Uptake of Synthetic Low Density Lipoprotein by leukemic stem cells – a potential stem cell targeted drug delivery strategy

Peixun Zhou[a], Sophia Hatziieremia[a], Moira A. Elliott[b], Linda Scobie[c], Claire Crossan[c], Alison M. Michie[a], Tessa L. Holyoake[a], Gavin W. Halbert[b] and Heather G Jørgensen [a]

[a] Paul O'Gorman Leukemia Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Gartnavel General Hospital, 1053 Great Western Road, Glasgow, G12 0NY; [b] Strathclyde Institute for Pharmacy and Biomedical Sciences, University of Strathclyde, John Arbuthnott Building, 27 Taylor Street, Glasgow, G4 0NR; [c] Department of Biological & Biomedical Sciences, School of Life Sciences, Glasgow Caledonian University, Govan Mbeki Health Building, Cowcaddens Road, Glasgow, G4 0BA.

Corresponding author: Dr Heather Jørgensen, Paul O'Gorman Leukemia Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Gartnavel General Hospital, 1053 Great Western Road, Glasgow, G12 0NY*;* tel: +44 (0)141 301 7875; fax: +44 (0)141 301 7898; e-mail: heather.jorgensen@ glasgow.ac.uk

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Abstract

Chronic myeloid leukemia (CML) stem/progenitor cells, which over-express Bcr-Abl, respond to imatinib by a reversible block in proliferation without significant apoptosis. As a result, patients are unlikely to be cured owing to the persistence of leukemic quiescent stem cells (QSC) capable of initiating relapse. Previously, we have reported that intracellular levels of imatinib in primary primitive CML cells ($CD34^+38^{lo/-}$), are significantly lower than in CML progenitor cells (total $CD34^+$) and leukemic cell lines. The aim of this study was to determine if potentially sub-therapeutic intracellular drug concentrations in persistent leukemic QSC may be overcome by targeted drug delivery using synthetic Low Density Lipoprotein (sLDL) particles. As a first step towards this goal, however, the extent of uptake of sLDL by leukemic cell lines and CML patient stem/progenitor cells was investigated. Results with non-drug loaded particles have shown an increased and preferential uptake of sLDL by Bcr-Abl positive cell lines in comparison to Bcr-Abl negative. Furthermore, CML $CD34^+$ and primitive $CD34^+38^{lo/-}$ cells accumulated significantly higher levels of sLDL when compared with non-CML $CD34^+$ cells. Thus, drug-loading the sLDL nanoparticles could potentially enhance intracellular drug concentrations in primitive CML cells and thus aid their eradication.

Key words: low density lipoprotein; Chronic Myeloid Leukemia; CD34+; hemopoietic stem cells; nanoparticle

Introduction

Chronic Myeloid Leukemia (CML) is a blood stem cell disorder characterised by the Philadelphia (Ph) chromosome and the resulting Bcr-Abl oncoprotein. Imatinib mesylate (IM; Gleevec®, Novartis Pharma AG, Basle, Switzerland) is a molecularly targeted drug that has proven to be remarkably effective in treating patients with CML in early chronic phase, with impressive rates of complete hematological and cytogenetic response [1, 2]. However, most patients with CML still show molecular evidence of minimal residual disease (MRD) i.e. the majority is polymerase chain reaction (PCR) positive for *Bcr-Abl*, the causative oncogene, even after IM treatment [1, 2]. Furthermore, previous studies have demonstrated the existence of rare, primitive ($CD34^+$), leukemic (Ph^+), quiescent stem cells ('QSC') [3-5] that can regenerate CML populations in immunodeficient mice and are insensitive to 10μM IM *in vitro*. It is therefore probable that a population of CML stem cells may be responsible for the MRD detectable in patients [5, 6] and could support the long-term maintenance of the disease. Additionally, it is this malignant population that may become active at relapse or when drug treatment is stopped which would lead to a regeneration of the disease [7].

To approximate the *in vivo* QSC pool, we have studied the primitive $CD34^+38^{lo/-}$ subpopulation of CML cells *in vitro* in order to elucidate mechanisms of IM-insensitivity. In this subpopulation that comprises <5% of total $CD34^+$ cells, we have observed a significant (>10-fold) increase in *Bcr-Abl* mRNA transcript expression as well as Bcr-Abl protein with respect to mature mononuclear cells (MNC) [8]. Moreover, higher Bcr-Abl tyrosine kinase activity in $CD34^+38^{lo}$ cells with respect to total MNC fraction is evident from elevated levels of total phosphotyrosine and phosphorylation of CrkL, a fastidious downstream adaptor protein, as determined by flow cytometry and Western blotting [9, 10]. In these primitive cells, CrkL phosphorylation is not fully inhibited in response to *in vitro* IM treatment at a concentration that exceeds the peak steady state plasma level (approximately 5μM) achievable *in vivo* at 400mg once daily dosing. Furthermore, we have measured the actual drug levels in peripheral blood plasma from IM-treated patients and found them to be equivalent to the levels measured in matched bone marrow plasma (data not shown). However, one possible explanation for the failure of IM to kill these CML stem cells is a failure to achieve sufficiently high intracellular drug concentrations in this population [11]. Thus, if the $CD34^+38^{lo/-}$ cells require significantly higher intracellular levels of IM to overcome the increased oncoprotein expression and activity before initiating apoptosis, then, arguably the target cell population must be exposed to an increased intracellular drug concentration. However, this is not best achieved through simply augmenting the oral dose, as selective tumour cell kill may be lost when high dose IM becomes toxic to normal cells. Hence a targeted cell-specific drug delivery approach maybe applicable to over come this resistance mechanism.

