MASTER OF SCIENCE BY RESEARCH

An evaluation of the cytotoxicity of the dihydroartemisinin-acridine hybrids and tetra-oxane dimmers against leukaemia and colon cancer cells lines ‘1’

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Abstract

Background and purpose: Cancer is a leading cause of death worldwide. Artemisinin (ARS) and its derivatives are extremely effective against malarial infections and have also shown good activity against tumouric cells, with the greatest activity reported against leukaemia and colon cancer cell lines. The current study aimed to investigate the antitumour activities and underlying mechanisms of action of novel derivatives of dihydroartemisinin (DHA) designed to target DNA (such as LLP246, SL2935) were evaluated against cultured HT29-AK (colon cancer cells) and HL-60 (leukaemia cells) cell lines. The activity of newly synthesised tetraoxane dimers (RKA152M, RKA153, RKA154, RKA155, RKA157 and RKA160) were also evaluated against the cells.

Methods: The cancer cells were incubated with the test compounds and assessed for cytotoxicity by MTT assay and cellular levels of sTfR by ELISA. The effects of drug treatment on apoptosis were evaluated by measuring levels of cleaved caspase-3, P-AKT and survivin by flow cytometric analyses and ELISA, respectively. Their effects on DNA fragmentation was assessed by gel electrophoresis and a scratch assay backed by trypan blue dye exclusion were used to evaluate the effects of the lead compounds on cell migration/wound healing.

Key results: The test compounds showed potent cytotoxic effects against HT29-AK and HL-60 cells. LLP246 showed ~13.7 fold greater activity than DHA (IC_{50} of 4.47±0.39 µM vs 61.39±2.86 µM). Among the tetraoxane dimers, RKA160 (IC_{50} of 22.85±4.36 µM) displayed ~2.7 fold greater activity than DHA against HT29-AK cells. The tetraoxane dimers showed less cytotoxicity against HL-60 cells as compared to DHA (IC_{50} of 0.39±0.10 µM). There were measurable levels of sTfR in the cells, but the effects of the drugs were variable. Drug treatment caused increased activity of cleaved caspase-3 in the cell, P-AKT, DNA fragmentation, but decreased cellular survivin levels in the cells. All the test compounds showed a concentration-dependent inhibition of cell migration/wound healing supported by a concentration-dependent increase in dead cells.

Conclusions and Implications: Data from these studies suggest that the cytotoxicity of the test compounds is may be mediated by an iron-dependent

mechanism, stemming from the identification of sTfR in the cells. The agents caused apoptosis by an increase in cellular levels of cleaved caspase-3 and decreased survivin concentrations. A potent inhibitory activity against the capacity of the wound to heal was measured. Overall, these agents may have utility (alone or in combination with conventional chemotherapeutic agents) against a variety of cancer cell types.

Section 1

Introduction

1.1. Cancer is a scourge of man: Cancer is a leading cause of death worldwide. As can be seen in table 1, in 2011, it is estimated that lung cancer accounted for 1.38-1.61 million (12.7%-18.2% of the total) deaths; breast cancer, 1.38 million (10.9%); colorectal cancer, 1.23 million (9.7%); stomach, 0.74 million (9.7%) and liver cancers, 0.69 million (9.2%) (Jemal *et al*., 2011). The incidence of malignancy is increasing globally and it is estimated to reach 400,060 cases by 2015 and 530,371 cases in the next twenty years (Ferlay *et al.,* 2010).

Table 1: Percentage of deaths caused different types of cancers in 2011.

Type of cancer	*Percentage of deaths (%) in 2011*
Lung cancer	12.7%-18.2%
Breast cancer	10.9%
Colorectal cancer	9.7%
Stomach cancer	9.7%
Liver cancer	9.2%

Some chemotherapeutic agents are not fully effective and are limited due to drug resistance, unfavourable side effects, such as hypotrophy, leukopenia or stomatitis (Efferth, 2006; Ozben, 2006; Brown *et al*., 2007; Jhawer *et al.,* 2008; Shahrokni *et*

al., 2009; Raschi *et al*., 2010; Cheung *et al.,* 2010; Liu *et al.,* 2010) and many of the treatments are expensive. There is evidence that 5-Fluorouracil and its oral pro-drug capecitabine are used in treatment of a number of solid cancers including colorectal cancer, but exhibit side effects such as leukopenia, diarrhoea, stomatitis, nausea, vomiting and alopecia. Hand-foot syndrome is also a relatively common side effect of 5-Flourouracil (Shahrokni *et al.,* 2009). Moreover, drug-induced cardio-toxicity is emerging, with higher than expected incidence of cardiac dysfunction occurring in patients treated with a combination of old and new chemotherapeutics such as doxorubicin and trastuzumab (Raschi *et al*., 2010). There are a number of cytotoxic drugs that are most frequently associated with drug-resistance e.g. paclitaxel, vinorelbine, doxorubicin, methotrexate, dactinomycin and metomycin (Ozben, 2006).

To identify chemotherapeutic agents with improved profiles over existing ones, some of the current research is focussed on harnessing the chemotherapeutic potential of natural products (Efferth, 2005; Cheung *et al.,* 2010; Lee *et al.,* 2010; Hussain *et al.,* 2011). Traditional Chinese Medicine (TCM) has been reported to benefit one fifth of the world's population in treating many diseases and its acceptance as a real healing option by the west is of increasing interest (Parekh *et al*., 2009). It has been reported that there are a number of TCMs, such as *Scutellaria baicalensis* commonly referred to as '*Golden root'*, Takrisodokyeum (TRSDY)- comprising 12 herbs in various proportions which have shown promising activities against various cancer cell lines, such as PC-3 (prostate cancer), DU145 (prostate cancer), HL60, KML-562 (chronic myloid leukaemia), HeLa (cervical cancer) and HO-8910 (ovarian cancer) cell lines (Chan *et al.,* 2000; Li *et al.,* 2000; Chen, Zhou and Fan, 2003; Chi *et al.,* 2003; Efferth *et al.,* 2003; Kwon *et al.,* 2003; Tan and Vanitha, 2004; Yu *et al.,* 2006; Zhou *et al.,* 2007). 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). It has been reported that *Scutellaria baicalensi* causes simultaneous inhibition of Cox-2 (pro-inflammatory marker) and 12-lipooxygenase (tumour cell proliferatory marker) which result in both reduced inflammation and tumorigenesis (Pidgeon *et al.*, 2002; Burnett *et al.*, 2007). There is also evidence that *Scutellaria baicalensi* acts against cell proliferation in PC-3 and DU145 cells, causing cell cycle arrest (at G_0-G_1) while also inducing apoptosis as confirmed detection of caspase-3 (Chan *et al.,* 2000). Further evidence of TCMs acting via caspase-3 activation was demonstrated by Kwon and colleagues showing that treatment of these cells with TRSDY caused caspase-3-induced cytotoxicity (Kwon *et al.,* 2003), which causes DNA fragmentation, chromatin condensation and plasma membrane blebbing (Sahara *et al.,* 1999; Sebbagh *et al.,* 2001). Kwon and colleagues supported their conclusions by using a caspase-3 inhibitor in their assay which resulted in lack of detectable capsase-3 production and the downstream cleavage of cellular death substrates was also absent (Kwon *et al.,* 2006). Phytochemicals have long been used as ‘lead compounds’ to generate test compounds with better pharmacokinetic profiles and reduced toxicity *in vivo.* For example, the artemisinins are potent chemotherapeutic agents against malaria infections and have also shown good cytotoxic effects and anti-angiogenic properties (Efferth *et al.,* 2001; Efferth *et al.,* 2002; Chen *et al.,* 2004; Parekh *et al.,* 2009). It has been reported that TCM often possess quite distinct and specialised modes of action and consequently tumours that are normally resistant to conventional chemotherapy may show some susceptibility to TCM therapy (Shi and Tian, 2006).

There is evidence that herbal compounds such as paclitaxel and its derivatives suppress microtubule depolarization, thus terminating cell mitosis (Kaye, 1999). As a result, these and compounds including harringtonine (*Cephalotaxus hainanensis)* and camptothecin (*Camptotheca acuminate)* with similar mechanisms of action are already commonly used in the clinic as anticancer agents for a variety of cancers (Wang and Yang, 2001).

1.2. The artemisinins as antimalarials and their mode of action: Artemisinin also known as Qinghaosu, was isolated in 1971 from sweet wormwood (*Artemisia annua),* which has been used as a Chinese herbal medicine for over 2000 years (Singh and Lai, 2001). ARS and its semi- and synthetic derivatives such as DHA, artesunate (ART) and artemether (figure 1) are extremely effective against malarial infections (O'Neill, Barton and Ward, 2010; Kano, 2010; Krungkrai *et al.,* 2010; Gravett *et al.,* 2011; Soomro *et al.,* 2011; Wu *et al.,* 2011). It has been reported that the compounds have been effective against chloroquine and sulfadoxine-pyrimethamine resistant and sensitive *Plasmodium falciparum*, the causative agent of severe malaria infections (Kano, 2010), although there is evidence of emerging resistance against these agents in South East Asia (Noedl, Socheat and Satimai, 2009). ART is also found effective against *Plasmodium vivax,* showing broad specificity and rapid onset of action against early blood forms of the parasites (Efferth *et al.,* 2004; O'Neill, Barton and Ward, 2010; Wu *et al.,* 2011). Figure 1 shows the chemical structures of artemisinin and some of its derivatives.

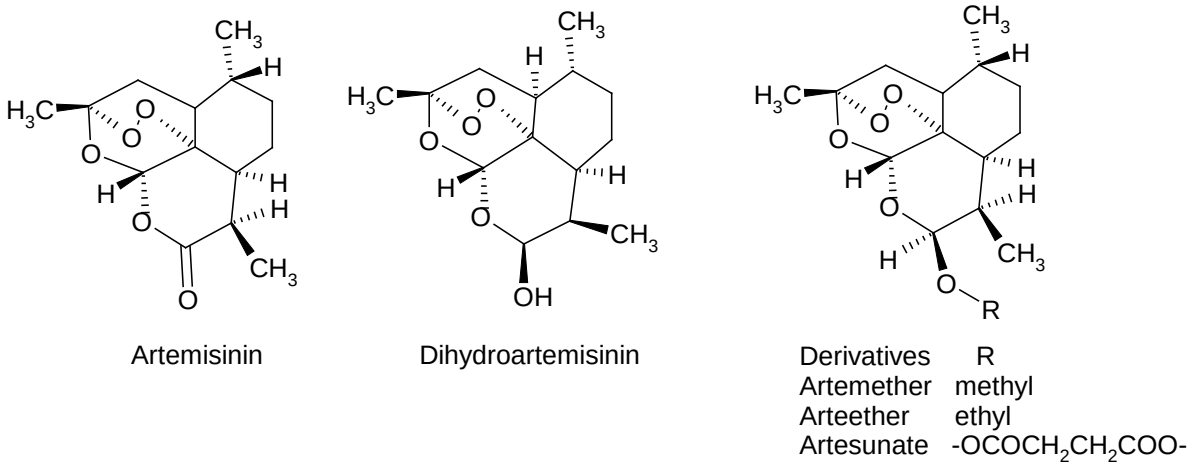


Figure 1: Chemical structure of artemisinin and some of its semi-synthetic derivatives.

ARS is a tetracyclic 1,2,4-trioxane sequesterpene lactone containing an endoperoxide bridge, which is essential for its activity (Efferth *et al.,* 2004; Mercer *et al*., 2007; Nakase *et al*., 2008*;* O'Neill, Barton and Ward, 2010; Alessandro *et al.,* 2011). Briefly, the antimalarial mode of action of ARS is based upon the formation of C-centred unstable free radicals [Fe(IV)=O], by reductive cleavage of the 1,2,4-trioxane, catalysed by the Fe (II) haem, generated as a by-product of the haemoglobin catabolism (Singh and Lai, 2001). The parasite degrades haemoglobin in its food vacuole, producing free haem which reacts with the endoperoxides and generates toxic reactive species (Robert *et al.,* 2001). The resulting free radical intermediate may then kill the parasite by alkylating essential malarial proteins, one of which is the translationally controlled tumour protein (TCTP) (Meshnick, 2002; Efferth *et al.,* 2004*;* Mercer *et al.,* 2007; Hommel, 2008; Nakase *et al.,* 2008; Jones *et al.,* 2009; Raghavamenon *et al.,* 2011). It has been reported that malarial parasites are rich in haemin; ARS's reactivity towards haemin may explain its selective toxicity to malarial parasites (Meshnick *et al.,* 1991) as iron chelators antagonise the antimalarial activity of the ARS (Eckstein-Ludwig et al., 2003; Weinberg and Moon, 2009). These agents have good antimalarial properties, reduce

parasite clearance rapidly, show limited significant toxicity, even for pregnant women, despite the fact that neurotoxicity was observed in animals with higher doses than used clinically (Robert *et al.,* 2001). However, there is worrying evidence of emerging resistance against the agents in SE Asia (Noedl, 2009; Noedl *et al*., 2009; Noedl *et al*., 2010).

1.3. The artemisinins as antitumour agents and their mode of action: Since the mid 1990s, subsequent studies have observed ARS and its analogues (1,2,4-trioxanes) to possess antitumor, antiproliferative and with some derivatives such as deoxoartemisinin amide trimer eliciting antiangiogenic properties (Moore *et al.,* 1995; Nakase *et al.,* 2008; Oh *et al.,* 2009; Zhang *et al.*, 2010), against many cancer cell lines including colorectal (HT29-AK, HCT-116 and SW-480), leukaemia (Jurkat and HL-60), hepatoma (HepG2 and Huh-7), lung (SPC-A-1), prostate (C4-2 and LNCaP), breast (MDA-MB-231 and MCF-7), endothelial (HMEC-1), osteosarcoma and pancreatic (BxPc3-RFP) cancer cells (Moore *et al.,* 1995; Posner *et al.,* 1997; Beekman *et al.,* 1997; Beekman *et al*., 1998; Mercer *et al.,* 2007; Parekh *et al.,* 2009; Morrissey *et al.,* 2010; Aung *et al.,* 2011). Unfortunately, many of the agents are effective against tumour cells within the micromolar range as opposed to the nanomolar range seen against *Plasmodium* parasites (Efferth, 2006), but it is hoped that via rational drug design this range can be reduced. This may be achieved by designing and synthesising new compounds with better pharmacokinetic profile, so that, they may represent alternatives to the currently used artemisinin derivatives. A collaborative research effort in own lab led to the development and evaluation of 1,2,4-trioxane-acridine hybrids which produced 2-4 fold enhanced cytotoxicity activity against a variety of cancer cell types including leukaemia (HL-60), colon (HT29-AK) and breast (MDA-MB-231 and MCF-7) compared to parent 1,2,4-trioxane. They have

been shown to induce cell death by apoptosis assessed by flow cytometric measurements of mitochondrial membrane depolarisation, DNA degradation and Western blot analysis of caspase-3 activation and to covalently bind to their intraparasitic cellular targets in the presence of iron (Jones *et al.,* 2009).

