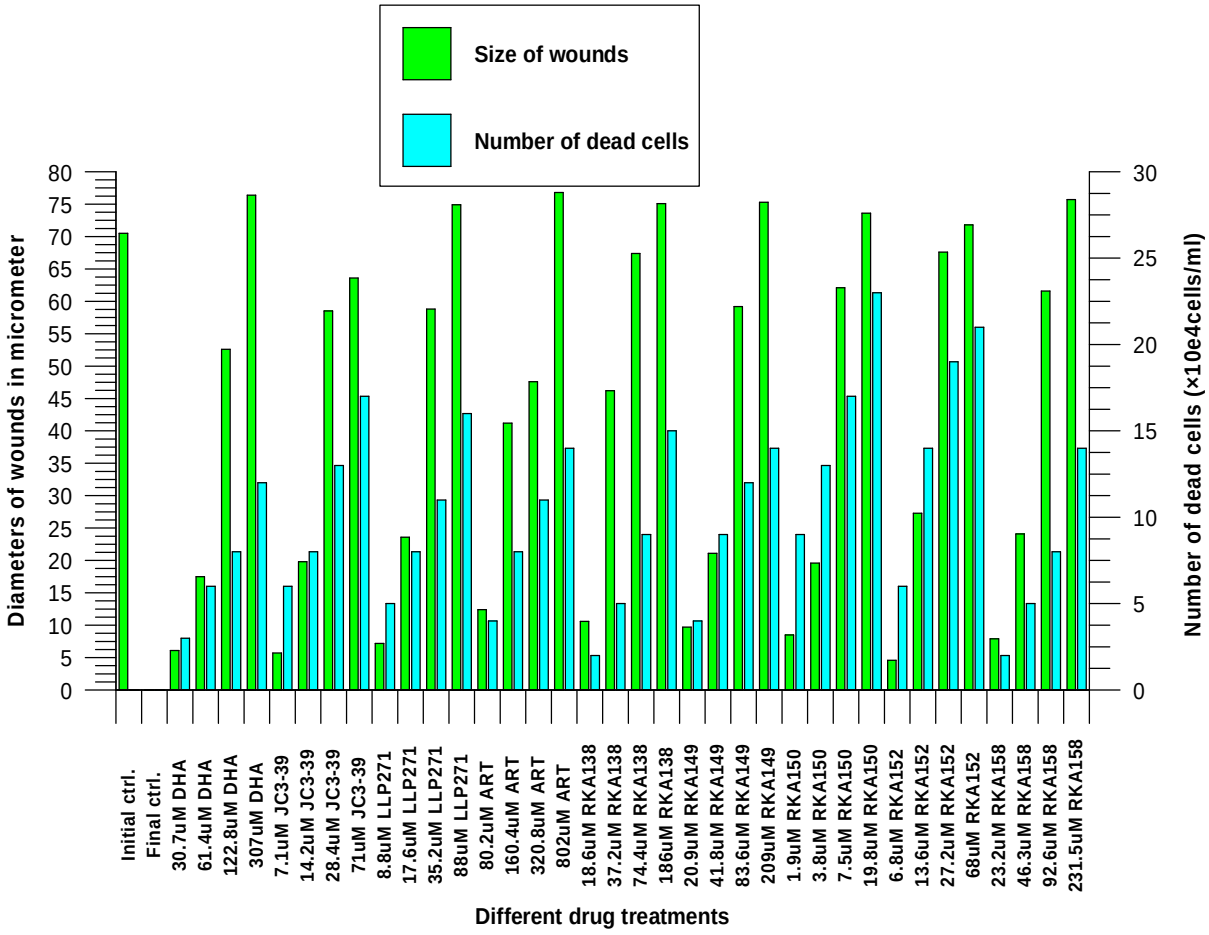


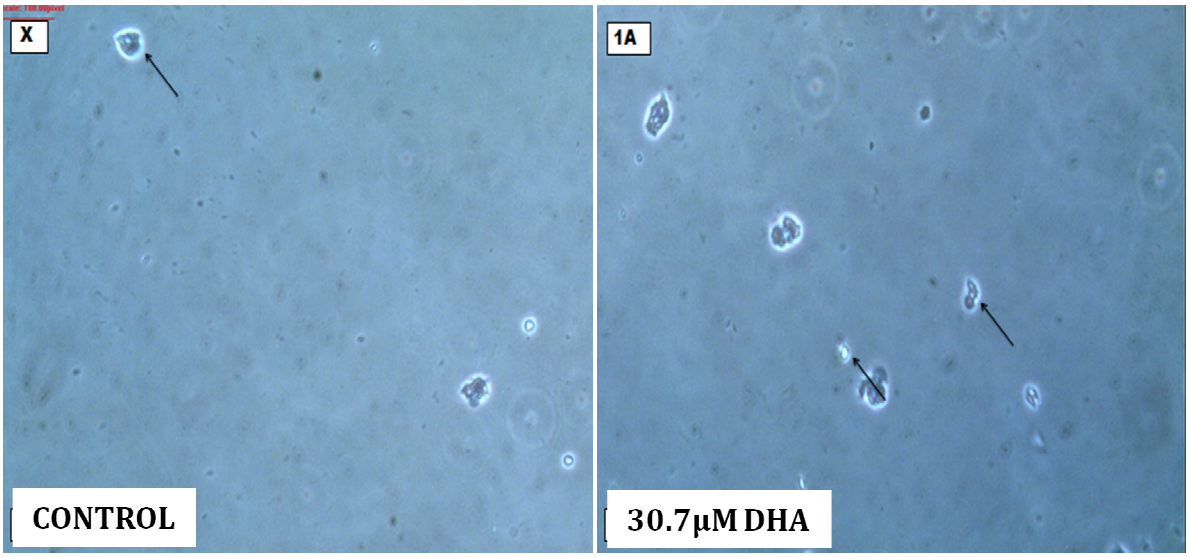
Figure 14 The concentration-dependent effects of the drugs on wound healing in HT29-AK cells after 7 days of treatment without or with the drugs as shown. Upon establishing confluency, wounds were created followed by cells being incubated without or with the drugs for 7 days before the assays were terminated and assessed as described in detail in the materials and methods section. Initial control is the wound size at the start of the experiment whereas final control is the wound size 7 days post incubation without drug but with media changed on day 2. Each bar represents the mean of 2 independent observations. X axis represents the different concentrations of the agents used in this experiment. The left Y axis represents the diameter of the

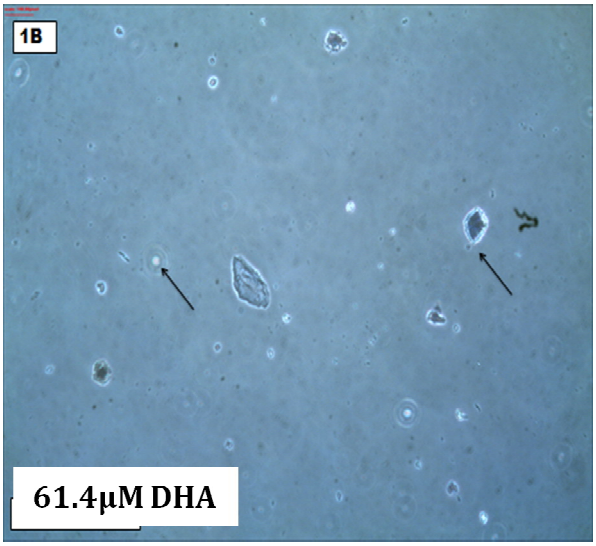
wounds in micrometer unit and the right Y axis represents the corresponding number of dead cells.

As the drugs are shown to be cytotoxic (Table 1and 2) and also decreased the ability of the wound to close, we were interested to find out if the cells that can be seen in the vicinity of the wounds in Figure 13 were dead or alive. By definition, cells undergoing apoptosis should not take up trypan blue. When these dies are taken up in vitro, it's usually due to a process called "secondary necrosis" which is detectable because cells are not engulfed by circulating phagocytes. Using trypan blue as a marker for apoptosis in vitro can be used as an estimate of apoptosis, but it may sometimes considerably underestimate the actual extent of apoptosis taking place, or alternatively, overestimate the amount of apoptosis if necrosis contributes to cell death.

