USE OF LOW DENSITY LIPOPROTEIN WITH CYTOTOXIC SUBSTANCES

The present invention relates to the use of cytotoxic substances in the preparation of agents with enhanced activity for the chemotherapy of cancer.

Cancer is a term used to describe the development of abnormal cells which are, in the main, indistinguishable from normal tissue cells except that they grow in a rapid and uncontrolled manner and are often invasive. The invasion of vital organs frequently results in the death of a patient.

Cancer is extremely widespread and, indeed, it is thought that about 30% of people are likely to contract cancer at some time in their lives with cancer being the cause of death in around 20%. Traditional treatments for cancer include surgery and radiotherapy but, more recently, attention has been turned to the development of chemotherapeutic or cytotoxic agents which can damage or kill cancer cells. However, the chemotherapy of cancer is problematic because of the low selectivity of these cytotoxic drugs which kill normal cells as well as cancer cells. This low selectivity leads, in many instances, to intolerable side-effects which may include damage to organs such as heart, liver, lungs and gastro-intestinal tract, as well as to bone marrow and hair follicles, giving rise to cardiac and respiratory problems, sensitivity to infections, nausea, vomiting, diarrhoea and hair loss.

Many attempts have been made to increase the selectivity of anti-cancer drugs and thus decrease the side-effects,

and recent methods have focused on the development of carriers which will act as vehicles for poorly soluble drugs and, more importantly, will direct drugs to the target malignant cells. The complexes formed between carrier and drug must, of necessity, be efficiently distributed throughout the body so that they encounter the target cells and they must be selective to ensure entry only into malignant cells. Complexes of cytotoxic drugs must also be stable, both before and after administration to the patient and until the target cell is reached.

Many different types of carrier for cytotoxic substances have been proposed and these include antibodies, hormones and liposomes. However, each of these types of carrier has limitations which has restricted its use in cancer chemotherapy. WO-A-8607540 describes an alternative carrier for biologically active substances consisting of low density lipoproteins (LDL) and a method for the preparation of LDL carriers loaded with a lipophilic biologically active substance.

The lipoproteins comprise a group of particles found in plasma with a physiological role in the transport of triglycerides and cholesterol. LDL transports cholesterol to actively dividing cells for the synthesis of cell membranes and as a substrate for the manufacture of steroid hormones. LDL particles consist of 75% lipids and 25% protein organised as a polar shell of cholesterol, phospholipids and protein surrounding a non-polar core of cholesteryl esters. The protein component exists as a single molecule of apolipoprotein B (apo B). Apo B combines specifically with LDL receptors carried on the membrane surface of many types of cell in "coated

pits". These are continually being pinched off to form vesicles within the cell in which the LDL is degraded into its component parts. Thus, the cholesterol is delivered to the interior of the cell and is available for the metabolic processes of the cell.

Many cancers, including leukaemic white cells, examined in vitro, have been shown to have high LDL receptor activity compared to normal tissues (Blood, **63**, 1186-1193 (1984)). It has also been shown that cells obtained from lung carcinomas will accumulate 200 to 300% more LDL labelled with radioactive ^{14}C sucrose than normal lung tissue (Cancer Research, **52**, 1-4 (1992)). Similar findings have been obtained in carcinomas from the gastro-intestinal tract.

As described in WO-A-8607540, a drug carrier can be prepared from LDL by incorporating a lipophilic drug into the core of the LDL particle. For some drugs, such as vincristine, it is preferred not to remove the cholesteryl esters from the core of the LDL particle but in other cases, the cholesteryl esters may be removed and replaced wholly or partially with a lipophilic drug. Suitable lipophilic drugs include cytotoxic agents of the type used in cancer chemotherapy. WO-A-8607540 also describes a way of preparing these LDL drug carriers without affecting the properties of the LDL so that it is rejected by the body. Thus, the LDL-drug complexes described in this document will persist in the plasma for longer than those prepared by other methods and, at a level similar to that of purified but non-modified LDL. Thus, it can be seen that the use of LDL as a carrier for a drug is extremely advantageous.

In our earlier work (Cancer Chemother. Pharmacol. **29**, 396-400 (1992)), we describe the administration to cancer patients of vincristine (VC) incorporated into an LDL carrier. VC is a cytotoxic substance which is widely used in cancer chemotherapy but which has some severe side-effects which necessitate cessation of the therapy. The side-effects include paraesthesia, muscle paralysis, constipation, paralytic ileus and alopecia. However, the paper just mentioned shows that when VC is formulated into LDL, many of the side-effects disappear and this is likely to lead to an increase in the use of the drug.