It has been reported that ARS and its derivatives are effective against cervical disorders associated with papillomavirus infection, such as cervical cancer and cervical dysplasia and is being used in vitro and in vivo against premalignant and malignant cells. In this study, ARS compounds were used against HPV-infected or trasformed cells and canine oral papillomavirus. In in vitro study, DHA-induced cell death involved activation of the mitochondrial caspase pathway with resultant apoptosis and in vivo study reported that tumour-negative dogs developed antibodies against the viral L1 capsid protein, suggesting that DHA had inhibited tumour growth (Disbrow *et al.,* 2005). It has been investigated that DHA analogues inhibited cell proliferation and tube formation of human umbilical vein endothelial cells (HUVECs) in a time- and dose-dependent manner, which resulted in inhibition of tumour growth in vitro and in vivo (Noori and Hassan, 2011). Recently, artemisone (ATM), a novel derivative of ARS was found to be more effective against cancer cells when used alone or in combination with other chemotherapeutic agents such as oxaliptin, gemcitabine and thalidomide (Gravett *et al.,* 2011). In a recent study, 10-substituted triazolyl ARS compounds were synthesized and have been observed to strongly inhibit the growth of cancer cell lines, such as, DLD-1(human colon carcinoma cells), U-87 (human primary glioblastoma cells), Hela, SiHa (cervical cancer cell line), A172 (human glioblastoma cell line) and B16 (mouse melanoma cell line) (Oh *et al.,* 2010).

The postulated antitumor mechanism is similar to the antimalarial mechanism of

ARS (Li and Zhou*,* 2007). Rapid proliferation is a basic feature of malignant cells, which requires high intracellular iron acts as a cofactor of DNA metabolism and continued cell proliferation (Mercer *et al.,* 2007; Oh *et al.,* 2009; Zhang *et al.,* 2010; O'Neill, Barton and Ward, 2010). Clearly, for the cells to utilise iron, they must express the receptors (transferrin receptors (TfRs)] to take it up (Beguin, 1992; Harford*,* 1994). To this end, TfR binds and internalises di-ferric transferrin to the cytosol. TfR expression is increased to facilitate iron uptake (Rao *et al.,* 1985; Mullner and Kuhn, 1988; Koeller *et al.,* 1989) and proteolysis of TfR leads to the formation of soluble transferrin receptor (sTfR) (Shih *et al.,* 1990; Baynes *et al.,* 1993) which can be measured. Measurement of sTfR is especially valuable as an indicator of iron deficiency in individuals with malignancy, inflammatory diseases and infections (Ferguson *et al.,* 1992; Erslev and Besarab, 1995). Thus a relationship has been exists between total TfR and sTfR and the concentration of sTfR has been demonstrated to be an indirect measure of total TfR (Beguin *et al.,* 1988). It has been reported that most of the cancer cells possess a high cell surface concentration of transferrin receptors (TfRs). For example, breast cancer cells have almost 5-15 times more TfRs compared to normal cells and hence have high rates of iron intake (Singh and Lai, 2001). The aggressiveness of tumour cells has also been shown to be positively correlated to the high surface expression of TfRs (Efferth, 2005*;* Singh and Lai, 2006; Cheung *et al.,* 2010; Xie *et al*., 2010). Interestingly, elevated iron levels allow the selective toxicity of ARS towards the cancer cells (Beekman *et al.,* 1998; Singh and Lai, 2005). Just as in malarial parasites, ARS becomes cytotoxic in the presence of ferrous iron which catalyses the reductive cleavage of the endoperoxide bridge leading to the formation of carbon-centred radicals, which cause cell death via macromolecular damage (Singh and Lai, 2006; O'Neill, Barton

and Ward, 2010). Plasmodium TCTP represents a known target of ARS and its derivatives in the malarial parasites. The microarray-based mRNA expression of human TCTP correlated with sensitivity to ART in tumour cells, suggesting that human TCTP contributes to response of tumour cells to the drug (Efferth, 2005).

Given that iron is central to the activity of ARSs, various manipulations have been attempted with the view to enhance the cytotoxic effects of the test compounds. For example, conjugation of ARS or DHA to transferrin has been shown to increase their targeted delivery and cytotoxic activity against tumouric cells such as DU145 (Lai *et al.,* 2005; Nakase *et al.,* 2008; Oh *et al.,* 2009; Xie *et al.,* 2010). The selective toxicity of the ARSs has also been demonstrated to be approximately 100 times more potent in killing human leukemia cells than normal lymphocytes (Singh and Lai, 2006), with the selectivity stemming from the differential levels of iron in the two cancer cell types. *In vitro* studies have verified the potent activity of ARS-transferrin conjugate against Molt-4-lymphoblastoid cells (Singh and Lai, 2001), while DHA-transferrin conjugate showed approximately 172 times more cytotoxicity against MCF-7 cells than DHA (Oh *et al.,* 2009; Xie *et al.,* 2010). The role of iron in the cytotoxicity of ARSs was further demonstrated by the observation that deferoxamine (an iron chelator) attenuated their cytotoxic effects (Mercer *et al.,* 2007; Huang *et al.,* 2007; Stockwin *et al.,* 2009; Raghavamenon *et al.,* 2011). Furthermore, a recent study found that oral co-administration of ART with ferrous sulphate induced greater apoptosis of cancer cells compared to ART alone (Fafowora *et al.,* 2011), suggesting that iron plays an important role in the cytotoxicity of ART.

The anti-oxidant stress genes (thioredoxin, catalase, and glutathione S-transferases) as well as the epidermal growth factors confer resistance to ART. Moreover, it has been reported that cell lines over-expressing genes that confer resistance to

established anti-tumour drugs (MDR, MRP1, dihydrofolate reductase), were not cross-resistant to ART, indicating that this drug has a different target and is not subject to multidrug resistance (Efferth, 2005).

1.4. Effects of artemisinins on apoptosis: There is evidence that ARS induces apoptosis in vitro and is lethal towards human leukemia and breast cancer cells (Efferth *et al.,* 2004; Singh and Lai, 2006). Clearly, apoptosis is controlled by a range of complex multi-step processes that consequently lead to the breakdown of cellular DNA leading to cell death (Parekh *et al.,* 2009). One of these multi-step events in cancer cells includes the induction of caspase-dependent apoptotic cell death. It is postulated that the activation of the endoperoxide bridge leads to apoptosis by caspase-dependent pathways, in which 'initiator' caspases (caspases 8, 9 and 10) have the primary role of processing and activating 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 events include induction of mitochondrial endoplasmic reticulum stress, release of cytochrome c, induction of cell cycle arrest at G_o/G_1 phase and subsequent DNA fragmentation (Parekh *et al.,* 2009). Indeed, there is evidence that ARS affect all these important processes such as apoptosis. ARS derivatives have been shown to induce caspase-dependent apoptotic cell death characterized by concentration- and time-dependent mitochondrial membrane depolarization, activation of caspase-3 and -7, sub- G_o/G_1 DNA formation and attenuation by benzyloxycarbonyl-VAD-fluoromethyl ketone, a caspase inhibitor (Mercer *et al.,* 2007; Shao *et al.,* 2008; Parekh *et al.,* 2009; Handrick *et al.,* 2010; Lu *et al.,* 2010; Zhang *et al.,* 2010; Morrissey *et al.,* 2010; Alessandro *et al.,* 2011). Gao and colleagues have

demonstrated that DHA induced apoptosis in a concentration- and time-dependent fashion in cultured human leukemia cells and it also demonstrated an anti-leukemic activity in vivo through a process that involves inactivation of MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated protein kinase), Mcl-1 (myloid leukaemia cell differentiation protein) down-regulation, culminating in cytochrome c release and caspase activation (Gao *et al.,* 2011). Furthermore, DHA is well documented to cause apoptosis in microvascular endothelial cells (HMEC-1) via increase in caspase 3 and 7 activation (Alessandro *et al.,* 2011), phosphatidylserine exposure and G_2 cell cycle arrest (Ji *et al.,* 2011).

There are a series of signalling pathways, for example, ARF(alternative reading frame)/Mdm-2/p53 pathway whose activation and suppression lead to a cascade of intracellular events which ultimately result in cell death (Green and Evan, 2002). For example, the mitochondrion plays a central role in cell survival and many of the triggers of apoptosis are known to act in mitochondria-dependent manner (Petit *et al.,* 1996; Bossy and Green, 1999; Parekh *et al.,* 2009; Fafowora *et al.,* 2011). DHA has been shown to cause apoptosis via the mitochondrial pathway, by reducing cellular iron influx. This is postulated to result in decreased expression of TfRs, which is thought 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 (B-cell lymphoma 2), VEGF (vascular endothelial growth factor) and up-regulating pro-apoptotic protein Bax (Bcl-2-associated x protein) in pancreatic cancer cells under normoxic, but not hypoxic conditions (Jiao *et al.,* 2007; Zhou, Wang and Li, 2008; Aung *et al.,* 2011).

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

oligonucleosomes (Mercer *et al*., 2007; Jiao *et al*., 2007; Lu *et al*., 2009). It is postulated that the carbon-centred free radicals generated by ARS and its derivatives upon reductive cleavage by iron alkylates into DNA (Jeyadevan *et al.,* 2004) which may cause DNA fragmentation.

Interestingly, it has been demonstrated that DHA-mediated apoptosis is correlated with inhibition of the PI3-kinase/AKT (phosphatidylinositol 3-kinase/serine-threonine protein kinase) and extracellular signal regulated kinase (ERK) survival pathway, in human prostate malignant cells and colorectal cancer cells (He *et al.,* 2010). There is also evidence that AKT (also a protein kinase) plays a vital role in a number of cell signalling pathways, such as mTOR (mammalian target of rapamycin) and regulates pro-survival genes such as PTEN (phosphatase and tensin homologue deleted on chromosome 10) in many cancers (O'Gorman *et al.,* 2000; Testa and Tsichlis, 2005; De Souza, Russell and Kearsley, 2009) and the effects of ARS on this molecule warrants investigating.

Clearly, all cells have to control the level of apoptosis that is occurring and as such there are many innate pathways involving inhibitors of apoptosis, such as survivin which promote cell survival (Wheatley *et al.,* 2005). Survivin is an inhibitor of apoptosis which is initiated by both intrinsic and extrinsic apoptotic pathways (Ambrosini *et al.,* 1997; Muchmore *et al.,* 2000; Salvesen and Duckett, 2002; Altieri, 2003; Schimmer, 2004; Wheatley *et al.,* 2005). Survivin suppresses cell death and therefore plays a vital role in cell division. There is also evidence that it plays a role in tumour formation and resistance to anti-cancer agents (Zaffaroni, Pennati and Daidone, 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) and may act as a marker and prognostic indicator for certain cancers including pancreatic and colorectal cancers (Zaffaroni *et al.,* 2005). The anti-apoptotic effect of survivin stems from its inhibitory action on caspase 3 and 7 (Tamm *et al.,* 1998) and it is known to play modulate the activity of a number of molecules important for cell growth and survival (Dohi *et al.,* 2004; McNeish *et al.,* 2005). Previous studies in 4 prostate cancer cell lines (C4-2, LNCaP, DU145 and PC-3) demonstrated that the ARS dimer, 2PY (at 15μM) significantly decreased the expression of survivin at the mRNA levels (Morrissey *et al.,* 2010).

1.5. Effects of artemisinins on metastasis: Metastasis and invasion are properties unique to malignant cells and they are a leading cause of morbidity and mortality (Hwang *et al.,* 2010). The invasion of lymphnodes and blood vessels are important steps in metastasis which requires increased expression of matrix metalloproteinases (MMPs), cell-extracellular matrix (ECM) interactions, disconnection of intercellular adhesion and degradation of ECM (Liotta, Steeg and Stetler-Stevenson, 1991; Deryugina *et al.,* 1997; Stamenkovic, 2000; Hwang *et al.,* 2010). MMPs have been implicated in malignancy and members of its family are involved in degradation of ECM (Westermarck and Kahari, 1999; Stamenkovic, 2000; Hwang *et al.,* 2010). The activity of MMPs is kept in check by TIMPs (tissue inhibitor of matrix metalloproteinases). It has been reported that inhibition of the expression of MPPs can be used as early targets for preventing cancer metastasis (Liabakk *et al.*, 1996; Hwang *et al.,* 2010; Wang *et al.,* 2011). ARS has been reported to have inhibitory effect on invasion and metastasis of human hepatocellular carcinoma (HCC) cell lines (HepG2 and SMMC-7721). The test compounds inhibited the invasion and migration of the cells *in vitro* and *in vivo* by reducing the levels of MMP2 and inducing cellular levels of TIMP2 (Du *et al.,* 2010; Weifeng *et al.,* 2011).

There is evidence that DHA is also acts as an effective anti-metastatic agent, down-regulates MMP-9 gene expression, through inhibition of PKCα (protein kinase C-alpha)/Raf (Ras factor)/ERK (extracellular signal-regulated kinase) and JNK (C-Jun N-terminal kinases) phosphorylation in human fibrosarcoma HT-1080 cells (Hwang *et al.,* 2010; Wang *et al.,* 2011).

1.6. Our Strategy – looking forward: The artemisinins have shown great promise as anticancer agents and may have potential clinical utility (Chen *et al.,* 2004; Efferth, 2007). However, a great deal of work remains to be done on them so as to bring their activity against tumouric cells to those seen in *P. falciparum*. Overall, the agents are active against tumour cells in the micromolar range as opposed to the nanomolar concentrations measured against *P. falciparum* (Efferth, 2006). In order to pharmacoenhance the activity of the agents, there is interest in the use of pyrrole-based reagents to target the minor groove of DNA in a sequence-selective manner (Jones *et al*., 2009). Interestingly, it has been reported minor groove binders such as 2,2'-dipyrrole derivatives have anti-tumour activity (Baraldi *et al.,* 2003 and 2006; Zaitsev *et al.,* 2006). Recent evidence showed that these molecues can be synthesised as hybrids with DHA with 2-4 fold greater against leukamia, colon and bvreast ceancer cells than DHA (Jones *et al.,* 2009). Furthermore, the synthesis of numerous hybrids from the dipyrrole framed netropsin tethered to benzoyl nitrogen mustard moiety also resulted in more cytotoxic agents than the parent agents (Baraldi *et al.,* 2003).

In order to increase the stockpile of our anticancer agents, interest is growing towards the use of 1,2,4,5-tetraoxanes as potential antimalarial and more recently anticancer agents (Singh and Lai, 2001; Efferth *et al*., 2004; Terzic *et al.,* 2007; Opsenica *et al.,* 2008; Atheaya *et al.,* 2008). They are purely synthetic and made

from readily available, cheap starting materials with identical mode of action as the ARS (Wu, 2002).