To investigate this we stained an aliquot of the incubation media/cell suspension from each of the respective wells with trypan blue and evaluated them for viable cell count. The results of this experiment are represented in Figure 15. As can be seen in figure 14 (y-axis on right hand side) there was a concentration dependent increase in the number of dead cells compared to control. The number of dead cells was 3×10^4, 6×10^4, 8×10^4 and 12×10^4 cells/ml when the cells were treated with 30.7μM, 61.4μM, 122.8μM and 307μM of DHA respectively. At the lowest concentration of JC3-39 (7.1μM) we obtained a dead cell count of 6×10^4cells/ml which gradually increased as the concentration increases. At the highest concentration of JC3-39 (71μM) there were 17×10^4 dead cells in each ml of the cell suspension. The dead cell densities were 5×10^4, 8×10^4, 11×10^4 and 16×10^4cells/ml when cells were treated with 8.8μM, 17.6μM, 35.2μM and 88μM of LLP271 respectively. We observed 4×10^4 dead cells in each ml of media when cells were treated with 80.2μM artesunate. As the concentrations of artesunate increased the

density of dead cells increased as well with the highest being 14×10^4cells/ml in case of 802µM treatment. The number of dead cells was 2×10^4, 5×10^4, 9×10^4 and 15×10^4 cells/ml when the cells were treated with 18.6µM, 37.2µM, 74.4µM and 186µM of RKA138 respectively. Number of dead cells was 4×10^4, 9×10^4, 12×10^4 and 14×10^4 cells/ml when the cells were treated with 20.9µM, 41.4µM, 83.6µM and 209µM RKA149 respectively. We measured a dead cell density of 9×10^4cells/ml when the cells were exposed to 1.9µM RKA150. As the concentration of RKA150 increases the number of dead cells increases as well with the highest being 23×10^4 cells in each ml of suspension in case of 19.8µM treatment. The measured number of dead cells were 6×10^4, 14×10^4, 19×10^4 and 21×10^4 cells/ml when the cells were incubated with 6.8µM, 13.6µM, 27.2µM and 68µM of RKA152 respectively. At the lowest concentration of RKA158 (23.2µM), we measured a dead cell density of 2×10^4cells/ml. The density gradually increased to 14×10^4cells/ml when cells were treated with 231.5µM of RKA158.

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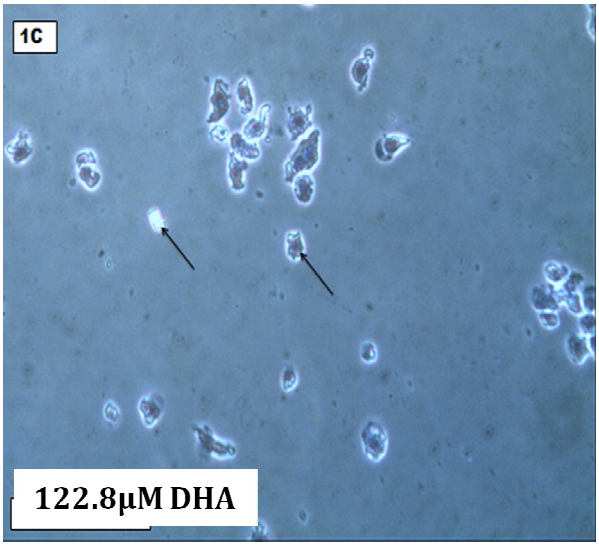
61.4µM DHA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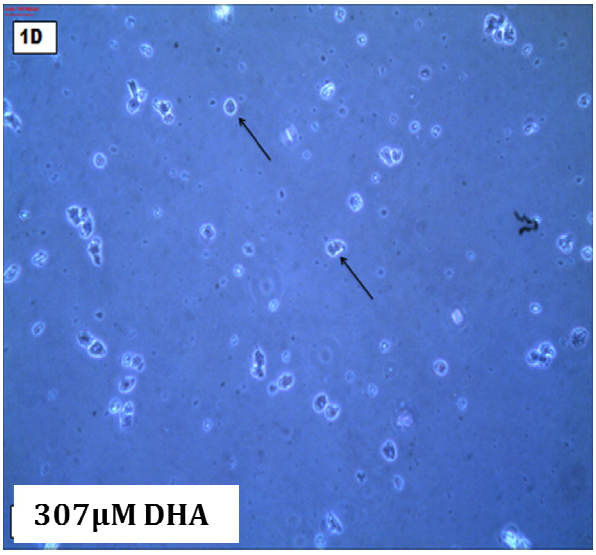
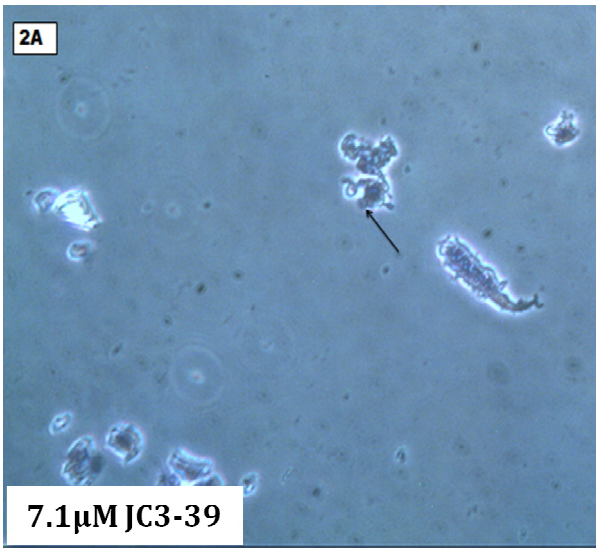
122.8µM DHA


1D
307µM DHA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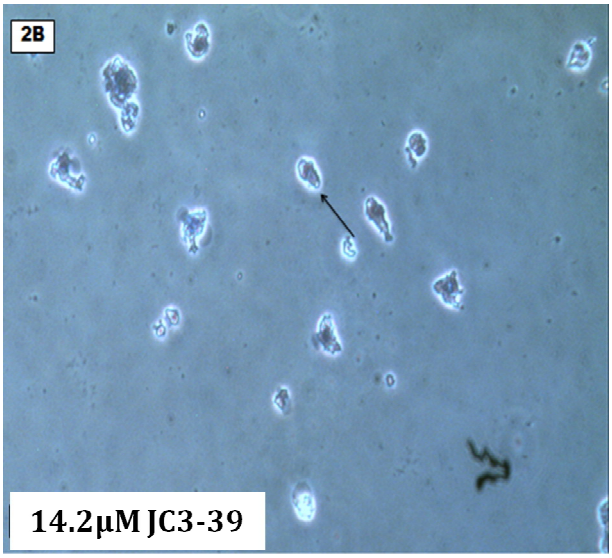
7.1µM JC3-39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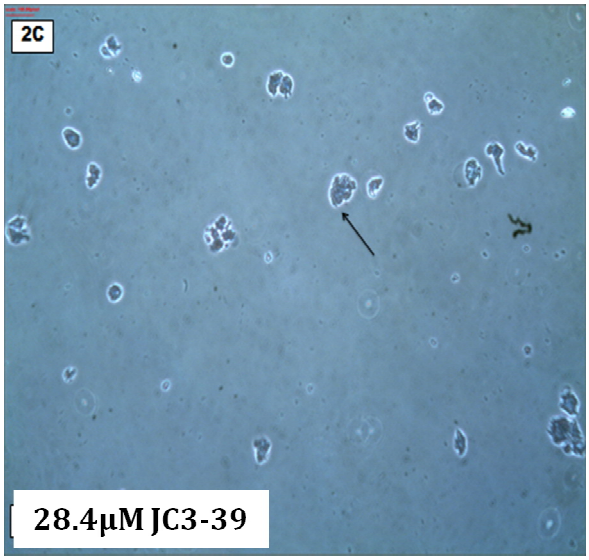
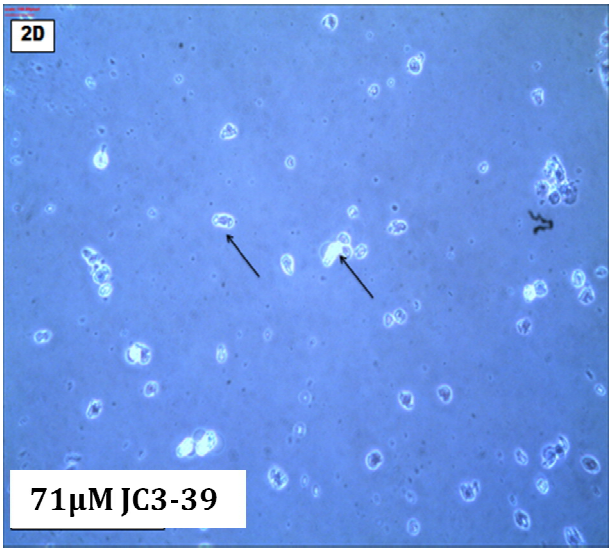
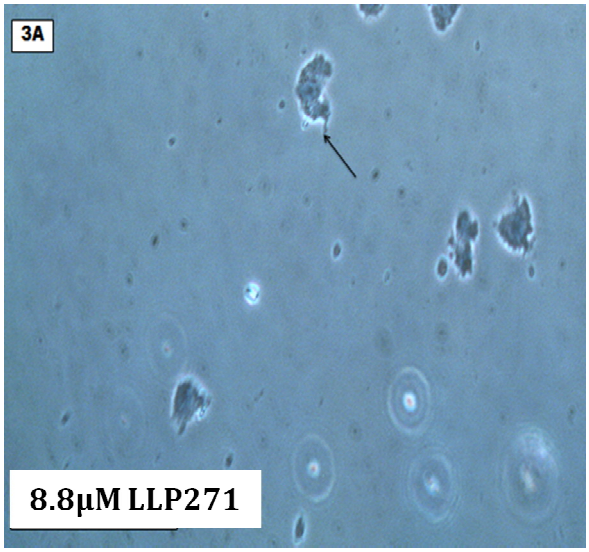
14.2µM JC3-39