Low Density Lipoprotein (LDL) is a plasma component involved in the transport of cholesterol and is a spherical particle (diameter 20-24nm) composed of an internal core of cholesterol ester containing small amounts of triglyceride, solubilised by a monolayer of phospholipid with small quantities of free cholesterol. Located in the monolayer is the receptor protein Apoprotein B which mediates receptor dependent uptake in the majority of cells [12, 13]. LDL has been of interest as a drug targeting vector in cancer chemotherapy since 1981 when Gal et al., [14] demonstrated an increased uptake in gynaecological cancer cells with respect to normal cells. This observation has been extended to multiple cancer types including leukemia [15] where elevated LDL receptor (LDLR) activity leads to an increased cellular LDL uptake and specifically in CML patients where poor prognosis is

linked to low plasma lipid concentrations [16]. However, the majority of these studies were conducted before the current knowledge on the importance of stem cells in leukemia was recognised [3]. Some recent studies have examined the effect of drug resistance efflux pump expression, such as P-glycoprotein (P-gp) that is known to be involved in cholesterol transport, on LDLR activity in human leukemic cell lines concluding that LDL uptake was sterol and P-gp independent [17]. Indeed this is a very different form of resistance to the intrinsic insensitivity observed in the primitive stem cell pool [4]. A re-evaluation of the LDL targeting paradigm with respect to leukemia, especially stem cell populations, is therefore warranted to determine if specific cell sub-types maybe targeted by LDL.

Native LDL requires to be sourced from serum by sequential ultracentrifugation [17] and suffers from the triple disadvantages of genetic and batch-to-batch variation coupled with limited availability. In order to avoid these issues, in this study we have employed synthetic LDL (sLDL) prepared using readily available lipid components coupled with a synthetic amphiphatic peptide molecule containing the Apoprotein B (Apo-B) receptor sequence [18, 19]. sLDL can be routinely produced [18] that is physicochemically [20] and biologically [21] equivalent to native LDL. In addition sLDL has been employed to incorporate a range of drug payloads [22, 23] and along with LDL therefore represent a proven class of drug targeting vector [24].

We ultimately aim to determine if sub-therapeutic intracellular drug concentrations in leukemic stem cells may be overcome by targeted drug delivery using drug loaded sLDL. However, in this paper as a first step towards this goal we have studied the extent of sLDL uptake by hemopoietic QSC (patient CML $CD34^{+}38^{lo/-}$) and progenitor cells ($CD34^{+}38^{hi/+}$), and compared our findings with Ph^{+} and Ph^{-} leukemia cell lines and human prostate cells, differential information that is currently not available in the literature. If hemopoietic QSC can be preferentially loaded with sLDL then this may become a paradigm differential targeting strategy for other cancers of stem cell origin e.g. breast, brain and prostate [25-27] information that is currently not available in the literature.

Materials and Methods

Materials

Cholesterol, cholesteryl oleate, glyceryl triolein, phosphatidylcholine, sodium oleate, dioctadecyloxacarbocyanine (DiO) and suramin were purchased from Sigma-Aldrich (Poole, UK). Dichloromethane was purchased from VWR International (Eastleigh, UK). Synthetic peptide (di-stearate-KGTTRLTRKRGLKL-cholesterol ester) was purchased from Thistle Research (Glasgow, UK) and Infinity™ cholesterol and triglyceride liquid stable reagent kits and phospholipid B assay kit were all purchased from Alpha Laboratories (Eastleigh, UK). Anti-human anti-CD34 and -CD38 antibodies were obtained from BD Biosciences, Oxford, UK. Carboxyeosin succinimidyl ester (CESE) was purchased from Molecular Probes, Invitrogen Ltd, Paisley, UK.

sLDL preparation

sLDL was prepared using a solvent evaporation method previously reported [18, 19], a mixture of phosphatidylcholine, triolein, cholesterol and cholesteryl oleate in the molar ratio 3:2:1:1 was dissolved in dichloromethane and the synthetic peptide (see Materials) added at a molar concentration of 0.03 per mole of total cholesterol. The dichloromethane was then added to a solution of sodium oleate in water for injection and homogenised using an ice-cooled EmusiFlex-C5 microfluidiser (Avestin, Canada) at pressures up to 30,000psi until the desired particle size was obtained. Residual organic solvent was then removed by

evaporation at room temperature. The organic to aqueous phase ratio was kept at 1:9. The resulting system was then filtered (0.2µm) and stored aseptically at 4°C in the dark under a nitrogen atmosphere. The concentrations of total cholesterol (free + esterified), triglyceride and phospholipid were measured using the Infinity™ cholesterol or triglyceride reagent and phospholipid B assay kits according to manufacturer's instructions. sLDL particle size was determined by photon correlation spectroscopy using the ZetaSizer Model 4 (Malvern Instruments, Malvern, UK). Samples were diluted in distilled water and measurements carried out at a fixed angle of 90°. Results are expressed as the number mean ± standard deviation (n = 10). Fully competent particles i.e. labelled with the tracer dye, DiO (added to the dichloromethane at a molar concentration of 0.006 per mole of total cholesterol added) and loaded with Apo-B peptide that binds LDLR that in turn initiates active cellular uptake, were prepared. Control particles lacking either the tracer dye or the peptide were also prepared.

Myeloid and prostate cell line culture

Ph^+ (K562, KCL22), Ph^- (HL60, AML3) myeloid cell lines and prostate cell lines PNT1A, PC3 were maintained at 37°C in 5% CO_2 in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) respectively supplemented with 10% (v/v) fetal calf serum (FCS) and 1mM each of glutamine, penicillin and streptomycin.