Through a collaborative research partnership with the Chemistry Department of the University of Liverpool, rationally designed and synthesised DHA-minor groove binding conjugates and a number of novel tetraoxane dimers (figure 2) with the view to identifying agents with activities over and above the parent artemisinins, were obtained. LLP246 and SL2935 were designed to incorporate metabolically stable 1,2,4 trioxane moiety. Given that the endoperoxide bridge is essential for activity, the tetraoxane dimers were synthesised with the hope that they would liberate more biologically reactive metabolites upon reductive cleavage by cellular iron. There is evidence that the highest cytotoxicity of the endoperoxides has been reported against human colon and leukaemia cancer cells which are most sensitive, rapidly proliferating cells with high expression of transferrin receptors needed for endocytosis-mediated iron intake (Mercer *et al.,* 2007; Jones *et al.,* 2009). Consistent with these findings, the cytotoxicity and mechanistic basis of activity of these agents against cultured human leukaemia (HL-60) and also colon (HT29-AK) cancer cell lines were evaluated.

Aims and Objectives:

1) To investigate the effects of the test compounds against HL-60 and HT29-AK cancer cells with the view to identifying lead molecules for development.

2) To evaluate the effects of the agents on sTfR, caspase-3, survivin, DNA fragmentation and P-AKT in order to identify the biochemical and molecular basis of toxicity of the test compounds.

3) The effects of test compounds on the migration of cancer cells in a scratch (wound healing) assay.

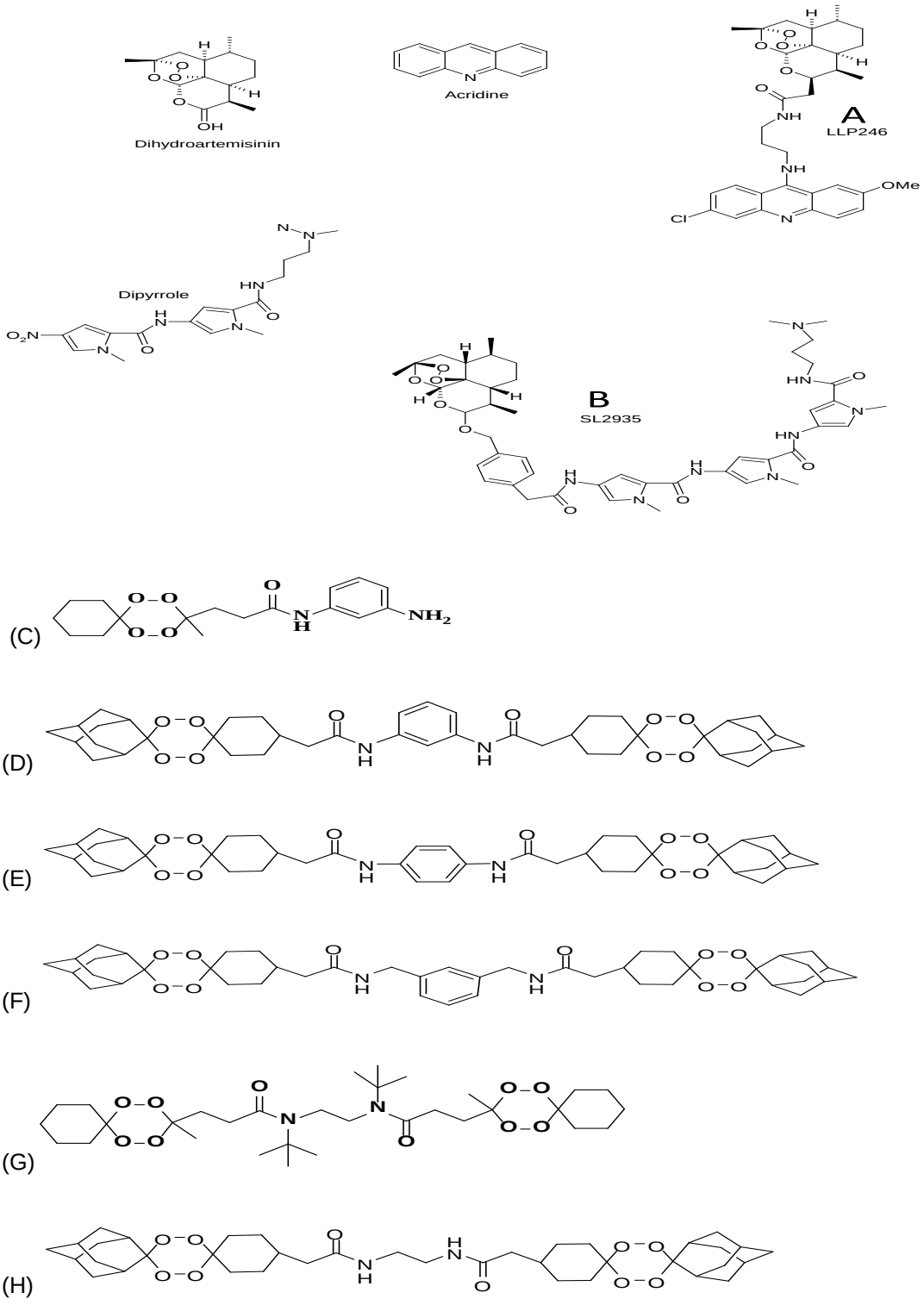


Figure 2: Shows the chemical structures of the test compounds used in this study. A (LLP246) is a dihydroartemisinin-acridine hybrid and B (SL2935) is a dihydroartemisinin-dipyrrole hybrid. The tetraoxane dimmers are (C) RKA152M; (D) RKA153; (E) RKA154; (F) RKA155; (G) RKA157 and (H) RKA160.

Section 2

Materials and methods

2.1. Materials

DHA was obtained from Dafra Pharma (Belgium). Hybrids of DHA (LLP246 and SL2935) and the tetraoxane dimers (RKA160, RKA153, RKA154, RKA155, RKA157 and RKA152M) were supplied by Prof Paul O'Neill (Liverpool University, UK) through a collaborated research project. Human leukaemia cells (HL60), colon cancer cells (HT29-AK) and cleaved caspase-3 and P-AKT Ser 473 antibodies were obtained from European Collection of Cell cultures and Cell Signalling, New England Biolabs (Herts, UK), respectively. All media were supplied by Biosera and Invitrogen, Paisley (UK) and chemicals were supplied by Fisher Scientific (Leicestershire, UK). All the stock solutions of test compounds were created using 100% dimethyl sulfoxide (DMSO). The final concentration of DMSO in each incubation was less than 0.5%, which did not affect the viability of the cells.

2.2. Cell culture

HL-60 cells were grown in RPMI1640 (Roswell Park Memorial Institute 1640 medium), supplemented with 10% FBS (fetal bovine serum). HT29-AK cells were cultured in EMEM (Eagle's minimal essential medium), supplemented with 10% fetal bovine serum and 20 mM glutamine (Lonza, Verviers, Belgium). Both cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and subcultured after every two days.

2.3. Trypsinisation (only for adherent HT29-AK cells)

To detach the adherent cells at the start or end of experiments, such as the test compounds screening assay and at the end of some incubations e.g. cleaved caspase-3 activity, trypsinisation process was used. At about a cell confluency of 70%, medium from culture flasks was aspirated and discarded. The attached cells were washed three times with 5 ml of sterile phosphate buffer saline (PBS) and the PBS discarded each time. After the final PBS wash, 1 ml of trypsin (Lonza, Verviers, Belgium) was added to the flask and incubated at 37 °C for about 5 minutes. Once the cells have detached, the trypsin was inactivated by the addition of 10-15 ml of MEM medium, supplemented with 10% FBS.

2.4. Cell counting

Before the start of any of the experiments reported here in, the cells were counted in order to fix the cell density in each assay. This was done using Nucleocounter (Chemometec, Allerod, Denmark). Briefly, 50 µl of reagent A (lysis buffer) was added to 50 µl of cell suspension in eppendorff and vortexed well for about 1 minute. Further, 50 µl of reagent B (stabilising buffer) was added to the lysed samples and vortexed. A small volume of the sample was then drawn into the nucleocassette which was preloaded with propidium iodide. The cells were then counted by loading the nucleocassette into cell counter socket.

2.5. MTT Cytotoxicity assay

Cytotoxicity of DHA, LLP246, SL2935 and RKA compounds were determined using the MTT cytotoxicity assay (Mosmann, 1983). The cells (1×10^4 cells) were seeded in 96-well flat-bottomed micro-plates and incubated them with varying concentrations of

the test compounds (0-100 µM) for 24 hr (at 37°C, 5% CO_2) as described previously (Jones et al., 2009). After incubation, the assays were terminated by adding 20 µl of 5 mg/ml MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) and incubated for 2 h. Then the cells were lysed by adding 100 µl of lysis buffer (20% sodium dodecyle sulphate in 50% dimethylformamide) and further incubated overnight. The optical density of the samples were then analysed at 492 nm in a plate reader (Anthos plate reader 2001, Anthos laboratory instruments, UK).

2.6. Data analysis to determine IC_{50} of the test compounds upon treatment

In order to allow the activity of the test compounds to be evaluated, the effects of test compounds treatment on cell growth versus control samples were estimated using the following equation:

$$\% \text{ of control cell growth} = \frac{\text{optical density of test compound-treated samples}}{\text{optical density of control samples}} \times 100$$

A plot of the percentage of cell growth compared to control against the test compound concentrations allows the estimation of the IC_{50} values using the 4 parameter logistic analysis (Grafit, Erithacus Software; UK). IC_{50} is the inhibitory concentration that kills/inhibits 50% of the cell growth.

2.7. Effect of test compounds treatment on sTfR

Enzyme-Linked Immunosorbent Assays (ELISAs) were used to investigate the effects of the test compounds on sTfR. HT29-AK cells (5×10^6 cells/flask) were incubated for 24 h (37 °C, 5% CO_2) in the absence and presence of LLP246 (4.5 µM, 2.2 µM, 1.1 µM); SL2935 (64.8 µM, 32.4 µM, 16.2 µM) and DHA (61.4 µM, 30.7 µM, 15.3 µM). Thereafter, the cells were collected by centrifugation following

trypsinisation and the cell lysates were prepared for the estimation of sTfR following the manufacturer's protocols (R&D Systems, UK).

For the determination of sTfR, 100 µl of sTfR assay diluent was added to each well, followed by the addition of equal volume of lysates/standard solutions. The samples were incubated for about 1 h at room temperature, before the wells were aspirated and washed three times with wash buffer. Then 100 µl of sTfR conjugate was added to each well and incubated for 1 h. Thereafter, the wash step was repeated, followed by the addition of 100 µl/well of substrate solution. The plates were incubated for 30 minutes at room temperature, followed by the addition of stop solution (100 µl/well). Finally, the plates were read at 450 nm within 30 minutes and the level of sTfR determined from the sTfR standard curve.

2.8. Effect of test compounds treatment on cleaved caspase-3 activity and P-AKT phosphorylation

A flow cytometery technique was performed in order to determine the effect of test compounds treatment on the activity of cleaved caspase-3 and P-AKT. HT29-AK cells (5×10^6 cells/flask) were incubated with and without LLP246 (1.1 µM, 2.2µM and 4.5µM) and DHA (15.3 µM, 30.7 µM and 61.4µM).

In order to investigate the effect of tetraoxane dimers on cleaved caspase-3 activity HL-60 cells (5×10^6 cells/flask) were incubated with and without RKA155 (28.2 µM, 9.4 µM, 18.8 µM and 28.2µM); RKA160 (10.1 µM, 20.2µM and 30.3µM) and DHA (0.2 µM, 0.4µM and 0.6µM).

The assays were incubated for 24 h at 37 °C, 5% CO_2 before being terminated by centrifugation (1200 rpm, 2 minutes, room temperature). The supernatant was

discarded and the cell pellets were resuspended in 250 µl PBS and centrifuged (1200 rpm, 2 minutes). The samples were fixed in 250 µl of 6% formaldehyde (37 °C, 10 minutes), followed by cell permeabilisation in 1 ml of 90% methanol. Thereafter, the samples were centrifuged (1200 rpm, 2 minutes), followed by 2 wash steps with 200 µl of incubation buffer (0.5% BSA [bovine serum albumin] in PBS). Then the samples were further washed in 100 µl of incubation buffer and incubated at room temperature for 10 minutes. Thereafter, the samples were incubated for 1 h in 100 µl/tube of the primary antibody (1:100 final dilution of antibody) for the pro-apoptotic marker cleaved caspase-3 or P-AKT antibody. The samples were centrifuged and the cell pellets were then washed in 200 µl of incubation buffer, followed by resuspension of cell pellets in 100 µl of incubation buffer and analysed on the BD FACS Calibur® flow cytometer on the FL-1 channel. Histograms were plotted for each of the groups showing the mean fluorescence for 10,000 counts.

2.9. Effect of test compounds treatment on DNA damage

HT29-AK cells (5×10^6 cells/flask) were incubated in the absence and presence of LLP246 (1.1 µM, 2.2µM and 4.5µM); SL2935 (16.2 µM, 32.4µM and 64.8µM) and DHA (15.3 µM, 30.7µM and 61.4µM).

The flasks were incubated for 24 h at 37 °C, 5% CO_2. Thereafter, the assays were terminated by centrifugation (8000 rpm, 10 seconds). The cell pellets were resuspended in 200 µl of media, ensuring each sample did not contain more than 1×10^7 cells. DNA was isolated following DNA isolation protocol of QIAGEN (Maryland, USA). Briefly, 200 µl of a well-mixed sample was added to the capture column tube by gently touching the tip of micropipette to the centre of the matrix, followed by incubating the capture column along with sample for about 30 minutes to

1 h at room temperature, which allows the DNA to adsorb onto the matrix. Then 400 µl DNA purification solution 1 was added to the capture column and further incubated for 1 minute at room temperature, followed by centrifugation (8000 rpm, 10 seconds). Thereafter, 400 µl of purification solution 1 was added to the capture column and incubated for 1 minute at room temperature. The collection tube was centrifuged at 8000 rpm for 10 seconds. Then, 200 µl of DNA elution solution 2 was immediately added followed by centrifugation (8000 rpm, 10 seconds). A 100 µl DNA elution solution 2 was then added and incubated at 99 ºC for 10 minutes followed by centrifugation (8000 rpm, 20 seconds) to finally obtain the purified DNA.