2C
28.4µM JC3-39


2D
71µM JC3-39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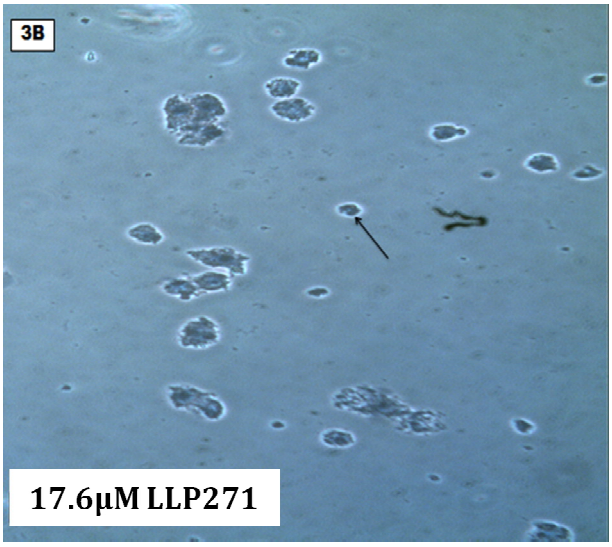
8.8µM LLP271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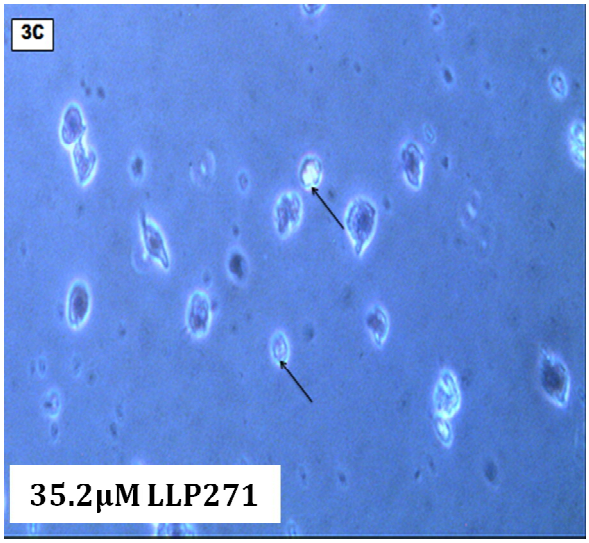
17.6µM LLP271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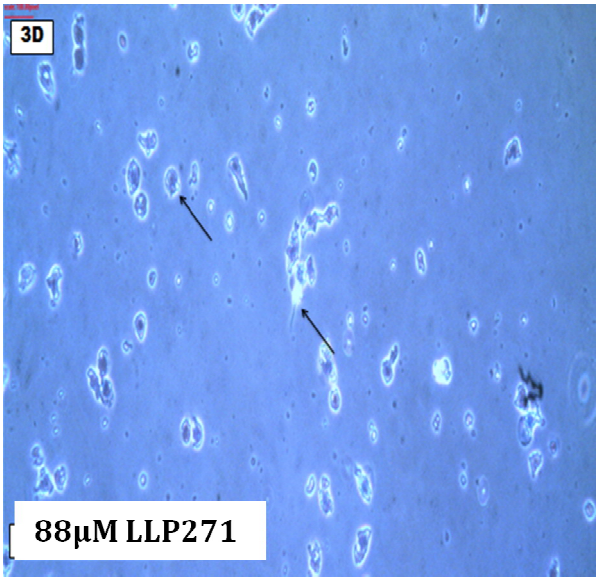
35.2µM LLP271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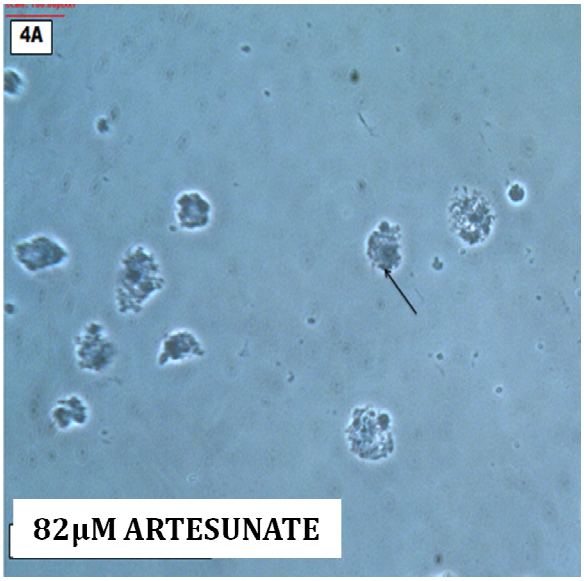
88µM LLP271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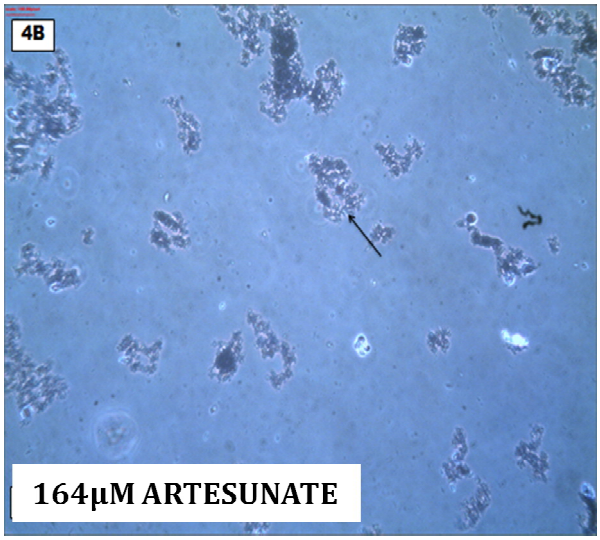
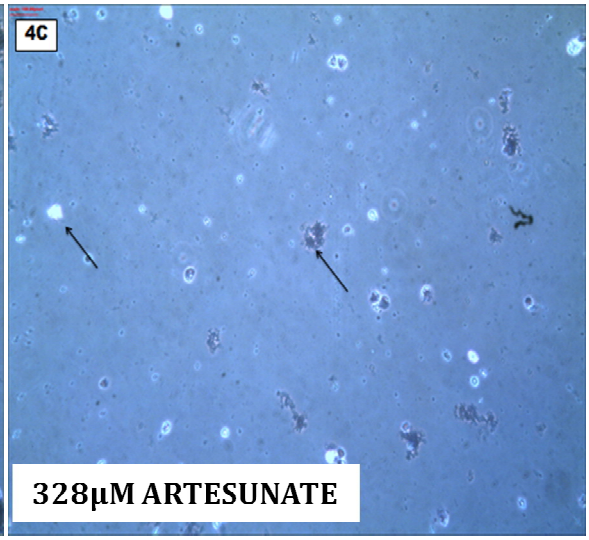
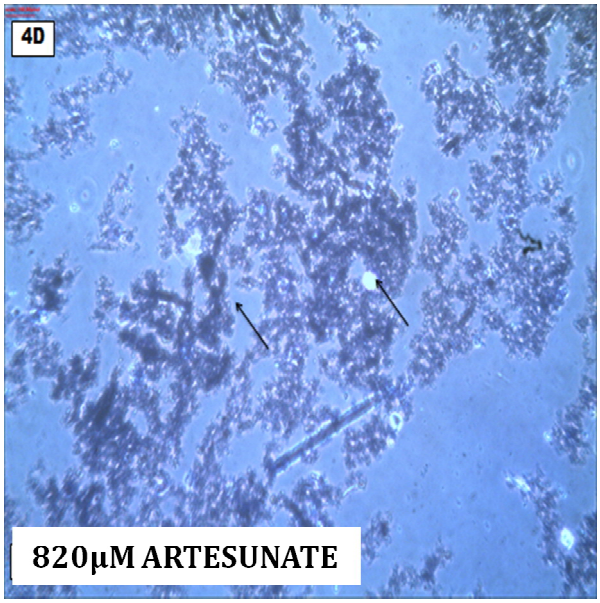
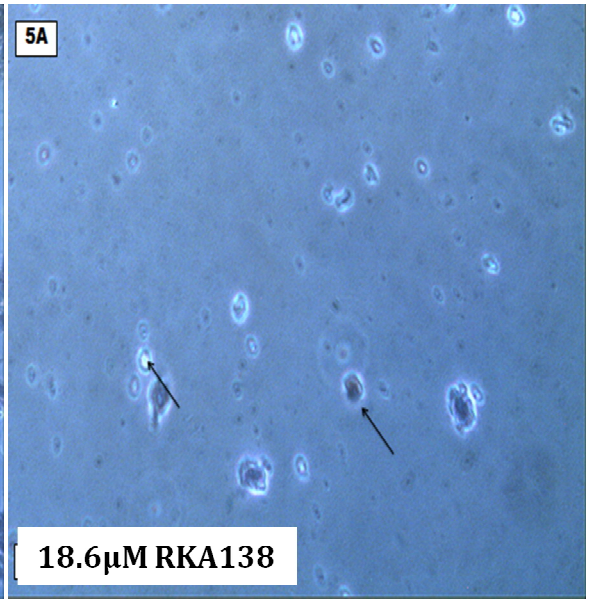
82µM ARTESUNATE