Primary CD34 cell enrichment and culture

Leukapheresis samples were obtained with informed consent as part of the routine assessment of untreated, newly diagnosed patients with chronic phase CML. Non-CML leukapheresis collections were processed as Ph^- controls. Samples were enriched to >90% $CD34^+$ progenitors by positive selection (CliniMACS) and cryopreserved. The $CD34^+$ enriched cells were stained with anti-human anti-CD34-APC and anti-CD38-PE antibodies before cell sorting using a FACS Aria (BD Biosciences, Oxford, UK) into two populations: $CD34^+38^{hi/+}$ and $CD34^+38^{lo/-}$. The $CD34^+38^{lo/-}$ fraction approximates the most primitive QSC pool (<5% total $CD34^+$ cells). Cells were analysed using a BD FACS CantoII instrument with Diva software. Non-CML (Ph^-) and CML $CD34^+$ cells were cultured at 37°C in 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with serum substitute (bovine serum albumin, insulin and transferrin: 'BIT', Stem Cell Technologies, Vancouver, British Columbia, Canada), glutamine, penicillin/streptomycin and 5 growth factors (IL-3, IL-6, G-CSF, flt3-L and SCF) as previously described [28].

Labelling of $CD34^+$ cells with CESE

Total primary $CD34^+$ cells were labelled with the vital cell division tracking dye, CESE, according to the maunfacturer's instructions, in a similar way to CFSE labelling as previously described [4] with minor modifications. Briefly, cell suspension was stained with a final concentration of 1µM CESE for 10mins at 37°C in a waterbath before stopping the reaction with ice cold 20% FCS/phosphate buffered saline (PBS), and washing twice. One well of cells was incubated with 100µg/mL Colcemid® (Sigma) to arrest cell division and allow identification of the cells that had remained undivided, i.e. $CESE^{max}$ by flow cytometry.

Isolation of normal mononuclear cell (MNC) and subset identification

Normal peripheral blood mononuclear cells (MNC) were isolated from blood donations of healthy individuals by density gradient centrifugation. Briefly, Leucosep tubes (Greiner Bio-one, UK) were filled with 15mL Histopaque 1077 (Sigma-Aldrich) and centrifuged for 30secs at 1000g. 10mL of anticoagulated blood was added to the tube and centrifuged for 10mins at

1000g. After centrifugation the enriched lymphocyte/peripheral blood MNC were monocyte depleted by plastic adherence, then washed twice with PBS and suspended in RPMI 1640 prior to addition of sLDL. For the identification of the subset cell populations, anti-CD3-PE was used for T-cells while anti-CD14-APC was used for monocyte/macrophages.

Uptake of sLDL by cell lines and primary cells

Myeloid cell lines K562, KCL22, HL60, AML3, enriched MNCs ($2x10^5$ cells/mL), prostate cell lines PNT1A, PC3 and primary CML and non-CML patient cells ($1x10^5$ cells/mL) were incubated in the presence or absence of increasing amount of sLDL (0.5, 5 and 50μg of total cholesterol per mL of cell suspension equivalent to a cholesterol concentration of 0.0013, 0.013 and 0.13 mmol) for 24h. The inhibitory effect of suramin on sLDL uptake was measured by incubating cells with 500μg/mL (3.8mmol) of suramin for 1h prior to addition of sLDL. Cells were washed twice with ice-cold PBS prior to re-suspension. Uptake of sLDL by the cells was measured by flow cyotmetry as mean fluorescence intensity in three independent experiments.

Immunofluorescence microscopy

K562 and CML $CD34^{+}38^{lo/-}$ cells were fixed onto poly-L-lysine (Sigma, St. Louis, USA) coated multi-spot microscope slides (CA Hendley, Essex, UK) in 3.65% formaldehyde (Sigma, St. Louis, USA) in PBS for 15mins at room temperature. After fixation cells were washed twice in PBS for 5mins each time. Dried cells were mounted in VECTASHIELD containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Cells were examined by a Zeiss Axio Imager M1 fluorescence microscope (Carl Zeiss, Jena, Germany). Images captured were subjected to deconvolution (AxioVision software; Carl Zeiss, Jena, Germany) to remove non-specific fluorescence.

Transmission electron microscopy (TEM)

Cells were fixed in suspension in 2ml fixative (2.5% glutaraldehyde) cells were then pelleted by centrifugation, supernatant removed and pellet resuspended in fresh fixative and stored overnight at 4^oC. Cells were then centrifuged and washed in 0.1M cacodylate buffer, pH7.4 3 times over 30min then after another centrifugation, secondary fixation was achieved by resuspending the pellet in 1% osmium tetroxide in 0.1M Sodium cacodylate buffer, pH7.4 for 1h before washing 3x in distilled water. Cells were then resuspended in 2% aqeuous Uranyl Acetate for 1h before centrifugation for dehydration of sample. Fixed cells were dehydrated through EM grade alcohol of increasing concentration (75 to 100%) and cleared by resuspension in 1ml of propylene oxide for 5mins at RT. Sample was then infiltrated with araldite resin overnight before embedding in new araldite resin followed by polymerization at 60°C for 20h. Ultra thin sections were cut using a LKB ultramicrotome (LKB Bromma, Sweden), mounted on copper grids and images were obtained using a FEI Morgagni electron microscope (FEI, Cambridge, UK) equipped with a SIS Megaview III camera with SIS iTEM software (Olympus, UK).