The DNA concentration in each sample was measured using Nano-Drop (ND-1000 3.3 software, Labtech International, UK). DNA concentration of each sample with LLP246 (93.0 ng/µl, 127.9 ng/µl, 307.9 ng/µl); SL2935 (69.6 ng/µl, 136.2 ng/µl, 207.9 ng/µl) and DHA (121.1 ng/µl, 156.6 ng/µl, 256.2 ng/µl) including control (507.0 ng/µl) were separated for 30 minutes on a 1.0% agarose gel stained with 2 µl of ethidium bromide. The samples were separated with parallel molecular marker, lamda Eco 1301. In this experiment 507.0 ng/µl of the heat-treated control, being control samples treated at 95 °C for 20 minutes to serve as positive control was used. The samples were visualized and photographed under a UV-light in GEL-DOC.

2.10. Effect of test compounds treatment on cellular survivin concentration

HT29-AK cells (2×10^6 cells/well) were incubated for 24 h (37 °C, 5% CO_2) in 6-well flat bottomed plates in the absence and presence of LLP246 (41.1 µM, 2.2µM and 4.5µM); SL2935 (16.2 µM, 32.4µM and 64.8µM) and DHA (15.3 µM, 30.7µM and 61.4µM). Thereafter, the assays were terminated by centrifugation (1200 rpm, 2 minutes). The cells were washed in PBS (2 ml) followed by resuspension in lysis

buffer (1 ml/1×10^7 cells) and the samples stored at -20 ºC for batch analysis. The cellular survivin concentration without or upon test compound treatment was done by ELISA following the manufacturer's instructions (R&D Systems Europe, Ltd., Abingdon, UK) with minor modifications. Briefly, the samples were thawed and centrifuged (1200 rpm, 5 minutes) and 80-90 µl of the supernatants transferred into clean eppendorff tubes. The samples were then diluted (1:2 dilutions) with assay diluent prior to the assay. Each of the wells were loaded with 100 µl samples/standard solutions and incubated for 2 h at room temperature. After incubation, each well was aspirated and washed three times with wash buffer, followed by the addition into each well of 100 µl of diluted Total Survivin Detection Antibody (1:15 dilution with reagent diluent) and incubating the plates for 2 h at room temperature. Then the wells were aspirated of the samples and washed three times with wash buffer. Thereafter, 100 µl diluted Streptavidin-HRP (1:200 dilutions) was added to each well and incubated for 20 minutes at room temperature, followed by three wash steps with wash buffer. Then, 100 µl of Substrate solution (1:1 substrate solution A and B) was added to each well and incubated for 20 minutes at room temperature, before 50 µl of Stop solution was added to each well and the plates read at 450 nm within 30 minutes. The concentration of cellular survivin without or upon test compound treatment was estimated from the standard curve using Grafit (Erithacus, UK).

2.11. Effect of test compounds treatment on migration and invasion/wound healing

For the wound healing assay, the cells were required to be confluent before a scratch (wound) is created. Here, HT29-AK cells (1×10^6 cells/well) were cultured in 12-well flat-bottomed plates and incubated (37 °C, 5% CO_2) without test compounds

for 5-7 days to allow the cells reach to confluency. A wound was created by using the tip of a sterile 10 µl pipette. This was followed by washing away of the non-adherent cells with MEM media supplemented with 10% FBS three times. Thereafter, the cells were incubated (37 °C, 5% CO_2) in absence and presence of LLP246 (1.1 µM, 2.2 µM and 4.5 µM); SL2935 (16.2 µM, 32.4 µM and 64.8 µM) and DHA (15.3 µM, 30.7 µM and 61.4 µM).

In the case of the tetraoxane dimers, the cells were incubated in absence and presence of RKA152M (37.9 µM, 75.8 µM, 151.5 µM and 378.8 µM); RKA157 (34.4 µM, 68.9 µM, 137.8 µM and 344.4 µM); RKA160 (11.4 µM, 22.8 µM, 45.7 µM and 114.2 µM,) and DHA (30.7 µM, 61.4 µM, 122.9 µM and 306.9 µM).

Following changes of the incubation media (every 2 days) the diameters of the wounds were measured under a light microscope at 10x magnification. This process was continued every 2 days for about 9 days. In order to reduce variability between measurements, a dot was applied under each well of the plates so that the diameter of same diameter area is measured every time. Photographs were taken using TS view so that we can compare test compounds-treated samples with control samples to enable the effect of test compounds treatment on wound closure to be assessed.

2.12. 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 test compound-treated) prior to changing the incubation media and assayed for cell viability. Briefly, equal volumes 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 taken. Along with this, the numbers of dead cells were counted using a haemocytometer, following the equation:

Number of death cells/ml = average number of death cells in large corner square X $1x10^4$ cells/ml X 2

2.13. Statistical analysis

Data are expressed as mean ± SD; 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 StatsDirect statistical software, version 2.6.3, 2007 (Cheshire, UK). Significance between controls and test samples was assumed if $P \leq 0.05$.

2.14. Safety and ethical considerations

Ethics exemption and COSHH forms were approved by Coventry University. Any data acquired from these studies cannot be related to any individual.

Section 3

Results

3.1. Cytotoxicity of the test compounds against HT29-AK and HL-60 cells

The cytotoxicity of the DNA intercalators, LLP246 and SL2935 and the tetraoxane dimers: RKA152M, RKA153, RKA154, RKA155, RKA157, RKA160 relative to DHA (parent compound) against HT29-AK and HL-60 cancer cells were evaluated using MTT assay. The compounds were cytotoxic against HT29-AK cell lines (table 2). LLP246 was significantly ($P<0.001$) more potent than DHA, with an IC_{50} of 4.47±0.39 µM vs 61.39±2.86 µM. LLP246 displayed ~13.7 fold greater activity compared to DHA. In contrast, SL2935 was of similar cytotoxicity compared to DHA (table 2).

Of the tetraoxane dimers, RKA160 was significantly ($P<0.01$) more cytotoxic than the parent drug DHA, being ~2.7 fold more potent than the parent compound DHA. All of the other tetraoxane dimers displayed only moderate cytotoxic activity against HT29-AK cancer cells (table 2). The rank orders of potencies of the dimers are as follows: RKA160 > RKA157 > RKA152M > RKA154 > RKA155 > RKA153 (table 2).

The tetraoxane dimers were evaluated for their in vitro activity against leukaemia-derived HL-60 cancer cells relative to DHA as a control. The agents showed cytotoxic effects against the cells, but they were all significantly ($P<0.001$) less active compared to DHA (table 2). They showed the following rank orders of activity: RKA155 > RKA160 > RKA152M > RKA157 > RKA153 = RKA154 (table 2). The IC_{50} of RKA153 and RKA154 compounds were greater than the highest concentration (100 µM) used in the assay (table 2).

*Table 2: The measured IC_{50} of the test compounds against HT29-AK and HL-60 cancer cells. Figures represent mean±SD of 3 separate experiments (n=3). **P<0.01 and ***P<0.001. ND-Not Determined*

Compounds	*HT29-AK*	*HL-60*
	IC_{50} ± SD (μM)	
DHA	61.39±2.86	0.39±0.10
LLP246	4.47±0.39***	ND
SL2935	64.81±0.36	ND
RKA152M	75.77±0.77	31.20±1.81
RKA153	92.46±1.60	>100
RKA154	77.58±5.46	>100
RKA155	90.16±2.12	18.80±2.30
RKA157	68.88±0.71	50.02±6.42
RKA160	22.85±4.36**	20.17±2.37

3.2. Test compound treatment altered the cellular concentration of sTfR in HT29-AK cells

As iron is central to the cytotoxicity of the artemisinins, the effects of the test compounds on cellular sTfR levels were measured to give an indication of cellular transferrin levels and its relationship to the cytotoxic effects of the agents. Here, an investigation was made on the effects of test compounds treatment on cellular sTfR levels in HT29-AK cells. As can be seen in figure 3, the cells express sTfR (~8pg/ml), but the test agents showed variable effects. DHA at 15.3 μM and 30.7 μM had no effects on sTfR levels in the HT29-AK cells (figure 3A). However, DHA (at 61.4 μM) decreased sTfR concentration by ~1.3 fold (5.8 pg/ml) compared to control (7.8 pg/ml). In contrast, LLP246 (at 4.5 μM) significantly (P<0.01) increased sTfR concentration, whereas no alterations in sTfR levels were measured at 1.1 μM and

2.2 µM of LLP246 (figure 3B). Similarly, treatment with SL2935 resulted in variable patterns of sTfR secretion, with 16.2 µM causing significantly (P<0.001) increased levels of sTfR, while 32.4 µM and 64.8 µM of SL2935 demonstrated no marked effects compared to control (figure 3C).

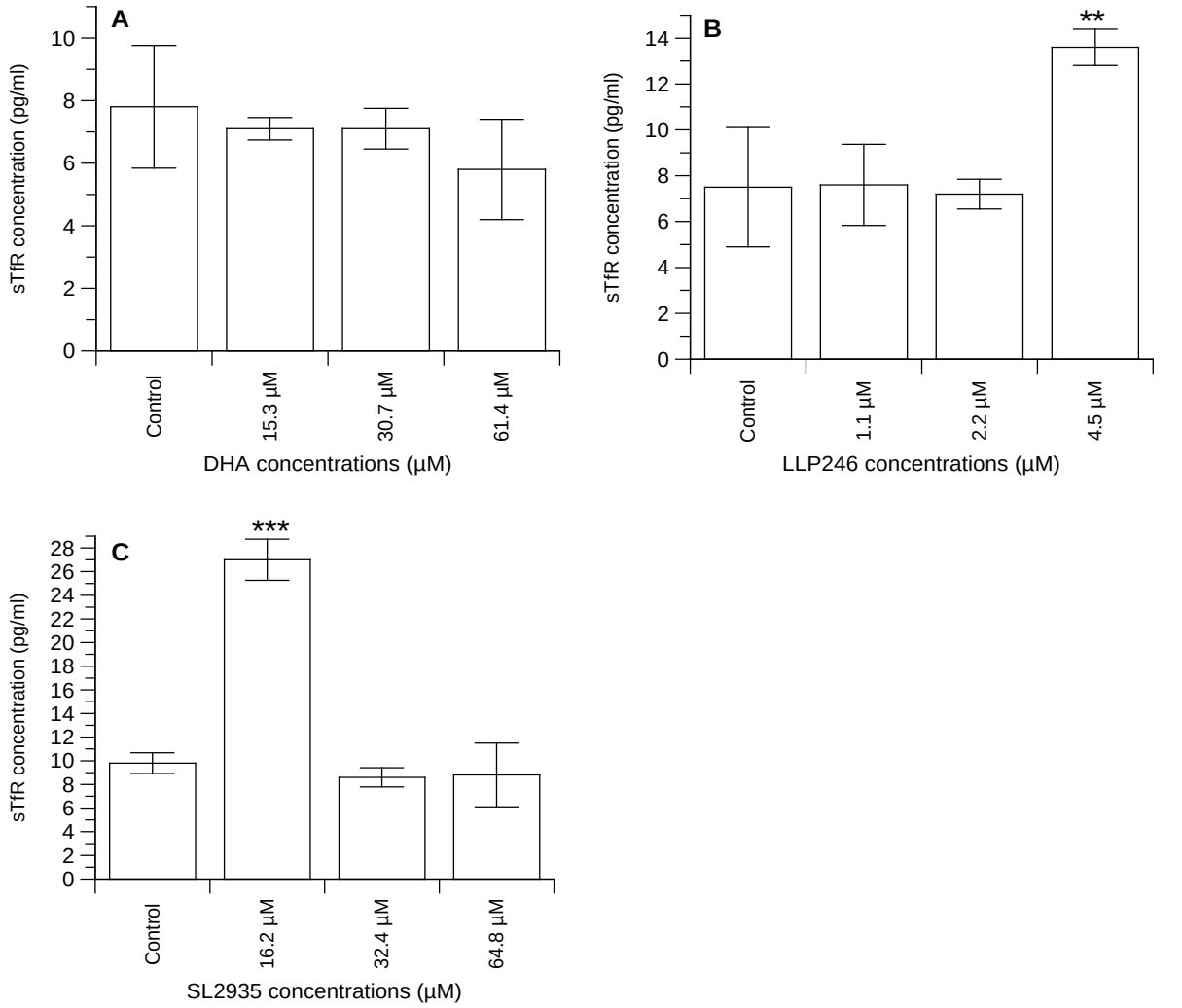


Figure 3: The effects of test compounds treatment upon the cellular concentration of sTfR in HT29-AK cells. Cells were treated with different concentrations of DHA, LLP246 and SL2935 as shown for 24 hours before sTfR levels were measured by ELISA. ***A)*** *Effects of 15.3 µM, 30.7 µM and 61.4 µM of DHA;* ***B)*** *Effects of 1.1 µM, 2.2 µM and 4.5 µM of LLP246 and* ***C)*** *Effects of 16.2 µM, 32.4 µM and 64.8 µM of SL2935 on sTfR levels in HT29-AK cells. Results are the mean ± SD of three independent experiments (n=3). **P<0.01 and ***P<0.001 as tested by the Mann-Whitney U test.*

3.3. LLP246 and RKA compounds tested induced the release of catalytically active cleaved caspase-3 in HT29-AK and HL-60 cells, respectively

The catalytically active cleaved caspase-3 levels were measured upon test compounds treatment to further investigate the mechanistic basis of LLP246-induced cell death. Compared to control, all of the DHA-treated cells showed a significant ($P<0.001$) increase in cleaved caspase-3 activity (figure 4A). There was a ~2.4 fold, ~2.5 fold and ~2.6 fold increase in fluorescence upon treating cells with 15.3 µM, 30.7 µM and 61.4 µM DHA, respectively (figure 4A). Similarly, LLP246 significantly ($P\leq0.05$) induced an increase in the release of cleaved caspase-3 compared to control in HT29-AK cells. Control cells showed cleaved caspase-3 activity of 50.53 RFU which was increased by ~2 fold (75.36 RFU) upon treating the cells with 4.5 µM LLP246 (figure 4B).

The cleaved caspase-3 activity of HL-60 cells treated with tetraoxanes dimers was also measured. Compared to control, all of the DHA-treated cells showed a significant ($P<0.001$) concentration-dependent increase in cleaved caspase-3 activity (figure 4C). There was a ~2.6 fold, ~2.9 fold and ~3.2 fold increase in fluorescence upon treating the cells with 0.2 µM, 0.4 µM and 0.6 µM of DHA, respectively (figure 4C). Similarly, RKA155 significantly ($P\leq0.001$) induced an increase in catalytically active cleaved caspase-3 as compared to DHA in HL-60 cells. Control cells showed cleaved caspase-3 levels of 16.3 RFU which was increased by ~3.2 fold (52.63 RFU) upon treating the cells with 9.4 µM RKA155 (figure 4C). Similarly, treatment of cells with RKA160 (at all concentrations) resulted in significant ($P<0.001$) increase in cleaved caspase-3 activity (figure 4C).