4B
164µM ARTESUNATE


4C
328µM ARTESUNATE


4D
820µM ARTESUNATE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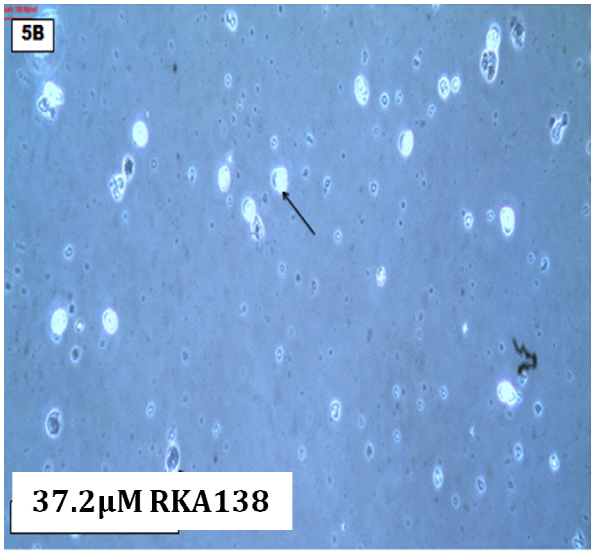
18.6µM RKA138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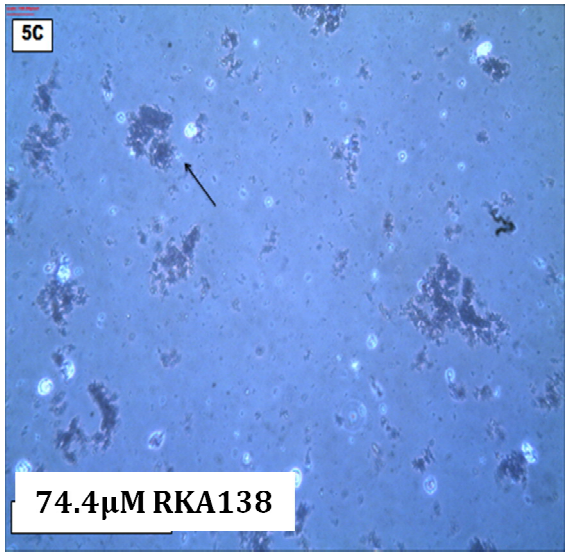
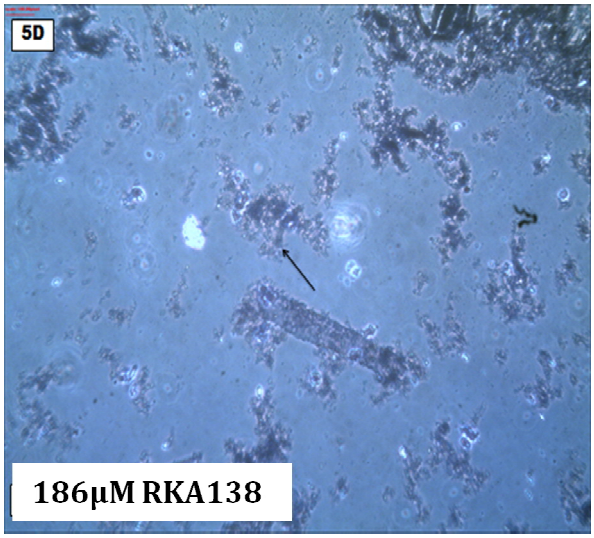
37.2µM RKA138


5C
74.4µM RKA138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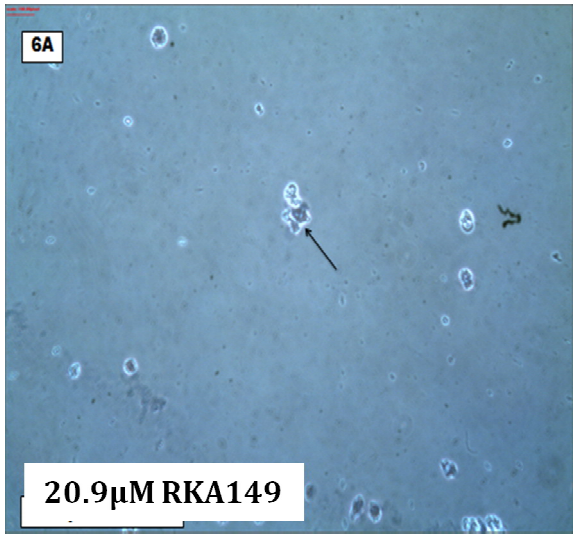
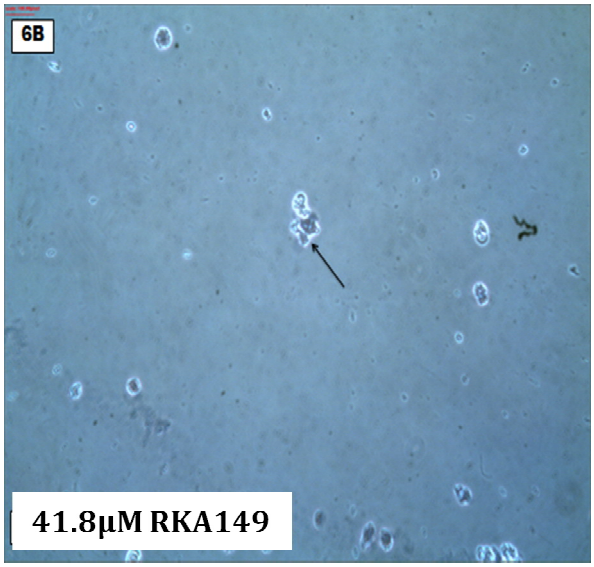
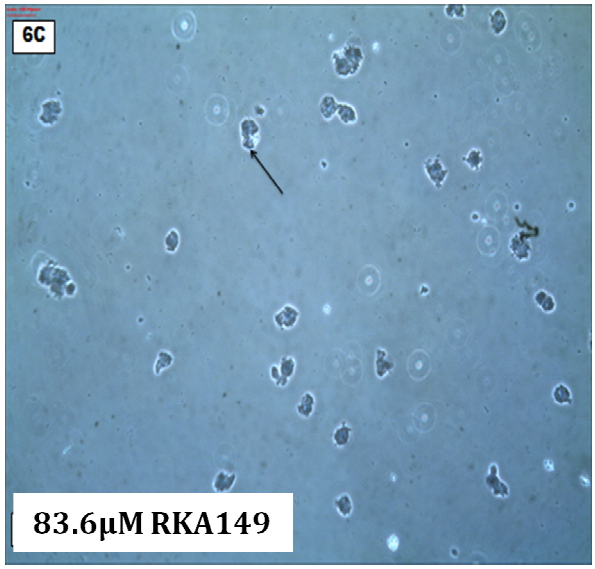
186µM RKA138