Results

sLDL Materials

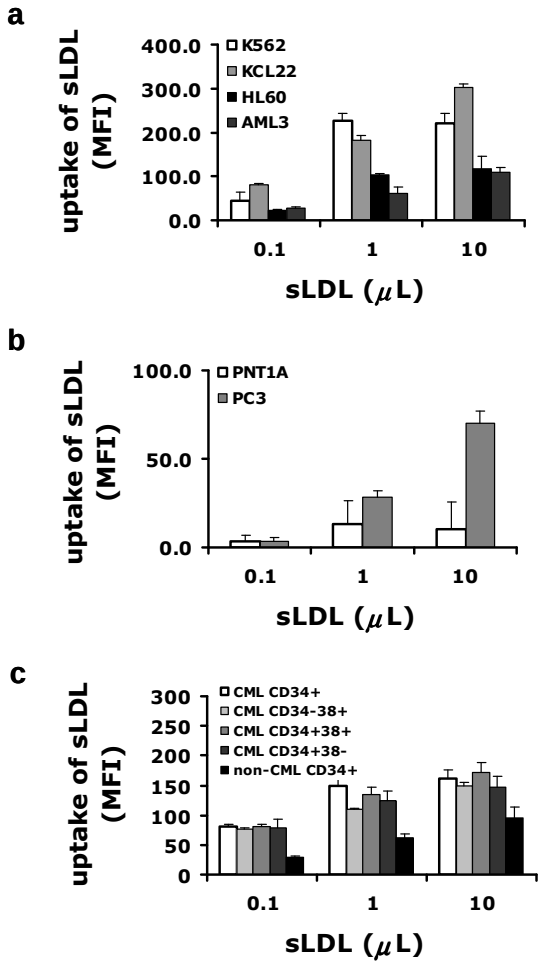
sLDL equivalent to native LDL (receptor peptide, no DiO) had a measured diameter of 19±0.7 nm, with a cholesterol content of 4.2mg/mL, phospholipid at 9mg/mL and triglyceride at 9mg/mL. The incorporation of DiO increased the diameter to 36±0.2 nm with a measured zeta potential of -54.8mV and resulting lipid concentrations of 4.8mg/mL, 9mg/mL and 8mg/mL respectively. A peptide free DiO containing system had a diameter of 25±0.6 nm,

and lipid concentrations of 4.2mg/mL, 5.4mg/mL and 9.4mg/mL respectively. The physicochemical and analysis results for these materials are similar to those already reported in the literature [18]. The larger diameter of DiO containing sLDL with respect to DiO free samples has been previously observed [18, 19] and does not affect cellular uptake.

sLDL uptake by myeloid and prostate cell lines in vitro

Rapidly dividing myeloid cell lines were found to take up DiO-sLDL in a dose dependent manner reaching a plateau at 50µg sLDL cholesterol/mL. Optimal uptake with minimal cellular toxicity (that is >85% viability) was seen within 24h when 5µg sLDL cholesterol/mL was added per $2x10^5$ cells in 1mL of culture medium (data not shown). Uptake was more avid in Ph^+ cell lines K562 and KCL22 (K562: 226.91±11.74, n=3; KCL22: 180.8±30.33, n=3) than in Ph^- cells HL60 and AML3 (HL60: 104.03±12.24, n=3; AML3: 61.40±10.69, $p<0.01$, n=3) as measured by FITC mean fluorescence intensity (MFI) for the same concentration of DiO-sLDL (5 µg cholesterol) added to the same number of cells (Figure 1a). Uptake of sLDL in the prostate cell lines was significantly lower than in myeloid cell lines. Furthermore, decreased levels of sLDL were observed in the non-tumourigenic prostate cell line PNT1A (13.3±2.1, n=3) in comparison to the tumour prostate cells PC3 (28.6 ± 6.6, n=3) (Figure 1b) verifying the notion that tumour cells have a higher need for cholesterol than normal non-tumour cells.

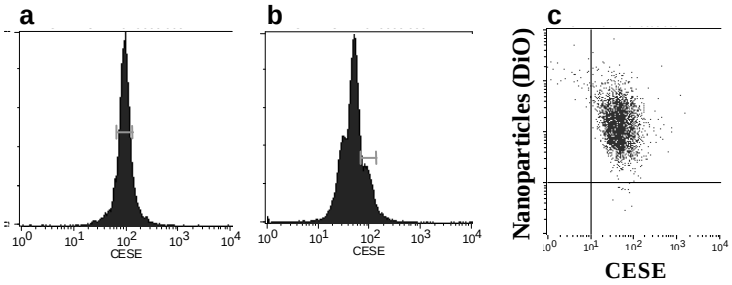
Figure 1



sLDL uptake by CD34$^+$ cells, including QSC

Primary CML CD34$^+$ cells also avidly took up DiO-sLDL *in vitro* in a similar manner to leukemic myeloid cell lines (Figure 1c). There was no statistically significant difference amongst the CD34/38 subpopulations observed, including the most primitive CD34$^+$38^{lo} population. However, the levels of sLDL taken up by non-CML CD34$^+$ cells (MFI 61.4 ± 5.1, n=3) were significantly lower than those observed in CML CD34$^+$ cells (MFI 148.8 ± 13.7, $p<0.01$, n=6). Significantly, QSC, as identified by their maximal CESE fluorescence, were also DiO-positive at 72h (Figure 2) after exposure to DiO-sLDL.