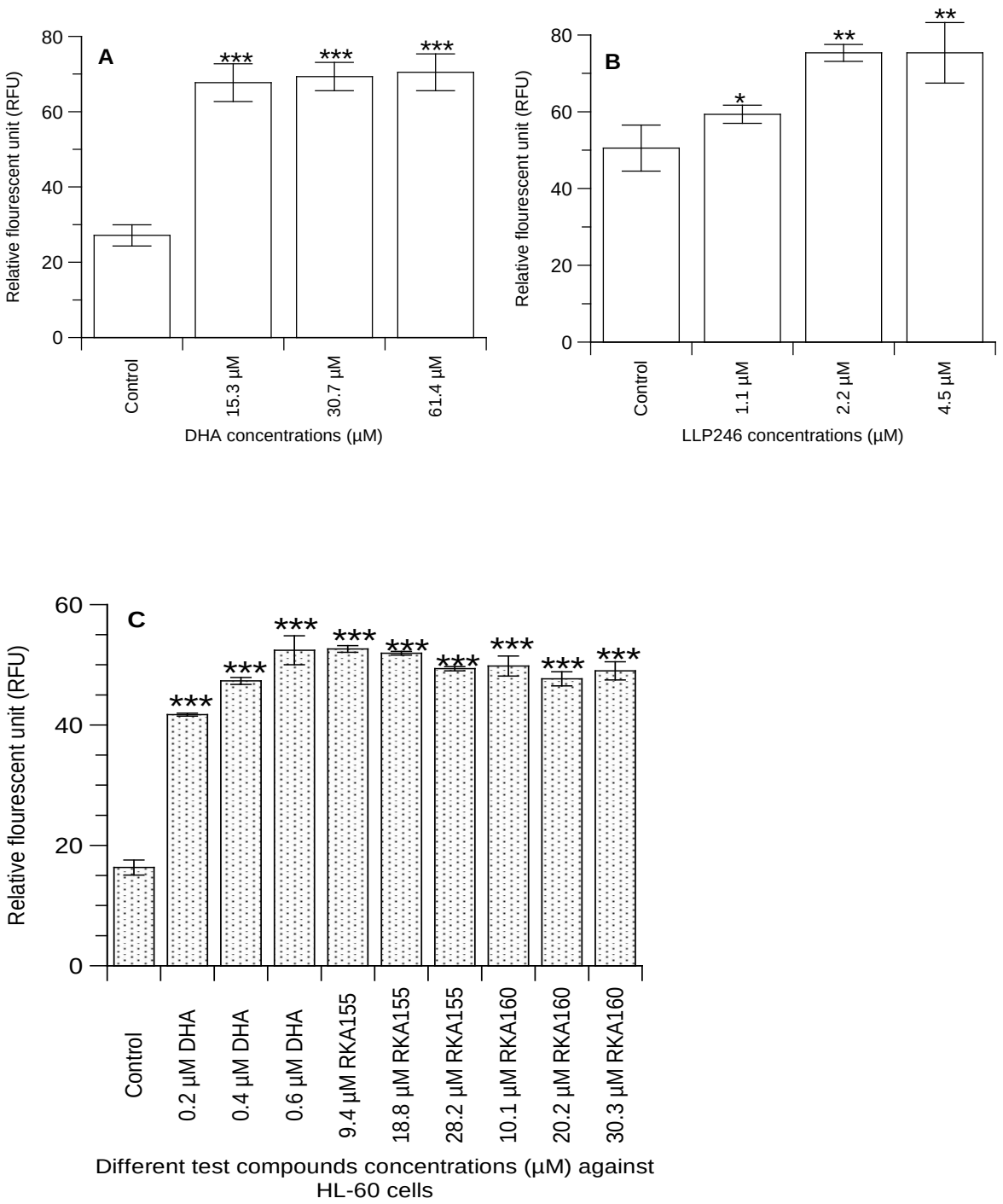


Figure 4: The effects of test compounds treatment on cleaved caspase-3 activity in HT29-AK cells. Cells were treated without and with different concentrations of LLP246 and DHA (as parent compound) for 24 hours before cleaved caspase-3 activity was measured by flow cytometric analysis. ***A)*** *Effects of 15.3 µM, 30.7 µM and 61.4 µM of DHA;* ***B)*** *Effects of 1.1 µM, 2.2 µM and 4.5 µM of LLP246 on cleaved caspase-3 activity compared to control in HT29-AK cells;* ***C)*** *Effects of 0.2 µM, 0.4 µM and 0.6 µM of DHA; 9.4 µM, 18.8 µM and 28.2 µM of RKA155 and 10.1 µM, 20.1 µM and 30.3 µM of RKA160 on cleaved caspase-3 activity in HL-60 cells.. Results are the mean ±SD of three independent experiments (n=3).* $^{*}P<0.05$, $^{**}P<0.01$ *and* $^{***}P<0.001$ *as tested by the Mann-Whitney U test.*

3.4. Test compounds treatment increased the cellular levels of AKT in HT29-AK cells

Compared to control (29.06 RFU), DHA treatment increased P-AKT activity by ~1.6 fold (45.59 RFU), ~1.7 fold (50.52 RFU) and ~1.8 fold (52.26 RFU) upon treating the HT29-AK cells with 15.3 µM, 30.7 µM and 61.4 µM of DHA, respectively (figure 5A). Similarly, LLP246 caused a significant (P≤0.05) increase in the activity of P-AKT as compared to control in HT29-AK cells (figure 5B). Here, control cells showed P-AKT activity of 45.75 RFU which was increased ~1.1 fold (49.66 RFU), ~1.4 fold (63.51 RFU) and ~1.6 fold (75.27 RFU) upon treating the cells with 1.1 µM, 2.2 µM and 4.5 µM LLP246, respectively (figure 5B).

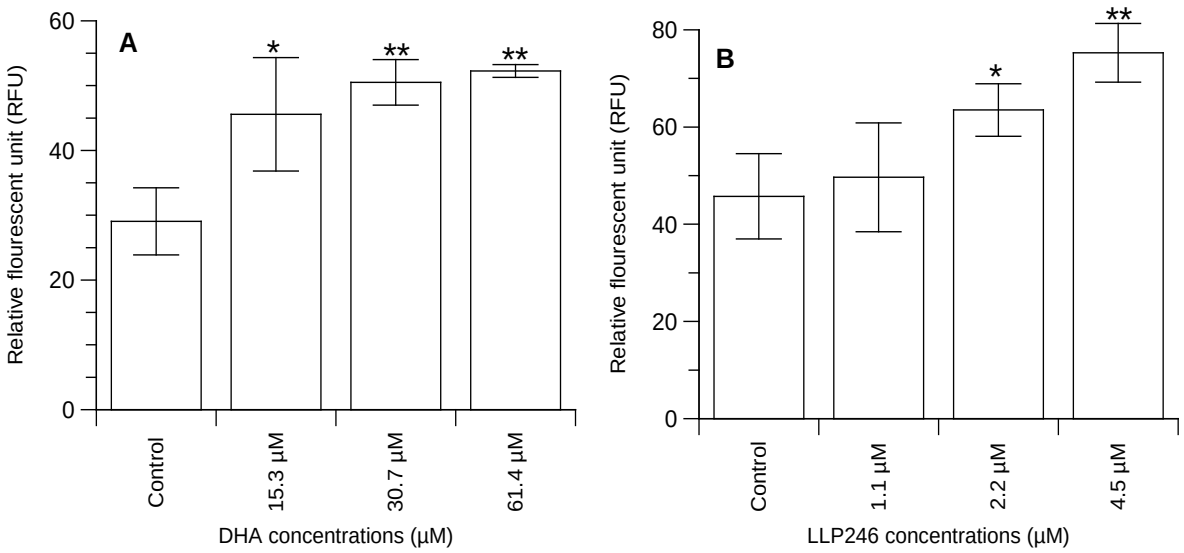


Figure 5: The effects of test compounds treatment upon the cellular levels of P-AKT in HT29-AK cells. Cells were treated without and with different concentrations of LLP246 and DHA (as control) for 24 hours before the P-AKT activity was measured by flow cytometric analysis. ***A)*** *Effects of 15.3 µM, 30.7 µM and 61.4 µM of DHA and* ***B)*** *Effects of 1.1 µM, 2.2 µM and 4.5 µM of LLP246 on the levels of P-AKT. Results are the mean ±SD of three independent experiments (n=3).* **P<0.05 and* ***P<0.01 as tested by the Mann-Whitney U test.*

3.5. DHA, LLP246 and SL2935 induced DNA fragmentation in HT29-AK cells

The analyses of induction of DNA fragmentation in HT29-AK cells was evaluated after incubating the cells with and without the test compounds for 24 h. As can be seen in figure 6, the drugs caused DNA damage which is illustrated in all the lanes by smearing. The effects of DHA on DNA damage was detectable in lanes 1, 2 and 3 which were treated with 61.4 µM, 30.7 µM and 15.3 µM of DHA, respectively. Similarly, the effects of LLP246 on DNA damage was detectable in lanes 4, 5 and 6 which were treated with 4.5 µM, 2.2 µM and 1.1 µM of LLP246. SL2935 treatment also caused DNA damage in lanes 7, 8 and 9 which were treated with 64.8 µM, 32.4 µM and 16.2 µM SL2935, respectively.

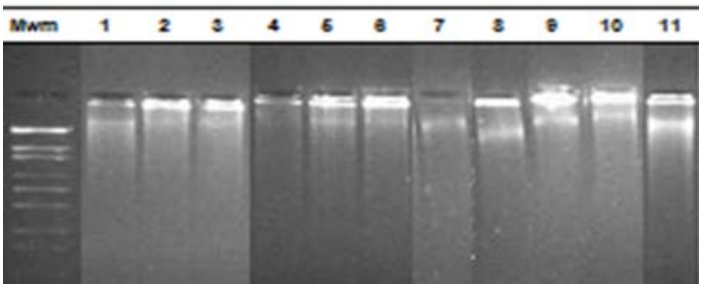


Figure 6: A representative image showing the effect of drug treatment on DNA fragmentation using agarose gel electrophoresis. HT29-AK cells were exposed to different concentrations of the test compounds for 24 hours. Purified DNA from control, heat treated and drug-treated samples were separated on 1.0 % agarose gel and visualized under UV light. Lamda Eco 1301 Molecular marker ladder (Bioline, UK) was used to determine DNA bands from 421 kDa to 19329 kDa, as shown in lane labelled Mwm. Lane 1=61.4 µM, lane 2=30.4 µM and lane 3=15.3 µM DHA-treated samples; lane 4=4.5 µM, lane 5=2.2 µM and lane 6= 1.1 µM LLP246-treated samples; lane 7=64.8 µM, lane 8=32.4 µM and lane 9=16.2 µM SL2935-treated samples, respectively. Lane 10 is control and lane 11 is a heat- treated control.

The characteristics DNA smear pattern in all the above mentioned lanes at different concentrations of the respective test compounds represent degradation of genomic DNA into smaller, low molecular weight fragments being definitive signs of induction

of apoptosis. Because different amounts of DNA were inadvertently added into each well (due to operator error), it is not possible to visualise the concentration-dependent effects of the test compounds on DNA damage. However, despite this, it can be observed that compared to control (lane 10) and heat-treated samples (lane 11), treatment of the cells with the test compounds caused observable DNA damage (figure 6).

3.6. Test compounds treatment decreased cellular survivin levels in HT29-AK cells

Given that survivin plays a pivotal role in the regulation of apoptosis, the effects of our novel agents on this unique molecule were investigated. As can be seen in figure 7A, DHA (at 15.3 µM and 30.7 µM) of the concentrations tested, significantly ($P<0.05$) decreased survivin concentration. In contrast, no alteration in the levels of survivin was observed upon treating the cells with any of the concentrations used of LLP246 (figure 7B). However, there was a significant ($P \leq 0.05$) in survivin concentration in HT29-AK cells, with a ~1.4 fold (4233.3 pg/ml) and ~2.7 fold (2128.3 pg/ml) decrease in levels as compared to control samples (5816.6 pg/ml) upon treatment with 32.4 µM and 64.8 µM SL2935, respectively (figure 7C).

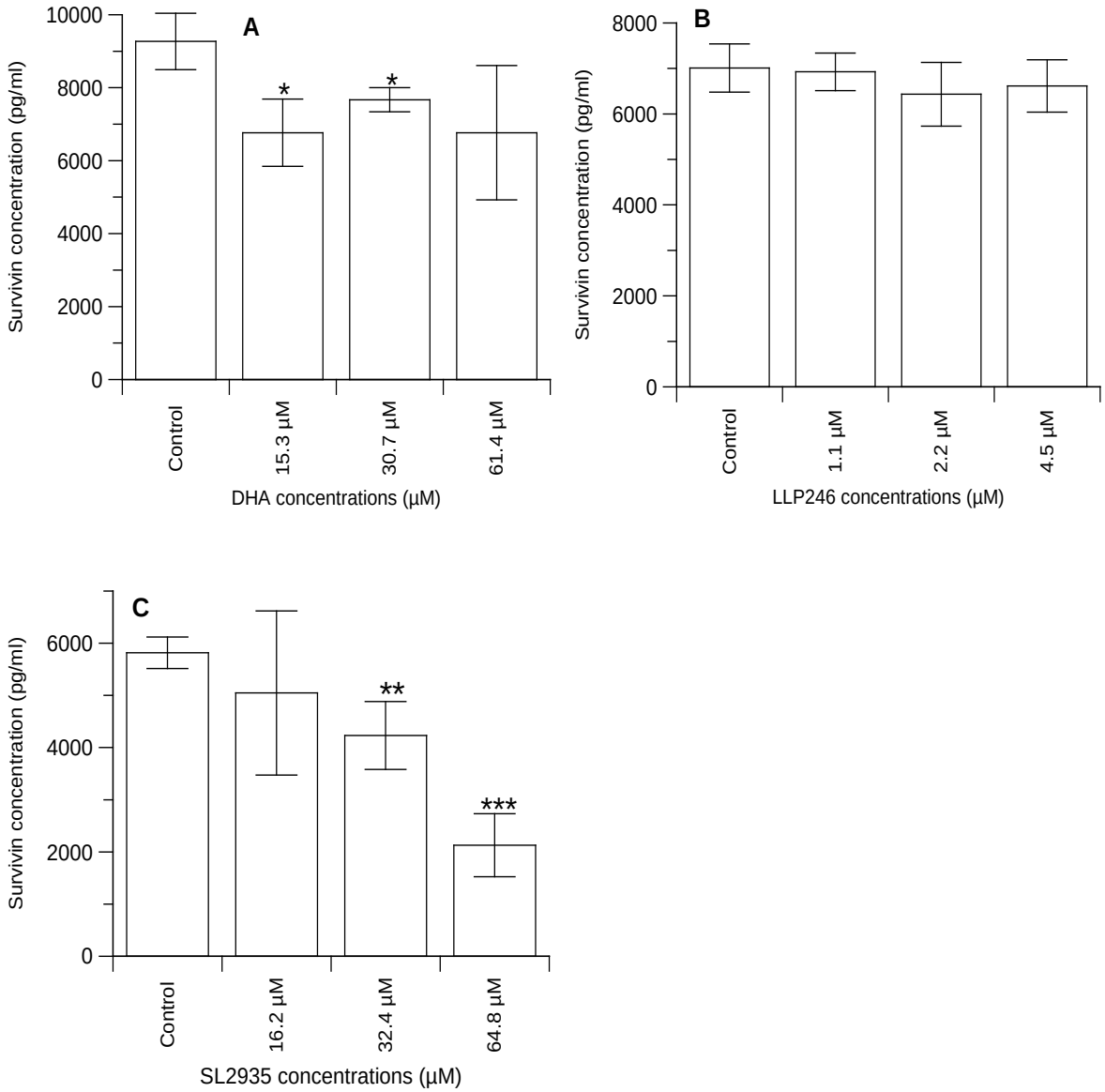


Figure 7: Showing the effects of test compounds treatment upon cellular survivin levels in HT29-AK cells. Cells were treated with different concentrations of DHA, LLP246 and SL2935 as shown for 24 hours before survivin levels were measured by ELISA. ***A)*** *Effects of 15.3 µM, 30.7 µM and 61.4 µM of DHA;* ***B)*** *Effects of 1.1 µM, 2.2 µM and 4.5 µM of LLP246 and* ***C)*** *Effects of 16.2 µM, 32.4 µM and 64.8 µM of SL2935 on survivin levels in HT29-AK cells. Results are the mean ± SD of three independent experiments (n=3).* **P<0.05,* ***P<0.01 and* ****P<0.001 as tested by the Mann-Whitney U test.*