6A
20.9µM RKA149


6B
41.8µM RKA149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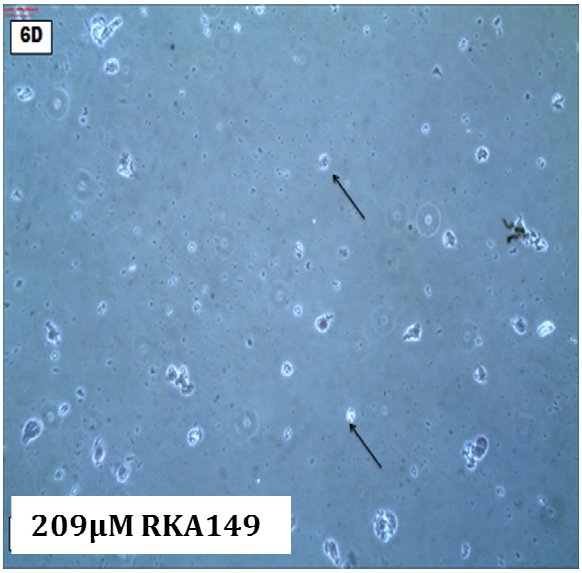
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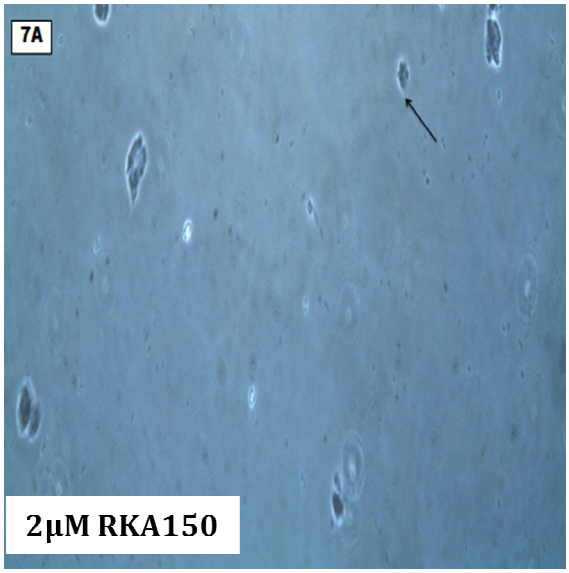
209µM RKA149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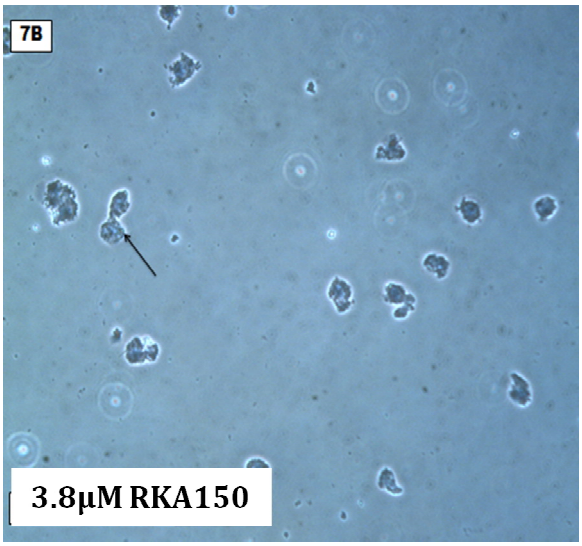
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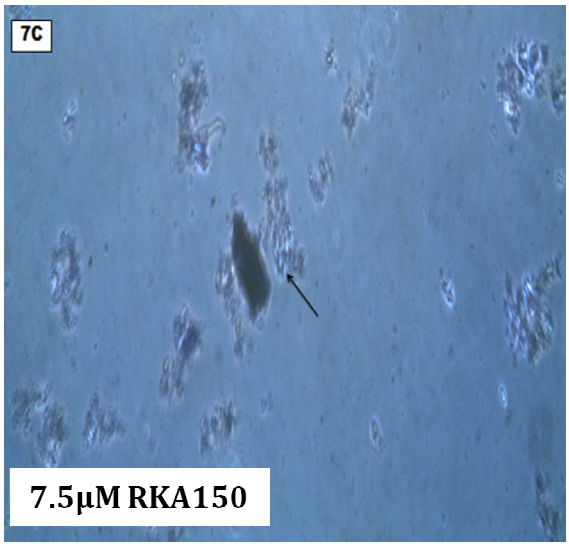
3.8µM RKA150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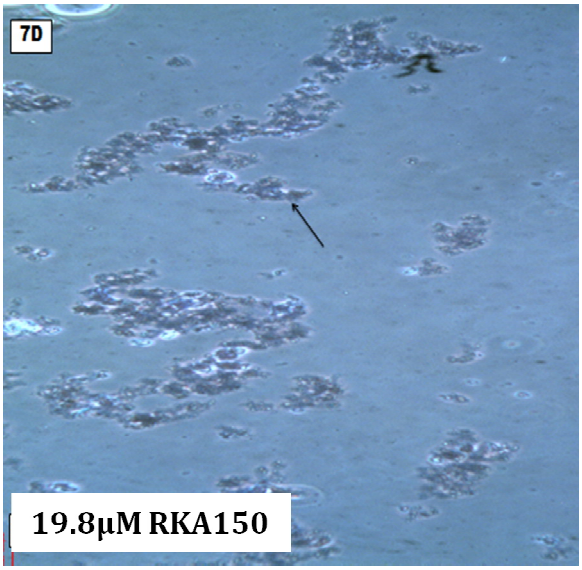
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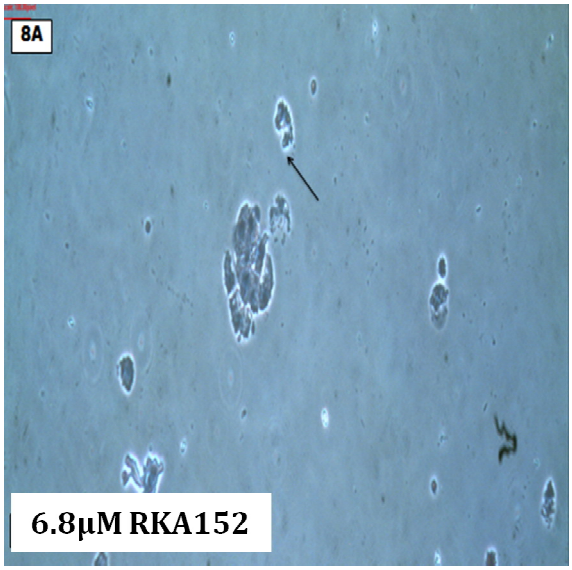
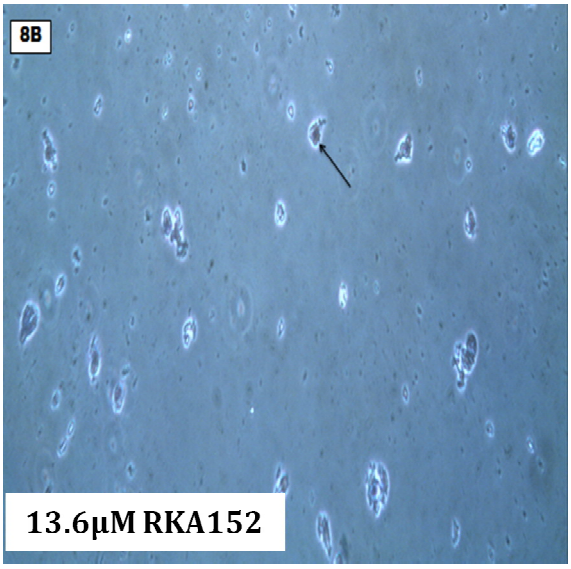
19.8µM RKA150


8A
6.8µM RKA152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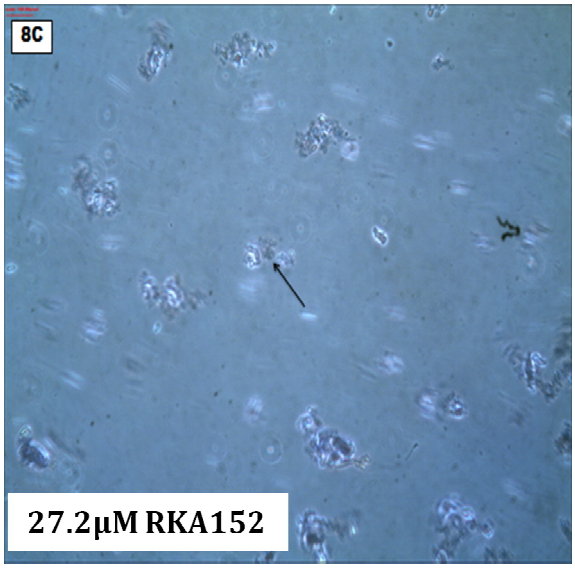
13.6µM RKA152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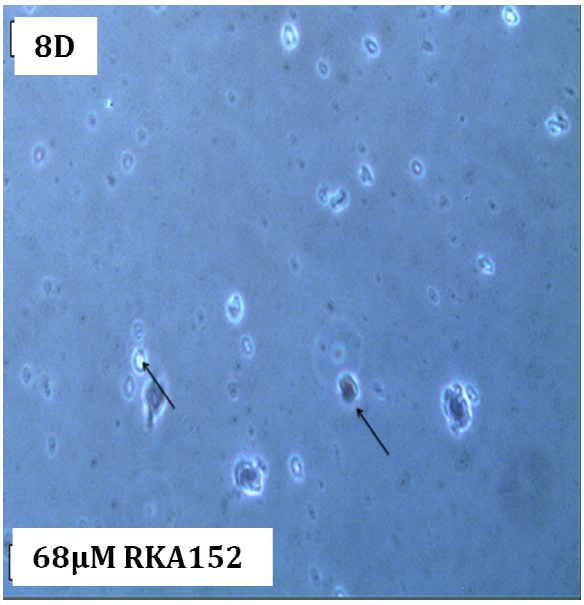
27.2µM RKA152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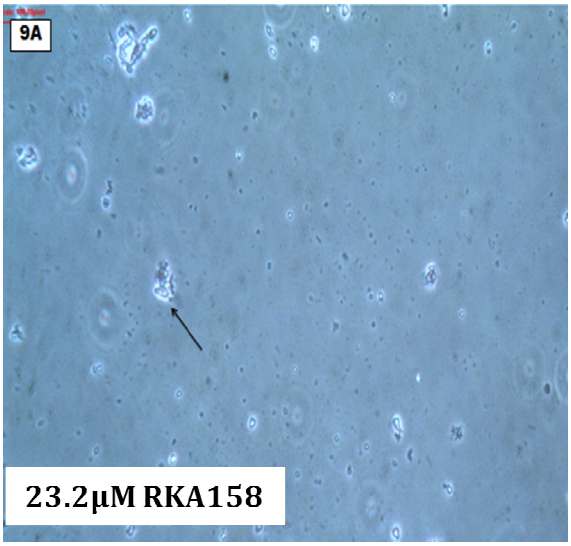
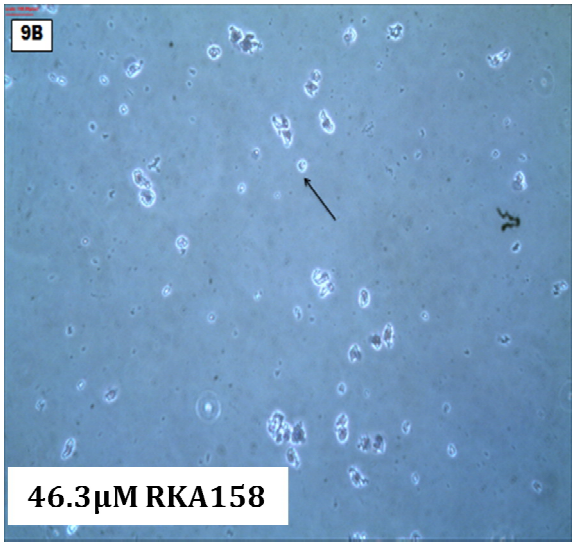
68µM RKA152