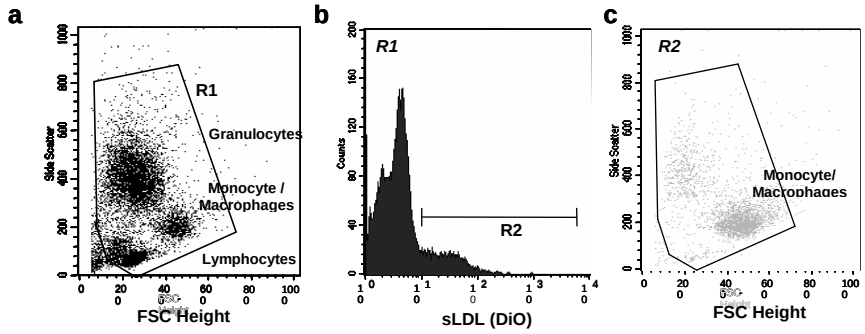
Figure 2



Uptake of sLDL by a subset of normal peripheral blood MNC

Based on size (forward scatter) and granularity (side scatter) determined by flow cytometry within total MNC, monocyte/macrophages were found to be positive for DiO-sLDL while granulocyte and T-cell subsets did not take up any particles within 24h (Figure 3). This is consistent with the monocyte/macrophage role as a scavenger with phagocytic function. To confirm this finding, total MNC were sorted based on their forward/side scatter parameters, treated with sLDL in vitro and phenotyped by surface staining. Non-phagocytic CD3$^+$ T-cells were negative over a range of concentrations of sLDL whilst CD14$^+$ monocyte/macrophages were positive for DiO-labelled sLDL.

Figure 3



sLDL is internalised and not surface adsorbed

Deconvolution microscopy of DiO-sLDL treated K562 and CML CD34$^+$38^{lo} cells confirmed the internalisation as opposed to surface adsorption of the sLDL *in vitro* at 24h. K562 and CML CD34$^+$38$^{lo/-}$ cells were treated with DiO-sLDL particles (5µg sLDL cholesterol per $5x10^5$ cells) for 24h, Z-Stack pictures were taken with 20 slices and then subjected to deconvolution to remove non-specific fluorescence (Figure 4). Intracellular DiO-sLDL particles were visualized in the cytoplasm of K562 and CML CD34$^+$38$^{lo/-}$ cells. No green fluorescence appeared in the untreated cells however a low level of green fluorescence was observed when the cells were treated with Apo-B peptide free sLDL. TEM

photomicrographs also confirmed the internalisation of the sLDL particles (Figure 5) associated with internal structures such as endosomes.