3.7. Test compounds treatment caused an inhibition of the capacity of the wound to heal in HT29-AK cells

To investigate the effects of test compounds on the migration/invasion of cells, the effects on wound healing were measured. Upon the cells reaching confluency (figure

8X) after 7 days a wound was created (figure 8Y) using the tip of sterile 10 µl pipette and the cells were treated without or with the test compounds as shown (figure 8A-I). It was observed that the wound in figure 8Y with diameter of 64.14 µm (figure 10) was closed after 9 days as shown in figure 8Z, while there is concentration-dependent inhibition of the ability of the wound to heal/close (figure 8A, B and C) upon treatment with DHA (at 30.7 µM, 61.4 µM and 122.8 µM, respectively) after 9 days with a wound diameter of 9.43 µm, 15.05 µm and 29.71 µm, respectively (figure 10).

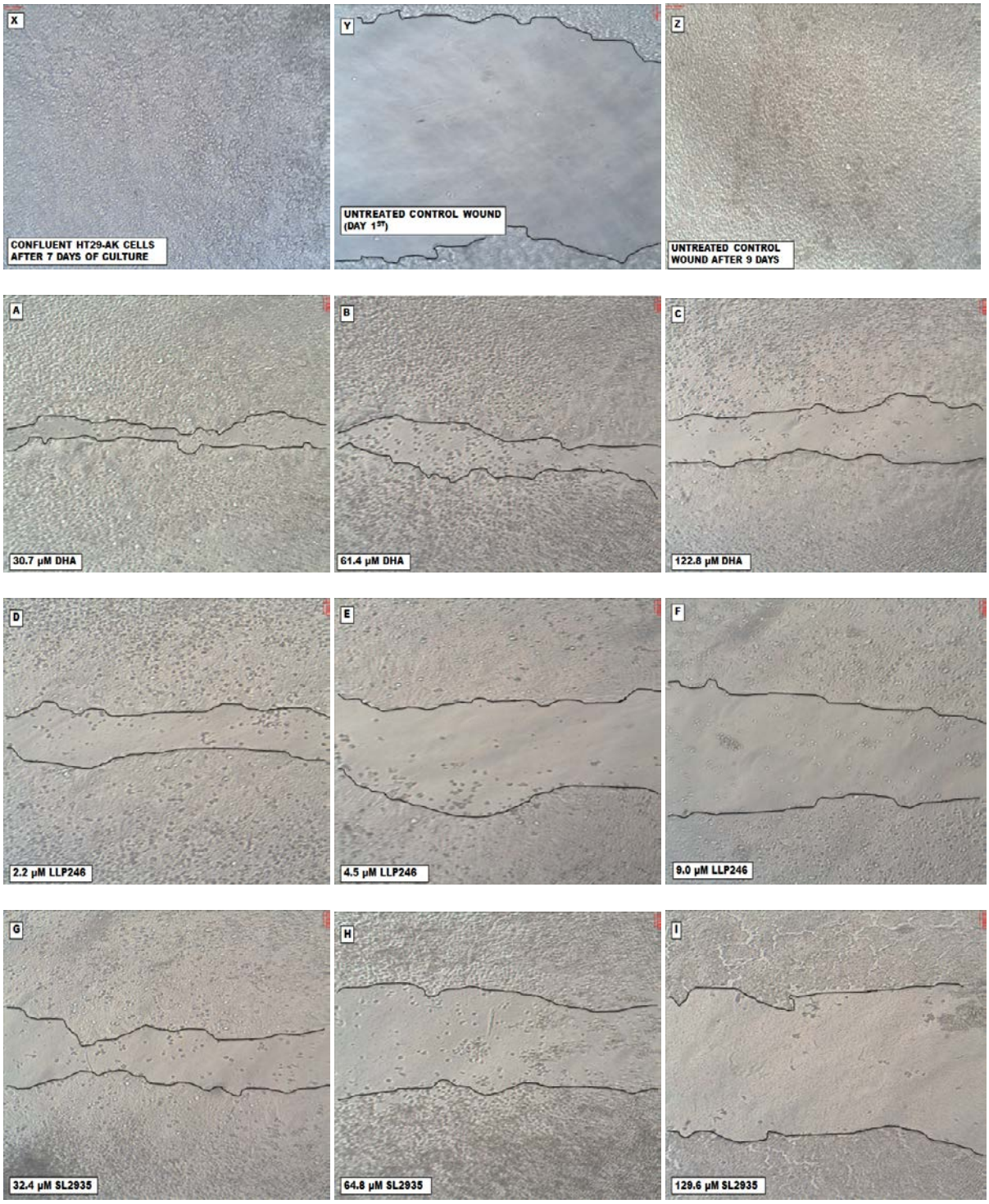
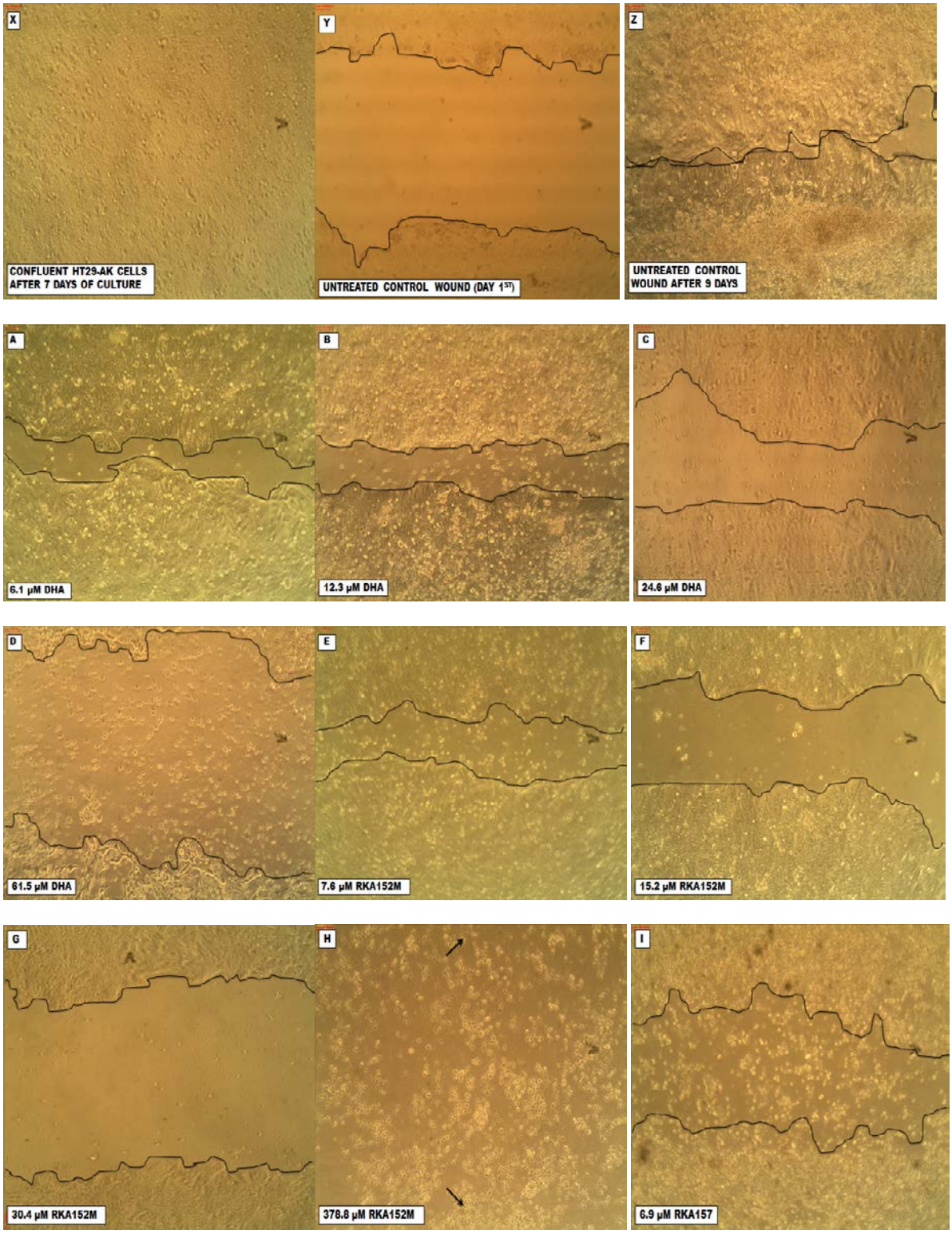


*Figure 8: Representative light-microscopic images of the concentration-dependent effects of the test compounds (DHA, LLP246 and SL2935) on wound healing in HT29-AK cells. Cells were cultured for 7 days to allow the establishment of confluency (X) and following the creation of a scratch (wound) using the tip of a sterile 10 µl pipette (Y). (Z) Shows the closure of the wound created in Y following 7 days of culture without test compound treatment. This serves as the positive control. Effects of **A)** 30.7 µM, **B)** 61.4 µM and **C)** 122.8 µM of DHA. Effects of **D)** 2.2 µM **E)** 4.5 µM and **F)** 9.0 µM of LLP246. Effects of **G)** 32.4 µM, **H)** 64.8 µM and **I)** 129.6 µM of SL2935 on wound healing in HT29-AK cells after 9 days. Lines represents the wound area, which can be seen to have closed or widened in the images. Images are at 10x magnification.*

There was also a concentration dependent inhibition of wound healing with diameter of 23.42 µm, 47.06 µm and 53.57 µm, respectively (figure 10) following treatment with LLP246 (at 2.2 µM, 4.5 µM and 9.0 µM, respectively) (figure 8D, E and F). Similarly, upon treatment with SL2935 (at 32.4 µM, 64.8 µM and 129.6 µM), there was a concentration-dependent inhibition of wound healing with diameter of 22.10 µm, 44.90 µm and 71.33 µm, respectively, after 9 days of culture (figure 8G, H and I; figure 10).

Given that the tetraoxane dimers showed cytotoxic activity, their effects on wound healing in comparison to DHA were investigated. Upon the cells reaching confluency (figure 9X) after 7 days a wound was created (figure 9Y). After washing away the non-adherent cells, the plates were treated without or with the test compounds as shown (figure 9A-P). It was observed that the wound with diameter 50 µm (figure 11) in figure 9Y was almost closed with wound diameter 5 µm (figure 11) after 9 days of culture as shown in figure 9Z. There is concentration-dependent inhibition of the ability of the wound to heal/close after 9 days of culture with DHA (at 30.7 µM, 61.4 µM and 122.8 µM, respectively) with diameter of 18.2 µm, 10.43 µm and 28.14 µm, respectively (figure 9A, B and C; figure 11); RKA152M (at 37.9 µM, 75.8 µM and 151.5 µM, respectively) with diameter of 21.01 µm, 36.86 µm and 86.87 µm, respectively (figure 9E, F and G; figure 11); RKA157 (at 34.4 µM, 68.9 µM and 137.8 µM, respectively) with diameter of 44.29 µm, 42.28 µm and 58.3 µm, respectively (figure 9I, J and K; figure 11) and RKA160 (at 11.4 µM, 22.8 µM and 45.7 µM respectively) with diameter of 10.29 µm, 24.14 µm and 44.86 µm, respectively (figure 9M, N and O; figure 11).