9A
23.2µM RKA158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46.3µM RKA158

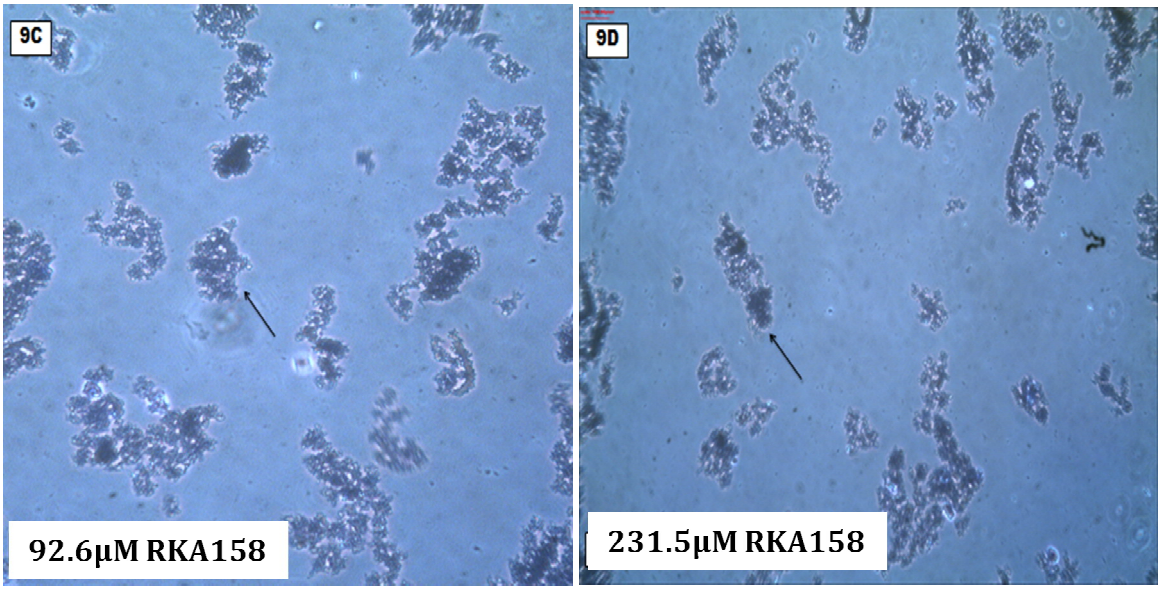

Figure 15 Representative light-microscopic (all pictures were taken using 10X magnification) images of the trypan blue exclusion test for DHA, JC3-39, LLP271, artesunate, RKA138, RKA149, RKA150, RKA152 and RKA158 against HT29-AK cells. Effects of 30.7µM, 61.4µM, 122.8µM and 307µM DHA (1A-1D); 7.1µM, 14.2µM, 28.4µM and 71µM JC3-39 (2A-2D); 8.8µM, 17.6µM, 35.2µM and 88µM LLP271 (3A-3D); 80.2µM, 160.4µM 320.8 µM and 802µM artesunate (4A-4D); 18.6µM, 37.2µM, 74.4µM and 186µM RKA138 (5A-5D); 20.9µM, 41.4µM, 83.6µM and 209µM RKA149 (6A-6D); 1.9µM, 3.8µM, 7.5µM and 19.8µM RKA150 (7A-7D); 6.8µM, 13.6µM, 27.2µM and 68µM RKA152 (8A-8D); 23.2µM, 46.3µM, 92.6µM and 231.5µM RKA158 (9A-9D) on wound healing. Arrows represent dead cells with dark blue colour and live cells with bright colour.

Effect of drug treatments on the secretion of pro-inflammatory cytokine TNF-α in HT29-AK cell:

To further investigate the mechanism of drug-induced cell death, the release of TNF-α was measured and the results are illustrated in Figure 16. The cells released TNF-α with

control values ranging from 24±0.9pg/ml to 64±1.04pg/ml. Overall, the effects of the test agents on TNF-α secretion were very variable. There were no significant alterations in TNF-α concentration when cells were treated with 15.3μM and 30.7μM of DHA as compared to control. However, the TNF-α concentration was significantly (P<0.05) increased as compared to control upon treating the cells with 61.4μM of DHA (Figure 16A). The cellular TNF-α concentration was significantly (P<0.001) increased in samples treated with 3.5μM of JC3-39 as compared to control (296±1.5pg/ml vs. 64±1.04pg/ml). On the other hand the cellular TNF-α concentration significantly (P<0.01) decreased to 15.2±0.3pg/ml in cells treated with 7.1μM of JC3-39 as compared to control. 14.2μM of JC3-39 treated samples did not alter the TNF-α concentration (Figure 16B). There was no significant change in the TNF-α level from control when cells were treated with 4.4μM of LLP271. However there was a ~3.5 and ~11.1 fold increase in cellular TNF-α concentration in cells treated with 8.8μM and 17.6μM LLP271 respectively as compared to control (Figure 16C).

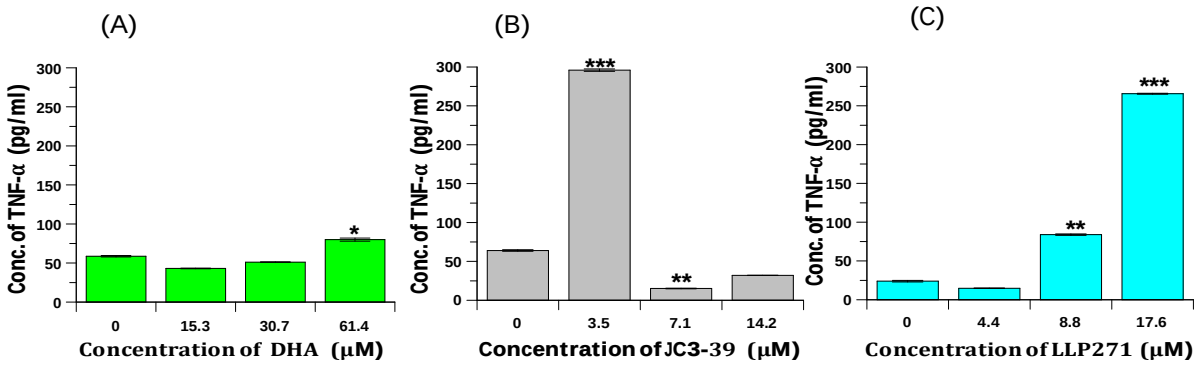

Figure 16 Representing pro-inflammatory cytokine TNF-α concentration in HT29-AK cells treated without or with DHA, JC3-39 and LLP271. HT29-AK cells were treated without or with different concentrations of drugs as shown above for 24 hours (at 37^0C and 5% CO_2) before TNF-α levels were measured by ELISA. Results are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test.

Effects of drug treatments on the secretion of IL-6 in HT29-AK cells:

The effects of drug treatment on the secretion of IL-6 in HT29-AK cells are illustrated in Figure 17. Although some IL-6 was detected (values ranging from 0.09±0.02pg/ml to 0.23±0.02pg/ml) in the control cells, none of the concentrations of DHA tested against HT29-AK cells significantly altered the cellular level of IL-6 as compared to control. Variable levels of cellular IL-6 concentrations of 0.09±0.02pg/ml, 0.12±0.06pg/ml, 0.078±0.01pg/ml and 0.11±0.03pg/ml were measured upon treating HT29-AK cells with 0μM, 15.3μM, 30.7μM and 61.4μM DHA respectively (Figure 17A). There was no

alteration in IL-6 concentration when cells were treated with 3.5μM JC3-39. However, the IL-6 concentration was significantly (P≤0.01) increased when cells were treated with 7.1μM and 14.2μM JC3-39 as compared to control (0.4±0.1pg/ml and 0.29±0.03pg/ml vs. 0.14±0.03pg/ml) (Figure 17B). When HT29-AK cells were treated with 4.4μM LLP271 the IL-6 concentration was significantly (P<0.01) increased as compared to control (0.3±0.1pg/ml vs. 0.23±0.02pg/ml). However, the IL-6 concentration was decreased by ~1.2-fold as compared to control when cells were treated with 8.8μM LLP271. There was a significant (P<0.01) increase in cellular IL-6 concentration in samples treated with 17.6μM LLP271 as compared to control (Figure 17C).