Figure 4

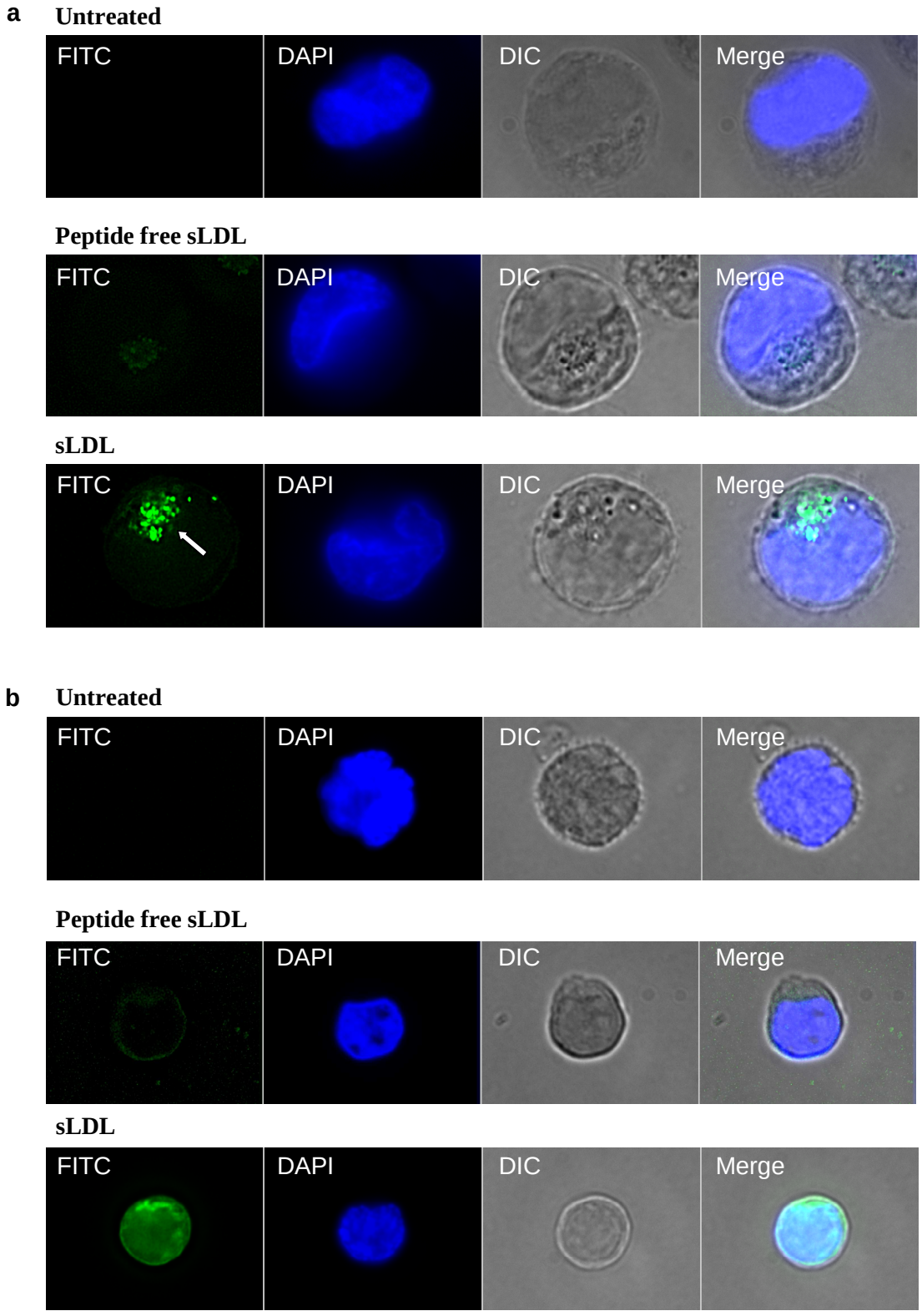


Figure 5

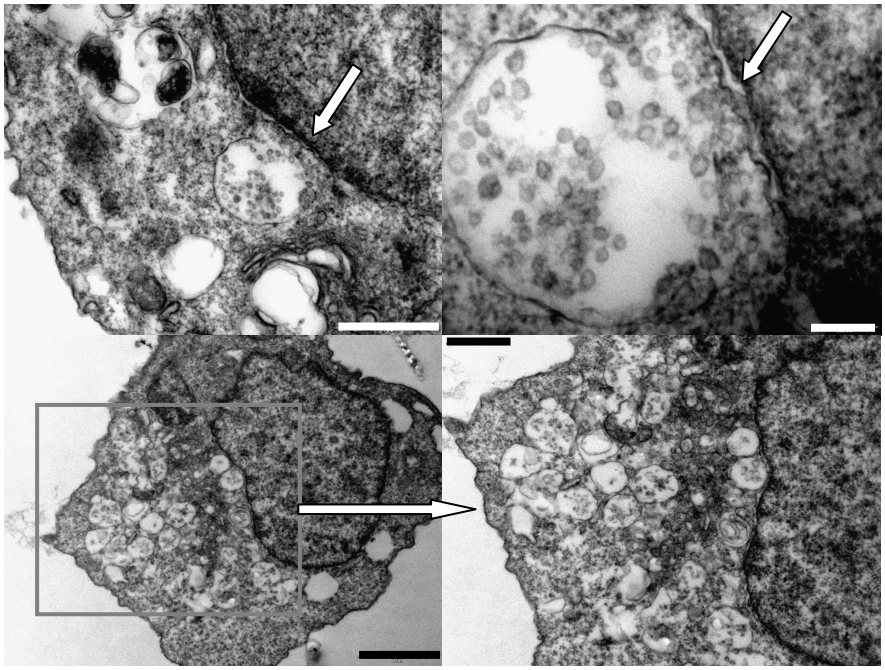
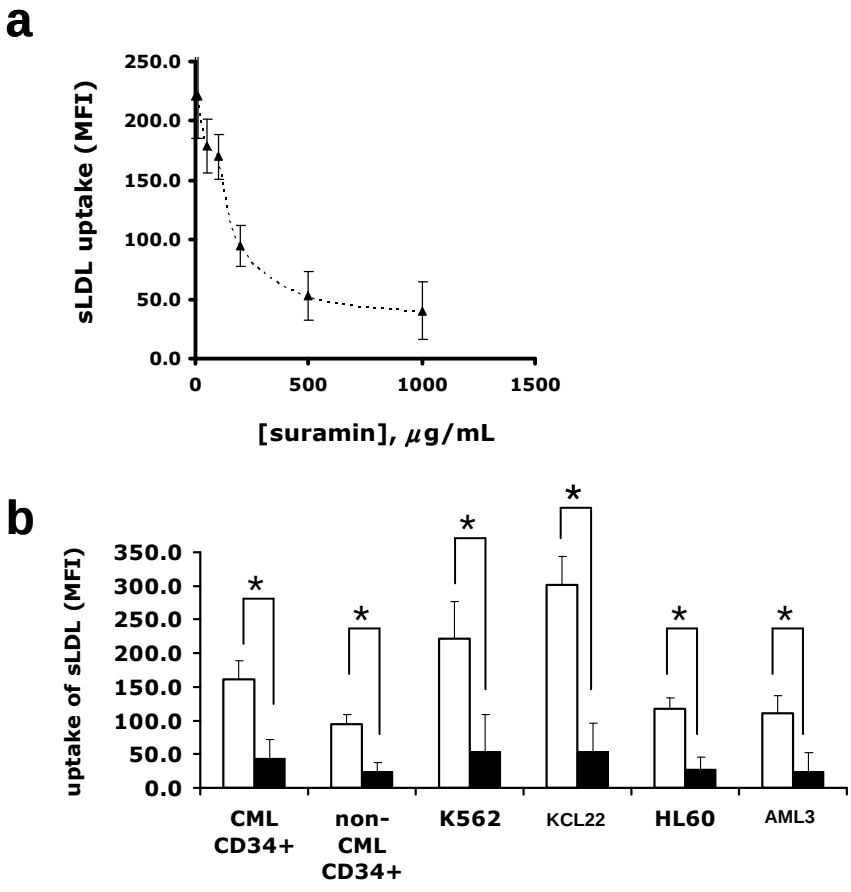


Figure 6



LDLR-dependent uptake specificity of sLDL

LDLR-dependent uptake of sLDL was examined by using the known LDLR inhibitor, suramin. Initial studies were performed in K562 cells in order to assign optimal suramin concentration that blocked sLDL uptake without affecting cell viability. Cells were treated with increasing concentrations of suramin (0, 50, 100, 200, 500, 1000μg/mL) for 1h prior to incubation with 5μg sLDL cholesterol for 24h. Higher concentrations of suramin were not assayed due to the profound toxic effect that 1000μg/mL had on cell viability (reduced to ~40%) (data not shown). Suramin's block of sLDL cellular uptake reached a nadir at 500μg/mL and plateaued with 1000μg/mL, but even at this high concentration complete inhibition of sLDL uptake could not be observed (Figure 6a). These data suggest that sLDL uptake was not only receptor mediated, a supposition further supported by the background level of cellular uptake of DiO-sLDL prepared without the Apo-B receptor peptide sequence (data not shown), in which case the fraction of uptake was most likely mediated by an adsorptive process.