X
CONFLUENT HT29-AK CELLS
AFTER 7 DAYS OF CULTURE
Y
UNTREATED CONTROL WOUND (DAY 1ST)
Z
UNTREATED CONTROL
WOUND AFTER 9 DAYS
A
6.1 µM DHA
B
12.3 µM DHA
C
24.6 µM DHA
D
61.5 µM DHA
E
7.6 µM RKA152M
F
15.2 µM RKA152M
G
30.4 µM RKA152M
H
378.8 µM RKA152M
I
6.9 µM RKA157

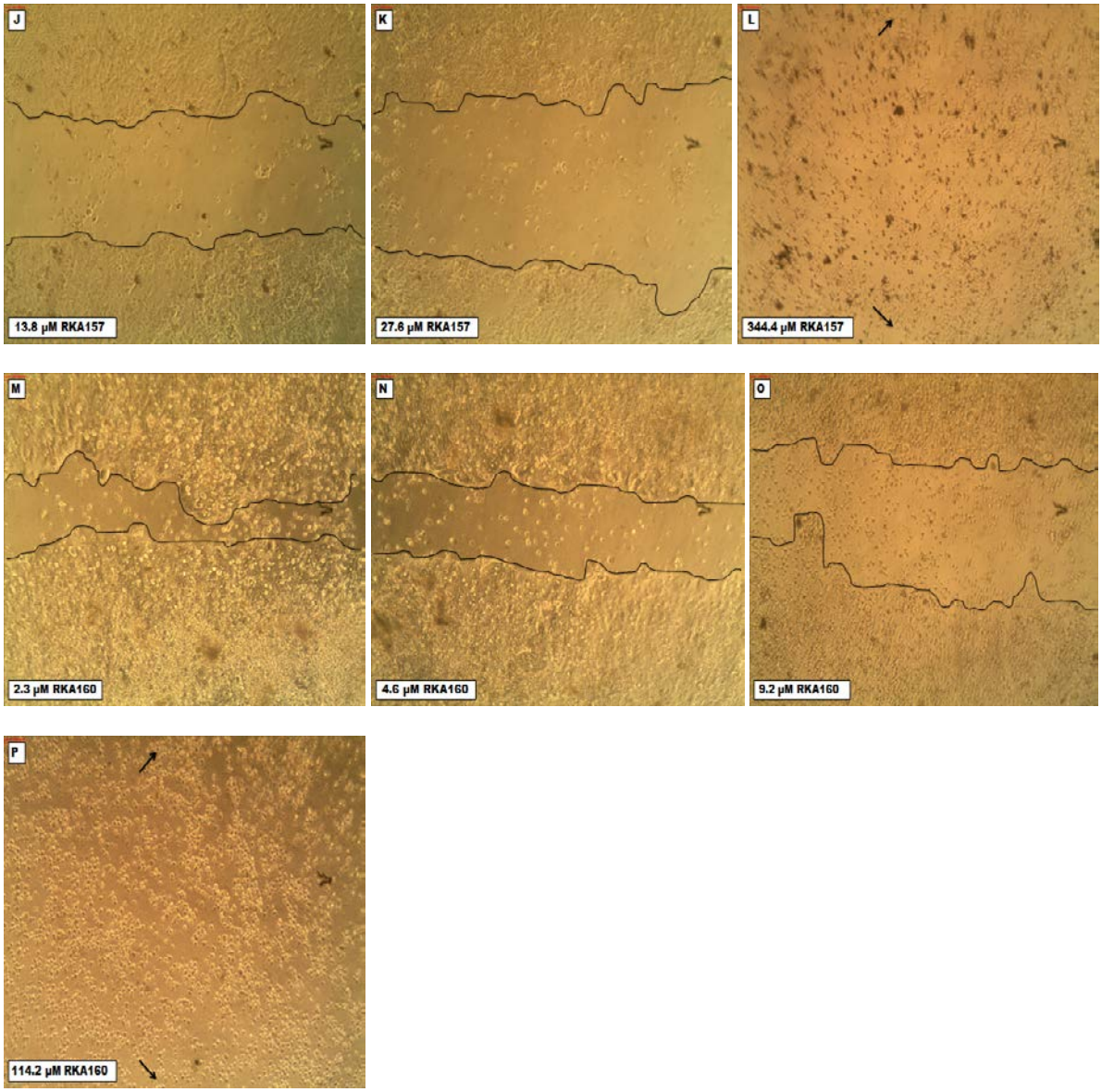


*Figure 9: Representative light-microscopic images of the concentration-dependent effects of the test compounds (DHA, RKA152M, RKA157 and RKA160) on wound healing in HT29-AK cells. Cells were cultured for 7 days to allow the establishment of confluency **(X)** and following the creation of a wound using the tip of a sterile 10 µl pipette **(Y)**. **(Z)** Shows the closure of the wound created in Y following 9 days of culture without drug treatment. This serves as the positive control. Effects of **A)** 30.7 µM, **B)** 61.4 µM, **C)** 122.8 µM and **D)** 306.9 µM of DHA. Effects of **E)** 37.9 µM, **F)** 75.8 µM **G)** 151.5 µM and **H)** 378.8 µM of RKA152M. Effects of **I)** 34.4 µM, **J)** 68.9 µM, **K)** 137.8 µM and **l)** 344.4 µM of RKA157. Effects of **M)** 11.4 µM, **N)** 22.8 µM, **O)** 45.7 µM and **P)** 114.2 µM of RKA160 on wound healing in HT29-AK cells after 9 days. Lines represent the wound area, which can be seen to have closed or widened in the images. Arrows shown in images H, L and P illustrate that the wound sizes are greater than the margin of the images. Images are at 10x magnification.*

It was observed that at the highest concentration of each test compound a much larger wound than that created in the control (figure 9Y) was left due to the

cytotoxicity of the agents, with DHA at 306.9 µM, RKA152M at 378.8 µM, RKA157 at 344.4 µM and RKA160 at 114.2 µM (figure 9 and 11). Overall, the RKA compounds were more effective in inhibition of wound as compared to DHA (figure 9 and 11).

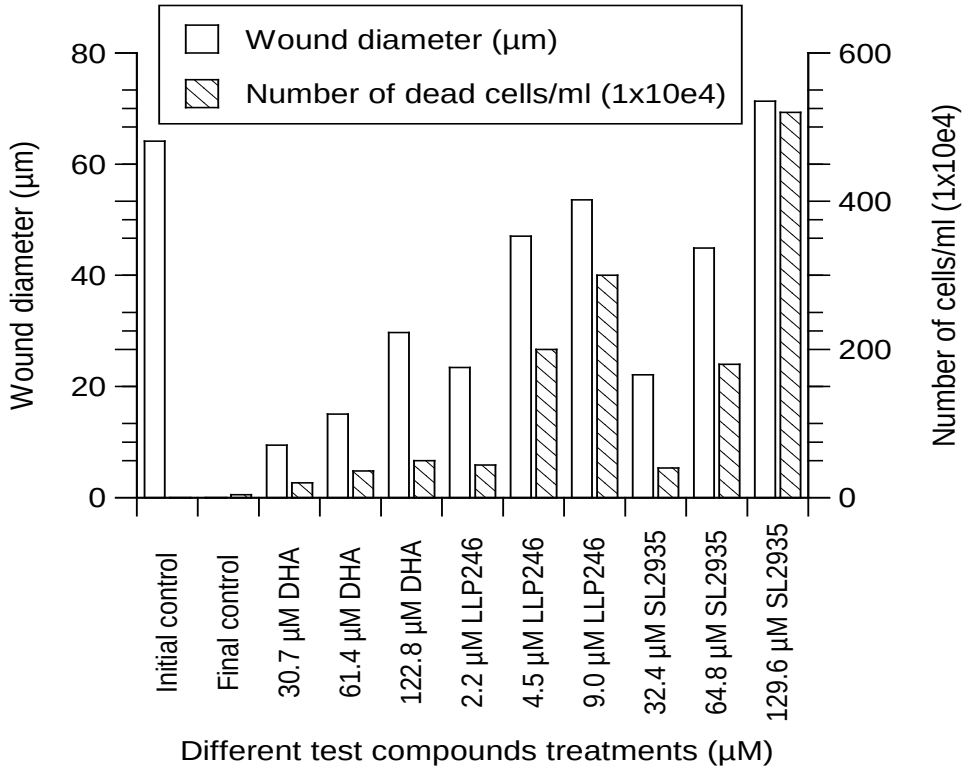


Figure 10: The graph showing effects of test compounds treatment on the capacity of the wound to heal via means of diameters and viability on HT29-AK cells. Cells were allowed to reach confluency before a scratch (wound) is created and the cells then treated without or with different concentrations of DHA: 30.7 µM, 61.4 µM and 122.8 µM; LLP246: 2.2 µM, 4.5 µM and 9.0 µM; SL2935: 32.4 µM, 64.8 µM and 129.6 µM for 9 days. Following incubation, the diameters of the wounds were estimated as described in the materials and methods section.

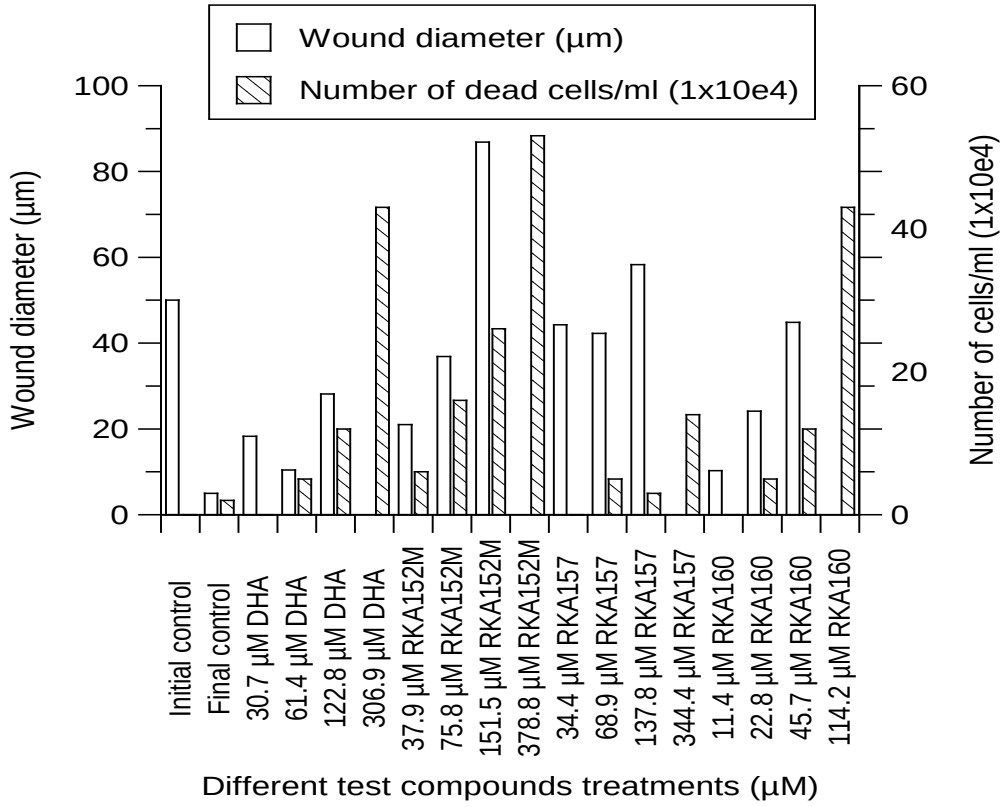


Figure 11: The graph showing effects of test compounds treatment on the capacity of the wound to heal via means of diameters and viability on HT29-AK cells. Cells were allowed to reach confluency before a scratch (wound) is created and the cells then treated without or with different concentrations of DHA: 30.7 µM, 61.4 µM and 122.8 µM; RKA152M: 37.9 µM, 75.8 µM and 151.5 µM; RKA157: 34.4 µM, 68.9 µM and 137.8 µM; RKA160: 11.4 µM, 22.8 µM and 45.7 µM for 9 days. Following incubation, the diameters of the wounds were estimated as described in the materials and methods section.

3.8. Trypan blue dye exclusion to test for viability

As the test compounds are shown to be cytotoxic (table 2) and also decreased the ability of the wound to close (figure 8 and 9), further studies were conducted to find out if the cells that can be seen in the vicinity of the wounds in figure 8 and 9 were dead or live cells, i.e. whether these was some migrations of the cells. Here, an aliquot of the cell suspension from each of the respective wells was stained with trypan blue and the results of this manipulation can be seen in figure 10 and 11. There was a concentration-dependent increase in the number of dead cells compared to control (figure 10 and 11).

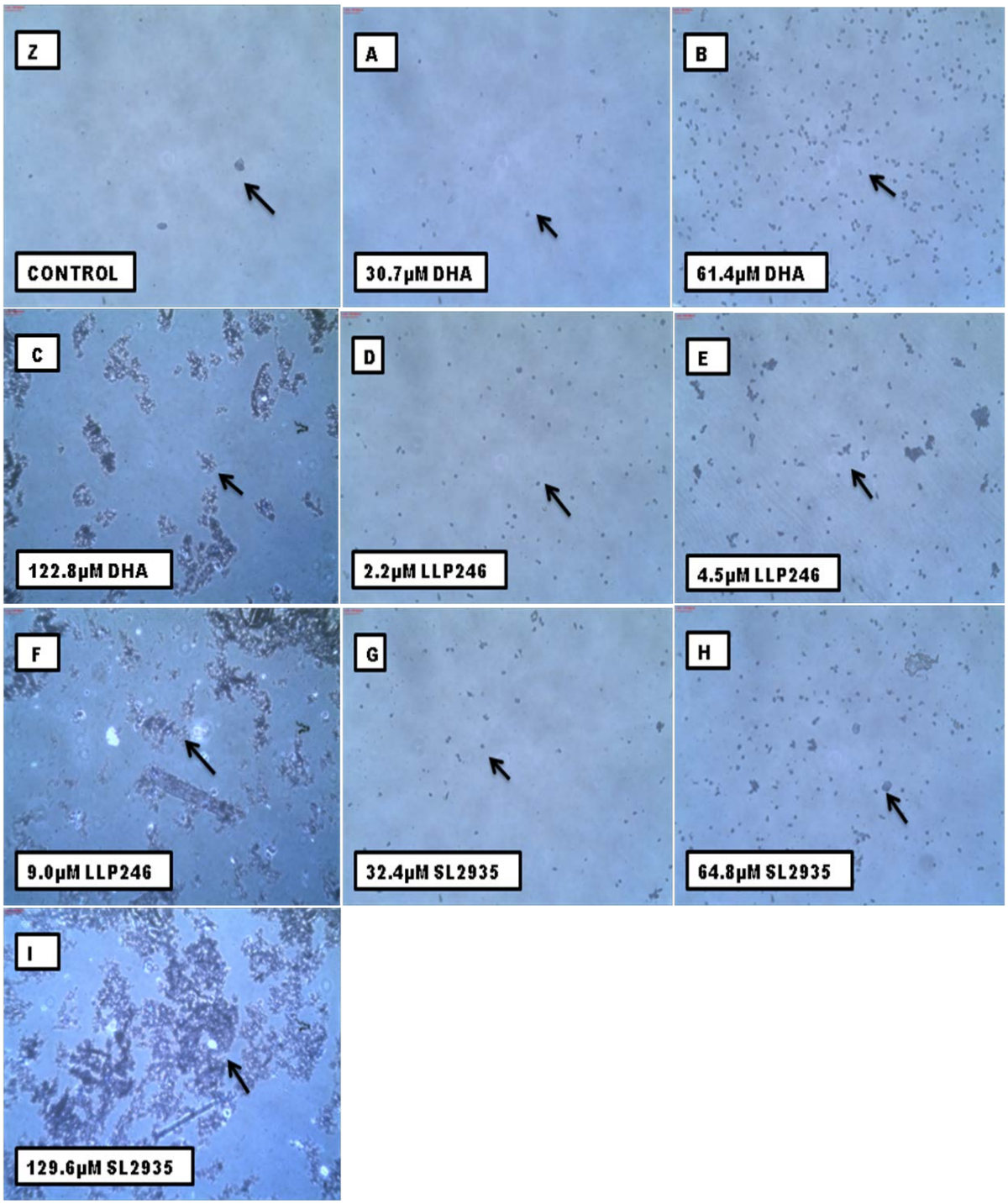


Figure 12: Representative light-microscopic images of the concentration-dependent effect of the test compounds (DHA, LLP246 and SL2935) on the viability of HT29-AK cells. ***(Z)*** *Represents control which is used to compare test compound-treated samples. Effects of* ***A)*** *15.3 µM,* ***B)*** *30.7 µM and* ***C)*** *61.4 µM of DHA. Effects of* ***D)*** *1.1 µM,* ***E)*** *2.2 µM and* ***F)*** *4.5 µM of LLP246. Effects of* ***G)*** *16.2 µM,* ***H)*** *32.4 µM and* ***I)*** *64.8 µM of SL2935 on HT29-AK cells. An aliquot of the cell suspension in figure 8 was taken from each well and stained with trypan blue. Arrows represent dead cells with dark blue colour and live cells with bright colour. Similar data were obtained for the cells treated with the tetraoxane dimers. Images are at 10x magnification.*

As can be seen in figure 10, there is concentration dependent increase in dead cells with 10 x 10^6, 16 x 10^6 and 29 x 10^6 cells/ml with DHA 30.7 µM, 61.4 µM and 122.8 µM treatment; 22 x 10^6, 103 x 10^6 and 150 x 10^6 cells/ml with LLP246 2.2 µM, 4.5 µM and 9.0 µM treatment; 20 x 10^6, 98 x 10^6 and 269 x 10^6 cells/ml with SL2935 32.4 µM, 64.8 µM and 129.6 µM treatment, respectively, compared to control (2 x 10^6 cells/ml) in suspension. These results were supported by images captured under light microscope (figure 12); showing the concentration-dependent increase in number of dead cells in suspension as compared to control (figure 12Z).