However, one problem with the cytotoxic substances used in cancer therapy, is that many cancers are or become resistant to them. The resistance may be primary resistance, in which the tumour never responds to therapy with the cytotoxic substance, or secondary resistance, in which, after initial regression of the tumour on treatment with the cytotoxic substance, the tumour starts to grow again. Attempts have been made to reverse the development of resistance by the use of a variety of non-cytotoxic drugs such as verapamil, tamoxifen and cyclosporin A. Verapamil, for example, is a calcium-channel blocking agent and reverses the enhanced efflux of doxorubicin from drug-resistant ovarian tumour cells and should, in theory, assist in the prevention of resistance to cytotoxic agents. However, this approach has met with limited success and, consequently, patients with tumours resistant to certain cytotoxic agents cannot be treated effectively with those agents.

A further problem with the use of VC and, indeed many other cytotoxic substances is that they have a small therapeutic window, that is, the dose at which they are

active in the treatment of cancer is not widely different from a dose which is toxic to the patient.

However, we have now unexpectedly found that, when a cytotoxic substance is incorporated into LDL, the composition has greater cytotoxic activity than the cytotoxic substance when used alone and this gives us the means to develop a method for the treatment of cancer, the method comprising administering to a patient an effective amount of a cytotoxic substance formulated with LDL, wherein the activity of the cytotoxic substance is greater than its activity when not in the presence of LDL.

Therefore, in a first aspect of the present invention, there is provided the use of a lipophilic cytotoxic substance and a low density lipoprotein (LDL), wherein the activity of the cytotoxic substance is greater than its activity when not in the presence of LDL, in the preparation of an agent for the treatment of a cancer.

Generally, a complex will be formed between the cytotoxic substance and LDL and the complex will be used to prepare the cancer treating agent.

In the present specification, the term "cancer" refers to carcinoma, leukaemia, lymphoma, blastoma and sarcoma.

The term "cytotoxic substance-LDL complex" refers to an entity in which the cytotoxic agent wholly or partially replaces the cholesteryl esters in an LDL particle.

When cytotoxic agents are complexed with LDL they may either be in the free acid or free base form or be used

as a physiologically acceptable salt such as a sulphate or hydrochloride. In the case of VC, it is preferred to use the free form of the drug.

It is preferred that the activity of the cytotoxic substance is at least 10%, preferably 15% and more preferably 20% greater than the activity of the cytotoxic substance when not administered with LDL.

The increase in the activity of the substance is measured by comparing the number of tumour cells remaining in samples treated with the cytotoxic substance alone and with the cytotoxic substance in the presence of LDL and this is described in more detail in the examples.

One possible advantage of this enhancement in the activity of the cytotoxic substance is that there is potential for the doses of cytotoxic substances administered to cancer patients to be significantly reduced. There would, in this case be an even greater decrease in side effects than was predicted from our previous work since, not only would the encapsulation of a cytotoxic substance in an LDL particle reduce the side effects observed for a similar dose of the drug, but, in addition, a smaller dose of drug could be administered when formulated in LDL and this, of course, would further reduce the adverse side effects. Furthermore, a decrease in the amount of cytotoxic drug necessary to treat a cancer patient would, of course, lead to a reduction in the cost of the treatment which the patient receives.

In some cases, however, it will not be desirable to reduce the dose of the cytotoxic substance since a greater cytotoxic effect may be required. Under some

circumstances, a clinician may find it useful to administer a higher dose of the complexed therapeutic substance than the standard dose for the same substance.

The reasons for the enhancement in the activity of cytotoxic substances when administered with LDL is not clear but this does not limit the effectiveness of the invention.

One further advantage of the use of the complexes of the present invention is that the enhancement in activity of the cytotoxic substance is even greater in the case of cancers which are resistant to the cytotoxic substance when administered alone. The complexes are therefore particularly useful for use in a method for the treatment of drug resistant tumours, the method comprising administering to a patient an effective amount of a cytotoxic substance formulated in LDL.

An additional advantage is that the use of the LDL-cytotoxic substance complex avoids the necessity of using toxic solvents such as dimethyl sulphoxide or Cremophor EL which, for some cytotoxic substances such as taxol, are the only available solvents for administration of these drugs to patients. Again, this means that increased doses of the drug may be feasible since the toxic side effects of solvents will no longer have to be treated.

In a second aspect of the present invention there is provided the use of a lipophilic cytotoxic substance and a low density lipoprotein (LDL) in the preparation of an agent for the treatment of a cancer which is resistant to the cytotoxic substance when administered without LDL.

Again, a complex will generally be formed between the cytotoxic substance and LDL and the complex will be used to prepare the cancer treating agent.