Discussion

All the myeloid leukemia cell lines that we tested in this study (K562, KCL22, HL60 and AML3) avidly accumulated fluorescently labelled (DiO-) sLDL particles, considerably more so than adherent epithelial prostate cell lines, whether SV40 immortalised normal (PNT1a) or adenocarcinoma (PC-3). Between the prostate cell lines, the transformed PC-3 cells took up significantly more sLDL with increasing concentration than their normal counterparts (PNT1a). This could reflect the increased cholesterol requirement of tumourigenic cells for their more rapid cell division. Indeed, within total normal donor derived peripheral blood leucocytes, only phagocytic monocyte/macrophages exhibited significant sLDL uptake, more than mature granulocytes, while lymphocyte subsets were universally negative for uptake after 24h. Both of these findings agree with previous literature studies and thus sLDL can achieve some level of cell targeting based on cellular metabolic requirements or specific (phagocytic) activity similar to that reported for the native material [14, 15, 24]. Of more significance to the treatment of myeloid leukemia such as CML, however, would be the ability to target leukemic QSC that by virtue of their quiescent nature, convention dictates should not require exogenous cholesterol [13].

sLDL uptake by QSC was studied in two ways. Cells were labelled with CESE, a red fluorescent vital stain that allows tracking of cell division history. Cells that are $CESE^{max}$ have remained undivided (quiescent) after a given treatment and/or period in culture. As the primary cells were cultured in the presence of a 5 growth factor cocktail which supports and encourages proliferation, $CESE^{max}$ may be identified as the most primitive QSC which clearly co-stained green indicating sLDL uptake. Furthermore, when primitive $CD34^+$ sub-populations were sorted based on their CD38 expression, again $CD34^+38^{lo/-}$ cells approximating the QSC pool, were found to take up sLDL as avidly as the total $CD34^+$ cells and the maturing $CD34^+38^{hi/+}$ cells indicating that the sLDL were not excluded from this primitive population owing to their quiescence. Importantly, uptake in CML $CD34^+$ cells was greater than in non-CML $CD34^+$ cells. Owing to the ethical concerns surrounding collection of $CD34^+$ cells from normal donors beyond the dose required for transplantation, patients with hematological conditions in which the $CD34^+$ stem cell is not involved in the pathogenesis of the disease such as lymphoma, are taken as non-CML 'normal' $CD34^+$ controls. Thus a greater uptake of sLDL in malignant CML $CD34^+$ cells suggests that normal $CD34^+$ would not be dosed with increased levels of drug carried by the sLDL

widening the therapeutic window. We believe this finding of QSC LDL uptake to be the first report in the literature.

We were interested to know if the sLDL uptake by leukemic QSC was a passive or active receptor mediated process. Apo-B is the ligand for the LDLR and is the presumed mechanism by which our sLDL was being internalised. Indeed, cells exposed to DiO-sLDL formulated without the Apo-B peptide did not give a green fluorescent signal indicating a failure to internalise particles. Furthermore, DiO-sLDL uptake by cells, both myeloid cell lines and primary $CD34^+$ cells, exhibited dose dependency and moreover, was saturable, being blocked by suramin, suggesting specific receptor mediated uptake. Immediately after labelling with CESE, approximately half of CML $CD34^+$ cells were also positive for CD36 whereas >90 % was CD13 positive. CD36 is a member of the class B scavenger receptor family of cell surface proteins which can bind oxidised LDL and Aminopeptidase-N (CD13), a molecular target for cholesterol absorption inhibitors [29] and leukemia antigen, may be implicated in cholesterol uptake which is likely a complex process involving several proteins. Although it was hypothesised that the expression of these molecules may be modulated on exposure to sLDL, neither significantly changed with time or on sLDL exposure, although CD36 was marginally up-regulated (+7%) with sLDL at 24h (data not shown).

We have previously shown CML QSC to be insensitive to molecularly targeted tyrosine kinase inhibitors (TKI) such as IM or nilotinib [4, 30]. Over-expression of *Bcr-Abl* message, protein and kinase activity in the more primitive $CD34^+38^{lo/-}$ cells [8] may explain this insensitivity to standard drug concentrations. One possible further explanation for such insensitivity may be that the higher level of Bcr-Abl protein in stem cells is not exposed to sufficient drug owing either to active efflux by antigen binding cassette (ABC) family proteins, or conversely, insufficient active uptake via pumps such as Oct-1 [31, 32]. In the former situation, a small xenobiotic molecule may be removed from the cell if it functions as a substrate for the efflux pump. We have found previously, however, that both IM and nilotinib function as inhibitors and not substrates for the ABCG2 family member [33, 34]. Moreover, it has been shown that although IM is a substrate for active uptake by Oct-1, CML stem and progenitor cells have relatively low expression of this protein [32]. Although trials of second generation TKI such as nilotinb and dasatinib may overcome some of these issues, it should be noted that the 20-times increase in potency of nilotinib over IM seen in Ph^+ cell lines [35] was not replicated in $CD34^+$ stem cells *in vitro* [30].