As can be seen in figure 11, all of the test compounds increased cell death in a concentration-dependent manner with RKA152M having the greatest effects. In case of RKA160, the numbers of dead cells were similar to that of DHA (figure 11). The rank order for the number of dead cells with respect to test compound used is: RKA157<RKA160=DHA<RKA152M. Despite this, it can be seen that the test compounds did not inhibit some of the cells from migrating into the vicinity of the wounds (figures 8 and 9).

Section 4

Discussion

The ARSs have already shown great potential as anti-cancer agents in vitro and in vivo (Chen *et al.,* 2004; Efferth, 2007; Soomro *et al.,* 2011). These compounds have been extensively studied as promising candidates for cancer therapy (Efferth, 2005; Mercer *et al*., 2007; Lu *et al*., 2008) and owing to their structure being amenable to modification. Rational drug design and synthesis are well-established disciplines in pharmaceutical research. The endoperoxides have been shown to possess potent cytotoxic activity against human colon and leukaemia cancer cells (Mercer *et al.,* 2007; Jones *et al.,* 2009). In contrast, the agents are lowly cytotoxic against human non-small lung neoplastic cells which depend on the lower proliferative and iron uptake status compared to the former cells types (Efferth, 2005; Mercer *et al.,* 2007).

The current study aimed at investigating the cytotoxicity and mode of action of rationally designed novel compounds based on the ARS backbone. In study by Mercer et al, it was demonstrated that ARS derivatives such as deoxy-10beta-(-P-flourophenoxy) DHA, which lacks the endoperoxide bridge, was 50- and 130-fold less active in HL-60 and Jurkat cells by using liquid chromatography-mass spectrometery analyses to monitor endoperoxide activation (Mercer *et al.,* 2007). Given that the presence of the endoperoxide bridge is important for antitumour activity, it was investigated whether the presence of more –C-O-O-C in the structure would result in improved activity. To this end, the activity of tetraoxane dimers was investigated against the cells. Our initial studies investigated the cytotoxicity of LLP246, SL2935 (derivatives of DHA, rationally designed from DHA and dipyrrole) against HT29-AK. In addition, the activity of tetraoxane dimers (RKA 152M, RKA

153, RKA 154, RKA 155, RKA 157 and RKA 160) were evaluated against HT29-AK and HL-60.

It was observed that LLP246 (with an IC_{50} of 4.47±0.39 µM) is significantly ($P<0.001$) more potent than DHA (IC_{50} of 61.39±2.86 µM), whereas, SL2935 (IC_{50} of 64.81±0.36 µM) is of identical potency to DHA against HT29-AK cells. The improved cytotoxicity of LLP246 over DHA may be due to the presence of the acridine moiety within the structure. There is evidence that 1,2,4-trioxanes interact with DNA (Jones *et al.,* 2009) and this may be further enhanced by the acridine skeleton, making LLP246 even more DNA-selective thus cytotoxicity (Baraldi *et al.,* 2003; Baraldi *et al.,* 2006; Jones *et al.,* 2009). Furthermore, several in vitro studies suggest that 1,2,4-trioxanes growth inhibition activities against tumour cells are associated with cell-cycle obstruction (Mercer *et al.,* 2007; Parekh *et al.,* 2009; Zhang *et al.,* 2010). It has been reported that DHA and its ether-linked dimers inhibit cell cycle progression through G_1 phase arrest by accumulation of cancer cells in G_1 phase and up-regulation of GADD 153, which is an ER-stress molecule (Efferth, 2005; Mercer *et al.,* 2007; Lu *et al.,* 2010). It is reasonable to postulate that LLP246 may interfere with cell-cycle progression which ultimately results in growth inhibition.

Although, 1,2,4,5-tetraoxanes have demonstrated inhibition of cancer cell proliferation at micromolar and submicromolar levels through an apoptotic mechanism (Zizak *et al.,* 2009; Kumar, Sharma and Rawat, 2011), it was observed that dimerization of the tetraoxanes did not enhance their anti-tumour effects against the cell types studied. Of the tetraoxane dimers evaluated, RKA 160 (IC_{50} of 22.85±4.36 µM) is the most potent, being 2.7 times more potent than DHA against HT29-AK cell line (table 2). In contrast, none of the other dimers were as potent as DHA (IC_{50} of 0.39±0.10 µM) against HL-60 cells (table 2). The endoperoxide bridge

is central to antimalarial and antitumour activity (Mercer *et al.,* 2007). Evidence in support of this stems from the observation that oral co-administration of ART with ferrous sulphate resulted in greater cytotoxicity compared to ART alone (Fafowora *et al.,* 2011). Similarly, iron tagged artemisinins demonstrated improved cytotoxic activity over artemisinin alone (Singh and Lai, 2001; Lai *et al*., 2005). Following these findings, It can be hypothesised that the presence of more endoperoxide bridges does not necessarily translate into greater cytotoxicity (figure 2C-H). This may 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. Future experiments may confirm these findings by tagging the test compounds with transferrin or evaluating their effects in the presence of ferrous sulphate.

Iron is an important co-factor for DNA metabolism and cancer cells need to take it up via TfR for growth and proliferation (Mercer *et al.,* 2007; Oh *et al.,* 2009). Thus, TfR expression is increased to facilitate iron intake, and its increased expression positively correlates with the aggressiveness of cancer cells (Efferth, 2005; Singh and Lai, 2006; Cheung *et al.,* 2010). There is a positive correlation between total TfR and sTfR; thus concentration of sTfR is an indirect measure of TfR levels (Beguin *et al.,* 1988; Singh and Lai, 2001; Lai *et al.,* 2005). In other words, the measurement of sTfR is not only valuable in determining prognosis but also in indirectly assessing the cellular levels of iron (Ferguson *et al.,* 1992; Erslev and Besarab, 1995), which is important in linking the antitumour effects of the agents. In the current study, the measured sTfR in both control and test compounds treated samples suggest the presence of iron in the cells. DHA decreased, in a concentration-dependent manner,

the levels of sTfR as compared to untreated HT29-AK cancer cells (figure 3A). In contrast, LLP246 and SL2935 demonstrated variable effects on sTfR (figure 3B and 3C). It warrants further investigating in order to decipher the true effects of the test compounds on sTfR levels. Overall, our data show the presence of sTfR which confirms the presence of iron within the cells and hence the observed cytotoxic effects of the test compounds (table 2).

Many studies have shown that some anticancer agents prevent tumour promotion and progression via the induction of apoptosis (Huang *et al.,* 2006; Mercer *et al.,* 2007; Handrick *et al.,* 2010; Morrissey *et al.,* 2010). Apoptosis is initiated by the induction of caspase-dependent apoptotic cell death, mitochondrial endoplasmic reticulum stress, activation of effector caspases-3 and -7, 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 pathways (Ghavami *et al.,* 2010). It has been reported that DHA induces apoptosis by up-regulation of pro-apoptotic protein Bax in pancreatic cancer cells leading to the downstream activation of caspase-3, a major final stage in the apoptotic pathway, causing apoptosis (Aung *et al.,* 2011). In addition, DHA induced caspase-dependent apoptotic cell death characterised by mitochondrial membrane depolarisation, activation of caspase-3 and -7, sub-G_0/G_1 DNA formation all of which were attenuated by treatment with benzyloxycarbonyl-VAD-fluoromethyl ketone, a caspase inhibitor (Efferth, 2006; Jiao *et al.,* 2007, Lu *et al.,* 2009). In human leukaemia HL-60 and HT29-AK cells, DHA induced apoptosis, which was associated with mitochondrial dysfunction and caspase-3 activation in a concentration-dependent manner (Li *et al.,* 2007; Lu *et al.,* 2008; Zhou *et al.,* 2008; Jones *et al.,* 2009). Data shown here demonstrate that DHA

and LLP246 elevated the levels of catalytically active caspase-3 in HT29-AK cell line (figure 4A and 4B). It is also demonstrated the tetraoxane dimers, RKA155 and RKA160 also elevated the levels of catalytically active caspase-3 in HL-60 cells (figure 4C). It may be postulated that the agents increase in cellular caspase-3 may cause the activation of death receptor DR5, initiator (apical) caspase-8, which will trigger activation of effector caspases -3, but also caspase 6 and 7 which may lead to DNA fragmentation and ultimately apoptosis (Mercer *et al.,* 2007; Lu *et al.,* 2008; Ghavami *et al.,* 2009; Lu *et al.,* 2009; He *at al.,* 2010). Although there was no evidence to suggest the involvement of either the intrinsic or/and extrinsic pathway in the increased levels of cleaved caspase-3, there is evidence that the intrinsic pathway (mitochondrial) may be initiated by several different stimuli, such as extra- or intracellular stress signals or upon drug therapy (Efferth, 2005; Ghavami *et al.,* 2009), it is probable that LLP246 treatment may be the activator of the pathway.

Data presented here suggest that test compounds treatment of the cells caused DNA fragmentation (figure 6). There were signs of DNA fragmentation in HT29-AK cells upon DHA, LLP246 and SL2935 treatment as compared to untreated samples (figure 6). The cytotoxicity of the agents may be explained by their effects on caspase-3 activity leading to DNA fragmentation and ultimately apoptosis (Efferth, 2005; Lu *et al.,* 2009; He *et al.,* 2010).

AKT promotes cell survival, 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; De Souza, Russell and Kearsley, 2009). An in-vitro study in human prostate malignant cells (PC-3, DU145 and LNCaP) has shown DHA-mediated apoptosis with the inhibition of the PI3-Kinase/AKT pathway and extracellular signal regulated kinase survival pathway (He *et al.,* 2010). Here, a concentration- dependent increase

in levels of P-AKT in HT29-AK cells upon both LLP246 and DHA treatment was measured (figure 5). Previous studies have demonstrated that PI3K/AKT pathway controls cell survival and drug resistance in HT29-AK and in drug resistant HT29RDB colon cancer cells (Ghani *et al.*, 2006; Chen, 2011). It can be speculated that the increase in P-AKT levels may be due to a reciprocal attempt of the cells to survive upon treatment of the agents or as independent stimuli to inhibit apoptosis induced upon test compounds treatment. It has been suggested that increase in P-AKT may involve mitogen signalling pathways mediated by Ras and PI3K/AKT in controlling multidrug resistance in colon cancer cells (Ghani *et al.,* 2006). Further studies 9in the presence and absence of PI3K/AKT inhibitors) would clarify the role of P-AKT in the cytotoxicity of the test compounds.

Survivin is over 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 study in 4 prostate cancer cell lines (C4-2, LNCaP, DU145 and PC-3) showed that ARS dimer (2PY) at 15µM significantly decreased the expression of survivin at the mRNA levels (Morrissey *et al.,* 2010). It is known that in tumourigenesis, survivin expression is inversely correlated with inhibition of apoptosis (Lu *et al.,* 2005; Ryan, O'Donovan and Duffy, 2009). Data reported here suggest a dysregulation of the cells ability to regulate apoptosis. There was a concentration-dependent decrease in levels of survivin upon SL2935 treatment (figure 7C). DHA and LLP246 also decreased survivin levels but this was not concentration-dependent (figure 7A and 7B). It can be speculated that the higher concentration of test compound did not cause any further drop in survivin levels because there may be a threshold beyond which no further drop can occur.

Metastasis and invasion are fundamental properties of malignant cancer cells which is a leading cause of death in patients with cancer (Hwang *et al.,* 2010). ARS has a concentration-dependent inhibitory effect on invasion and migration in human carcinoma cell lines HepG2 and SMMC-7721, both in vitro and in vivo (Du *et al.,* 2009; Weifeng *et al.,* 2011). DHA is also an effective anti-metastastic agent against human fibrosarcoma HT-1080 cells (Hwang *et al.,* 2010; Wang *et al.,* 2011). The scratch (wound healing) model used in our studies confirms these findings in HT29-AK cells. A concentration-dependent inhibition of wound healing upon treating HT29-AK cells with DHA, LLP246, SL2935 and RKA compounds as compared to untreated samples, demonstrated by an increase in wound diameter was measured (figure 8-11). The wound healing results were also supported by the trypan blue test, which showed a concentration-dependent increase in dead HT29-AK cells as compared to control (figure10-12). In previous studies 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 down-regulation of MMP-9, MMP-2 gene expression, through inhibition of PKCa/Raf/ERK and JNK phosphorylation, reduction of NF-kB and activation of Cdc42, which enhanced E-cadherin activity, resulting in greater cell-cell adhesion and consequently reduced metastasis. Further experiments, such as testing the effects of the test compounds on the expression of MMP-9 and MMP-2, which are the focus of current investigations, may accept or refute these claims.

Conclusions: The rationally designed and synthesised novel agents showed potent cytotoxic effects against HT29-AK and HL-60 cells. There is evidence of the presence of TfR, stemming from the measurement of sTfR in the samples. The

cytotoxicity of the test compounds is strongly mediated by increased levels of caspase-3, DNA fragmentation, decreased survivin levels and an inability of the wound to close. The results presented here provide evidence that further studies of test compounds may have cytotoxic activity (either alone or in combination with conventional chemotherapeutic agents) against other cancer cell types, such as breast cancer, prostate cancer, ovarian cancer and pancreatic cancer**.**

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