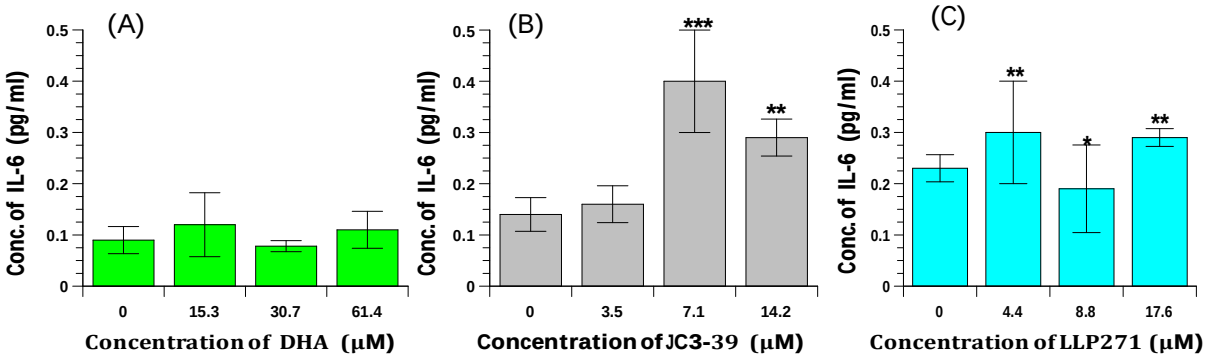

Figure 17 The effects of the test agents on IL-6 secretion in HT29-AK cells. Cells were treated without or with (A) DHA (15.3μM, 30.7μM, 61.4μM); (B) JC3-39 (3.5μM, 7.1μM and 14.2μM) and (C) LLP271 (4.4μM, 8.8μM and 17.6μM) for 24 hours before the cellular IL-6 levels were measured by ELISA following the manufacturer's

(www.rndsystems.com) protocol. Results are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 as tested by Mann-Whitney U test.

DISCUSSION

Artemisinin and its derivatives (artesunate, dihydroartemisinin, arteether, artemether, etc) are effective first-line anti-malarial agents for the treatment of both drug-sensitive and resistant strains of *P. falciparum* (Efferth *et al.,* 2002; Chen *et al.,* 2004; Soomro *et al.*; 2011). Interestingly, these agents have shown potent cytotoxic activities against many types of human cancer cell lines and they have been extensively studied as promising candidates for cancer therapy (Efferth, 2005; Mercer *et al*., 2007; Lu *et al*., 2008). Through collaborative research we obtained novel agents which have been rationally designed to incorporate metabolically stable endoperoxide moieties as well as improved DNA-targeting properties. This study demonstrates the evaluation of the cytotoxicity and mode of action of novel compounds LLP271, JC3-39 (hybrids of DHA which are rationally designed from DHA and dipyrrole) and tetraoxane dimers RKA compounds (RKA138, RKA149, RKA150, RKA151, RKA152, RKA155, RKA158, RKA160) against HT29-AK (colon cancer cells) and HL60 (human leukaemia cells). Given that iron is central to the mode of action of 1, 2, 4-trioxanes (the artemisinins) and the tetraoxane dimers (i.e. incorporating more C-O-O-C in the structure) they are designed

to liberate reactive biomolecules upon reductive cleavage by cellular iron. In pharmaceutical research there is a great improvement in the process of drug discovery and development by rational drug designing (Ette *et al.,* 2004; Galeazzi, 2009) and we recently reported the synthesis and antitumour activities of DHA-acridine hybrids/with improved activities over the parent drug DHA (Jones *et al*., 2009).

Iron has also been reported to be essential in anti-tumour activity of artemisinin and its derivatives in several human malignancy cell lines (Moore *et al.,* 1995; Posner *et al.,* 1997; Beekman *et al.,* 1997; Beekman *et al*., 1998; Efferth *et al.,* 2001; Jeyadevan *et al.,* 2004; Mercer *et al*., 2007; Parekh *et al.,* 2009). The highest cytotoxic activity of these endoperoxides were reported towards human colon and leukaemia malignancy cells which are most sensitive, rapidly proliferating cells with high expression of transferrin receptors needed for endocytosis-mediated iron intake (Disbrow *et al*., 2005).

DHA was the most potent among all the test agents against HL60 cells and the potency of the tetraoxane compound RKA150 was evaluated to be the highest of all the test agents against HT29-AK cells (Table 1 and 2) suggesting that the agents have differential cytotoxic effects against the cells. Although leukaemic cells are believed to contain more cellular iron (Li and Zhou, 2007*;* Mercer *et al*., 2007*;* Mu *et al*., 2007) and hence more susceptible to the cytotoxic effects of 1, 2, 4-trioxanes (Singh and Lai, 2001), it is probable that the presence of more endoperoxide bridges, as in the tetraoxane dimers, does not necessarily translate into greater cytotoxicity. This may possibly be due to the unavailability of sufficient cellular iron to reductively cleave all of the peroxide bridges, generating the carbon-centred radicals which ultimately kill the cells. Alternatively, it may be due to the reduced capacity of these agents to enter the cells, due to steric hindrance. The increased cytotoxicity of JC3-39 may be due to both,

potent DHA and minor groove binding dipyrrole. This cytotoxicity may be due to ROS-mediated damage or the damage may be independently associated with oxidative stress (Zizak *et al.,* 2009; Kumar, Sharma and Rawat, 2011). There is evidence that 1, 2, 4-trioxanes may interact with cellular DNA (Jones *et al*., 2009) and it is possible that the introduction of the dipyrrole skeleton of JC3-39 may have pharmacoenhanced DNA targeting and thus the cytotoxicity of JC3-39.

sTfR expression is increased to facilitate iron intake, and there is evidence that its levels positively correlates with the aggressiveness of tumouric cells (Beguin *et al.,* 1988; Singh and Lai, 2001; Lai *et al.,*2005). There is a constant relationship between total TfR and sTfR and concentration of sTfR is an indirect measure of TfR (Beguin *et al.,* 1988; Singh and Lai, 2001; Lai *et al.,* 2005). Thus measurement of sTfR may be a surrogate marker of the growth of the cells and may also explain the observed cytotoxic effects of the agents. The effects were variable in case of all the test agents in the current study. There were measurable amount of sTfR in HT29-AK cells with the concentrations ranging from 4.3±0.5nmol/L to 5.9±0.6nmol/L but the effects of the test agents were variable (Figure 7). However, DHA (at $15.3\mu M$) only showed a weak reductive effect on sTfR levels, with higher concentrations ($30.7\mu M$ and $61.4\mu M$) lacking effects. When cells were treated with LLP271, there was a decrease in sTfR level at lowest concentration ($4.4\mu M$) while at higher concentrations ($8.8\mu M$ and $17.6\mu M$) the sTfR level was increased (Figure 7C). At the highest concentration of JC3-39 ($14.2\mu M$) there is decrease in sTfR level as compared to the lower doses ($3.5\mu M$ and $7.1\mu M$) (Figure 7B). It may be that the addition of lysis buffer alters the sTfR concentration by over-diluting the cells and/or all the cells were not lysed properly to release sTfR. Other possibility is may be the cells are dead due to the effect of the drug, hence, showing less sTfR. The increase in

concentration of receptors may be to enhance the delivery of drug via endocytosis and decrease in receptor level may show that drug is targeted to block the receptors or lowering the level of receptors in order to kill the higher proliferative cancer cells (Efferth, 2005; Cheung *et al*., 2010; Lee *et al*., 2010). The further future studies of sTfR upon the drugs treatment may lead to better understanding of their cytotoxicity and mode of action.