To overcome this significant clinical problem of stem cell persistence in CML, we hypothesised that such cells must be exposed to an intracellular concentration of drug greater than is achieved with standard oral dosing. Simply augmenting the dose to the patient to saturate the uptake pump (Oct-1) risks side effects as every drug, even a potent molecularly targeted agent, has its therapeutic window beyond which non-specific effects are experienced. Nevertheless increasing intracellular concentrations in QSC may be made possible through pharmaceutical drug targeting strategies i.e. use of physical, biocompatible, drug-carrying vehicles, circumventing the problem of inadequate active uptake by influx proteins through low expression. We decided to investigate the known increased cholesterol requirements of cancer cells [14], specifically LDLR activity in the myeloid lineage [15, 36]. Thus the aim of the current study was to determine if sLDL maybe utilised as a drug targeting vector to ultimately deliver augmented doses of TKI into CML QSC to initiate apoptotic cell death. Interestingly, Nikanjam et al [23] demonstrated receptor mediated, targeted delivery of sLDL prepared according to our recipe to gliobastoma multiforme cell lines, following up with delivery of incorporated paclitaxel to the same brain tumour cell line model [37].

The results presented in this paper in combination with literature data demonstrate the utility of the sLDL system as a drug delivery vehicle for persistent leukemic stem cells. Although it is perhaps counter-intuitive that cells residing out of the cell cycle should be targeted by this system, we nonetheless have demonstrated the propensity for QSC to be loaded with our sLDL nanoparticles. Finally, 'smart' delivery of TKI in CML may not be relevant in the event that CML stem cell persistence and survival is shown to be Bcr-Abl independent such as through autophagy induction [38], or ligand-independent activation of growth factor signalling [39]. In this situation, non-TKI therapeutic modalities will need to be developed, nonetheless once a 'drug-able' target has been identified, like Bcr-Abl was with IM, the problem of delivering to the root cause of the disease, i.e. the cancer stem cell, and not simply tackling the bulk tumour remains, which is where drug delivery technologies may become invaluable.

Conclusion

In summary we have achieved our initial aim of determining the cell targeting capacity of a synthetic carrier system, sLDL, for its potential drug delivery to leukemic stem/progenitor cells. This result opens the door to a pharmaceutical solution to the medical problem of persistent CML QSC. We therefore plan to continue this study by loading TKI and /or other novel investigational agents into the nanoparticles and assessing the change in efficacy to drug induced apoptosis in leukemic QSC, a strategy that may have value in other stem cell derived cancers.

Disclosure Statement

All authors have no actual or potential conflict of interest to disclose including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) their work.

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Figure legends

Figure 1a-c: *sLDL uptake by Ph^+ and Ph^- cell lines as well as primary CML $CD34^+$ cells and subsets.* (**a**) Ph^+ K562 and KCL22 myeloid cell lines (white and light grey bars, respectively) exhibited a greater propensity to uptake of DiO-sLDL than Ph^- HL60 or AML3 (black and dark grey bars, respectively). (**b**) The uptake of DiO-sLDL by the tumourigenic prostate cell line, PNT1a (white bars) was greater as seen by MFI at 1 or 10μL (5 or 50μg cholesterol). (**c**) Although there was no significant difference amongst the CD34/38 subpopulations observed, including the most primitive $CD34^+38^{lo}$ population, the levels of sLDL taken up by non-CML $CD34^+$ cells were significantly lower than those observed in CML $CD34^+$ cells

Figure 2a-c: *Undivided ($CESE^{max}$) $CD34^+$ CML cells can be loaded with DiO-sLDL particles.* (**a**) $CD34^+$ CML cells were uniformly stained with CESE on day 0. (**b**) After 72h in culture, cells that remain undivided are identified by their unchanged CESE fluorescence, marked by 'H' gate. (**c**) Undivided ($CESE^{max}$) $CD34^+$ CML cells were also positive for DiO-sLDL uptake.

Figure 3: *sLDL uptake by Ph^- peripheral blood MNC*

Within non-leukemic total MNC (shown in gate R1 in panel a), some cells take up sLDL and can be seen as green positive cells in region R2 in panel b; the positive green signal was above any cell autofluorescence (data not shown). The DiO^+ cells in R2 are principally comprised of monocytes (panel c) based on forward scatter (FSC)/side scatter (SSC) characteristics. Thus sLDL is not as avidly taken up by non-dividing, non-phagocytic, mature, normal cells as leukemic stem cells that have a greater propensity for sLDL internalisation (see Figure 1).

Figure 4: *Internalisation of sLDL by (a) K562 and (b) $CD34^+38^{lo}$ CML cells* Cells fixed post-treatment with sLDL were visualised by fluorescence microscopy and Z-stacks were captured then subjected to deconvolution to remove non-specific fluorescence. Clearly the sLDL was internalised and the lipid soluble dye (DiO) became associated with intra-cellular structures such as the endoplasmic reticulum (indicated by arrowheads). The sLDL would have been expected to be routed to the ER following uptake via the LDL receptor mediated pathway. Thus, fluorescence signals by flow cytometry are not owing to sLDL simply adsorbed on the cell surface. FITC channel shows DiO-sLDL particles, DAPI channel shows cell nucleus. DIC differential interference contrast.

Figure 5: *Electron micrographs of $CD34^+$ CML cells after treatment with sLDL.* Upper left panel scale bar is 1 micron; upper right, scale bar is 200nM; lower left, scale bar is 2 microns; lower right, scale bar is 1 micron. White arrows in upper panels indicate engulfed sLDL particles within intracellular vesicles.

Figure 6a,b: (a) *Concentration-effect curve of suramin on sLDL uptake by K562.* An effective concentration of the LDLR inhibitor, suramin, was established in K562, a Ph^+ myeloid cell line which had been shown to avidly absorb DiO-sLDL particles. **(b)** *Effect of suramin on uptake of sLDL by various cell types.* DiO-sLDL uptake as measured by MFI in the FITC channel by flow cytometry was determined in the presence (black bars) and absence (white bars) of suramin. The uptake of DiO-sLDL by all cell lines tested was blocked by the action of 500μg/mL suramin.