P-AKT is the apoptotic inhibitor promoting the cell survival and proliferation and plays a vital role in a number of cell signalling pathways, regulating pro-survival genes in many cancers (O'Gorman *et al.,* 2000; Testa and Tsichlis, 2005; De Souza, Russell and Kearsley, 2009). An in-vitro study in human prostate malignant cells and colorectal cancer cells has correlated the DHA-mediated apoptosis with the inhibition of the P13-Kinase/P-AKT (an upstream component of P-AKT) and extracellular signal regulated kinase survival pathway (He *et al.,* 2010). In another study, artesunate in human rheumatoid arthritis cells suppressed P-AKT pathway (Ghani *et al*., 2006; Chen, 2011). P-AKT activation includes many stimuli, such as IL-6 or to most chemotherapeutical therapeutics and has been found to decrease apoptosis by alteration of NF-κb pathway (Efferth *et al*., 2004; Singh and Lai, 2006). At all of the concentrations of the test agents, there was a significant increase in levels of P-AKT as compared to control upon treating HT29-AK cells with DHA ,JC3-39 and LLP271 (Figure 8). This increase in P-AKT level may be due to the cell survival pathway via drug resistance. In-vitro studies determined that p13k/P-AKT pathway controls cell survival and drug resistance in HT29-AK and HT29-RDB (drug resistance) colon cancer cells (Tamm *et al*., 1998; Dohi *et al.,* 2004; McNeish *et al.,* 2005; Lu, Luo and Tao, 2007; Ryan,

O'Donovan and Duffy, 2009). Further studies of LLP271 and JC3-39 in the presence of P-AKT inhibitors may clarify the effects of these agents on P-AKT activity.

Many studies have shown that anticancer agents prevent tumour promotion and progression via the induction of apoptosis (Huang *et al.,* 2006). Apoptosis involve the induction of cleaved caspases-3 and 7-dependent apoptotic cell death, mitochondrial endoplasmic reticulum stress, induction of cell cycle arrest at G_O/G_1 phase and subsequent DNA fragmentation (Parekh *et al.,* 2009; Zhang, Chen and Gerhard, 2010; Morrissey *et al.,* 2010; Alessandro *et al.,* 2011). Caspase-3 is involved in both intrinsic and extrinsic mediated apoptosis (Ghavami *et al.,* 2010). There is evidence that DHA induces apoptosis by up-regulation of pro-apoptotic protein Bax in pancreatic cancer cells leading to downstream activation of caspase-3, causing apoptosis (Ghavami *et al.,* 2010, Aung *et al.,* 2011). These observations are consistent with the data acquired from the current studies. The novel anti-cancer agents JC3-39 and LLP271 elevated the level of caspase-3 in a concentration dependent manner in HT29-AK cell line (Figure 9). It could be postulated that LLP271-induced caspase-3 up-regulation may be related with activation of death receptor DR5, initiator (apical) caspase-8, which further triggered the activation of effector caspase -3 (Li *et al.,* 2007; Lu *et al.,* 2008; Zhou *et al.,* 2008). It is not possible to conclude from the current studies if the drug treatment leads to caspase-3 increases via intrinsic or/and extrinsic pathways, but as extracellular signals are the main regulators of the intrinsic pathway it is probable that the observed effects are mediated via intrinsic pathway.

The present study demonstrates the signs of DNA fragmentation in HT29-AK cells upon DHA, LLP271 and JC3-39 treatment as compared to untreated DNA (Figure 10 and 11). But there was no such clear visible difference in the smearing/laddering upon drug

treatments due to operator error. It can be suggested that LLP271 and JC3-39 mediated concentration dependent up-regulation of effector caspase-3 was leading to DNA fragmentation (Mercer *et al.,* 2007; Lu *et al.,* 2008; Ghavami *et al.,* 2009; Lu *et al.,*2009; He *et al.,* 2010). Unfortunately due to operator error it is not possible to evaluate if LLP271 and JC3-39 treated cells have greater DNA damaging effects compared to DHA alone. This may be expected due to the incorporation of dipyrrole, a DNA minor groove alkylating agent, into the structures of these agents (Efferth, 2005, Ghavami *et al.,* 2009). Therefore this DNA interaction may cause an increase in the effect of the drug on DNA fragmentation.

Survivin is highly expressed in human malignancies including colorectal cancer and leukaemia but rarely in healthy tissues and its down regulation may have a crucial role in cancer drug therapy (Lu, Luo and Tao, 2007; Ryan, O'Donovan and Duffy, 2009; Liu *et al.,* 2010). Previous studies related to DHA have shown to down-regulate expression of survivin in lung cancer cell line SPC-A-1, due to down-regulation of mRNA and deplete levels across 4 prostate cancer cell lines (Mu *et al.,* 2007; Morrissey *et al.,* 2010). Our DHA treatment data showed increase in survivin level at the highest concentration ($61.4\mu M$) but the lower concentrations ($15.3\mu M$ and $30.7\mu M$) could not significantly alter the level compared to control (Figure 12A). It is known that in tumorigenesis survivin expression is inversely correlated with apoptosis inhibition (Lu *et al.,* 2005; Ryan, O'Donovan and Duffy, 2009). JC3-39 and LLP271 produced identical effects with lower concentrations without effects whilst higher concentrations reduced cellular survivin concentrations. Overall the data showed variable effects on cellular survivin level. It may be due to the effect of the agents which caused death of the cells.

Metastasis and invasion are fundamental uncontrolled properties of malignant cancer cells, the leading cause of death in patients with cancer (Huang *et al.,* 2010). There is in vitro and in vivo evidence that artemisinin has an inhibitory effect on invasion and migration in human carcinoma cell lines HepG2 and SMMC-7721 (Du *et al.,* 2009; Weifeng *et al.,* 2011). DHA is also an effective anti-metastatic agent against human fibrosarcoma HT-1080 cells (Huang *et al.,* 2010; Wang *et al.,* 2011). In our study we observed a concentration-dependent inhibition of wound healing upon treating HT29-AK cells with DHA, LLP271, JC3-39 and RKA compounds as compared to untreated samples, demonstrated by an increase in wound diameter (Figure 13 and 14). The wound healing results were also supported by the trypan blue viability data, which showed a concentration-dependent increase in dead HT29-AK cells as compared to control (Figure 15). In previous studies, it has been reported that inhibition of MMP expression or enzyme activity can be used as early targets for preventing cancer metastasis (Liabakk, 1996; Wang *et al.,* 2011). It can be postulated that these compounds cause decreased capacity of the wounds to close via the down regulation of MMP-9 gene expression, through inhibition of PKCa/Raf/ERK and JNK phosphorylation and reduction of NF-kB. Nevertheless, the effects observed with the compounds support those of Wu and colleagues who showed a concentration-dependent inhibition in the migration of epithelial ovarian cancer cells by DHA (Mercer *et al.,* 2007). Further experiments, which are the focus of on-going investigations in the lab, may accept or refute these claims.

The NF-κb factor is involved in apoptosis inhibition and further regulates the production of pro-inflammatory cytokine, such as TNF-α or IL-6 (Efferth, 2005; Paule *et al.,* 2007; Wang *et al.,* 2009). It has been reported in vitro and in vivo studies that 1, 2, 4-

trioxanes block the signaling pathway of NF-κb and may therefore induce apoptosis (Efferth, 2005). Indeed, artesunate leads to the suppression of signaling pathway NF-kB and consequently, inhibits the tumour necrosis factor (TNF-α) induced production of pro-inflammatory cytokines, such as, interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8), in human rheumatoid arthritis (Xu *et al.,* 2007). Furthermore DHA has been shown to inhibit angiogenesis in human pancreatic cancer by inhibition of NF-kB DNA-binding activity and decreased the expression of NF-kB targeted pro-angiogenic gene products- VEGF, IL-8, COX-2 and MMP-9 in vitro and in vivo (Wang *et al.,* 2011; Ji *et al.,* 2011). There were detectable levels of TNF-α and IL-6 in the cells (Figure 16 and 17) with drug treatment showing variable effects, but tending towards an increase in levels. Further studies are warranted to decipher the true effects of the agents on these important cytokines.

As continuation of this research work further experiments can be done for each assessment. Iron dependency can be measured through the use of iron chelators like deferoxamine. DNA damage can also be evaluated using the COMET assay. Glutathione can be used to assess apoptotic pathways. Artemisinin and its derivatives are renowned for their potent anti malarial activity. They have found their way into clinical use in many areas where malaria is endemic. The fat-soluble derivatives artemether and arteether are approximately twice as active. The water-soluble dihydroartemisinin and artesunate are 4 to 5 times more active in vitro. Artemisinin is available only for oral and rectal administration. Absorption is incomplete and elimination is fast. Artesunate and artemether can be considered as prodrugs. Biotransformation into the active metabolite dihydro-artemisinin occurs rapidly almost immediately for artesunate. The pharmacokinetics of dihydroartemisinin is not yet completely clear.

In summary, the results of this study showed for the first time, that novel LLP271 and JC3-39 effectively induced growth-inhibition in colon cancer HT29-AK cells, JC3-39 being the most potent. The cytotoxicity of tetraoxane RKA compounds against HT29-AK cells were also compared with their cytotoxicity against HL-60 cells. It was observed that the drugs displayed differential sensitivity against the cells with the HL-60 cells being more sensitive towards the drugs. As we hypothesized, JC3-39 displayed more potent anti-cancer properties over parent DHA and this may be due to its increased sequence-selective DNA binding affinity. Furthermore, cytotoxicity of LLP271 and JC3-39 is strongly mediated by over-expression of P-AKT and cleaved caspase-3. These results provide evidence that LLP271 and JC3-39 may serve as an alternative candidate in the treatment of colon cancer alone or in combination with conventional therapeutic agents, and deserve to be further studied against other cancer cell types.

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