Clearly, the present invention may prove extremely useful when treating cancers since, even when the cancer has become resistant to a particular drug, it may still be possible to treat the patient using that drug, provided the drug is administered with LDL.

One possible explanation for the effectiveness of LDL complexed cytotoxic agents in treating cells which are resistant to the agent when administered alone is that the mechanism by which the drug enters the cells has been changed. It is usual for a cytotoxic agent to enter a cell via a diffusion process and it seems that, in some cases, resistance to the drug is caused by a permeability barrier which prevents the drug from entering the cancer cell. However, when a cytotoxic agent is encapsulated in an LDL particle, it enters the cell via the LDL receptor, not by diffusion and so the resistance of the cell to the cytotoxic agent is overcome.

An alternative explanation is that cancer cells which are resistant to a cytotoxic agent actually excrete the agent after it has entered the cell. However, when the agent is encapsulated in an LDL particle, it is not recognised by the cell sufficiently early for it to be excreted.

However, although both of these explanations are plausible, it must be stressed that the effectiveness of this aspect of the invention does not depend on their being correct.

Cytotoxic agents which may be useful in the present invention include doxorubicin and its lipophilic derivatives AD32 and AD312, chlorambucil, prednimustine, WB4291, aclacinomycin, nitrosoureas, VC, taxol and its analogues and other lipophilic drugs. However, VC is particularly suitable since it is widely used in cancer chemotherapy and, in addition, as discussed above, formulation of VC with LDL reduces its side-effects, allowing VC therapy to be continued for prolonged periods.

The cytotoxic substance is generally complexed with LDL as described in WO-A-8607540. The LDL particles can be reconstituted in such a way that the core contains only the lipophilic cytotoxic substance or, alternatively, some of the cholesteryl esters extracted from the core of the LDL particle can be reincorporated into the particle along with the cytotoxic substance. A third possibility, which is preferred in the cases of VC and taxol is to incorporate the therapeutic substance into the core of the LDL particle without removing any of the cholesteryl esters.

The LDL used in the present invention may be synthesised chemically or may be derived from animal sources, in particular mammalian or avian sources. Suitable avian sources of LDL include egg yolks, and mammalian LDL may be ovine, bovine, porcine, equine or human, with human LDL being especially preferred.

Although the present invention is of use for the treatment of any type of cancer, it is particularly suitable in the treatment of leukaemias and lymphomas.

The cytotoxic substance-LDL complex may be formulated for administration by any route. In the past, it has been usual to administer cytotoxic substances such as VC by methods such as intravenous bolus injection so that extravasation is avoided, since many cytotoxic drugs are corrosive. The cytotoxic substance-LDL complex can be administered by a variety of routes, although the parenteral route is especially suitable. A particularly preferred route is intravenous administration but oral, nasal, intraocular and intraperitoneal administration may also be used.

The amount of cytotoxic substance administered to the patient will, of course, depend on the individual subject and the nature of the cancer to be treated, but it is within the skill of the practitioner to determine an appropriate dose.

A typical dose of VC when given alone without LDL is 1.4 mg/m^2 body surface vincristine sulphate and an equivalent dose may be appropriate for an LDL-VC complex. Because of the enhanced activity of VC when complexed with LDL, it is possible that a rather lower dosage level may be used when VC is complexed with LDL but, of course, this may not be appropriate if higher cytotoxicity is required.

When the complexes are administered by the parenteral route, they may be formulated as a sterile solution, for example, in phosphate buffered saline (PBS) and will preferably be at approximately physiological pH. The pH is preferably in the range from 6 to 8.5, more preferably pH7 to pH8 and most preferably pH7.2 to pH7.6, with pH 7.4 being the optimal value. The concentration of the

cytotoxic drug in the final formulation will depend on the drug being used but, for VC, an appropriate concentration is in the range of 20 μg to 2 mg/ml, preferably 100 to 500 μg/ml, with about 200 μg/ml being particularly preferred. The concentration of LDL protein is usually in the range of 0.1 to 10 mg/ml, preferably 0.5-5.0 mg/ml and most preferably 1.0 to 2.0 mg/ml, with about 1.5 mg/ml being especially suitable.

The invention will now be described in more detail with reference to the Examples and to the drawings in which:

Figure 1 is a plot of tumour cell survival against VC concentration for non-Hodgkin's lymphoma (NHL) patients' cells treated with VC and LDL-VC;

Figure 2 is a plot of tumour cell survival against VC concentration for acute myeloid leukaemia (AML) patients' cells treated with VC and LDL-VC;

Figure 3 is a plot of tumour cell survival against VC concentration for cells from chronic lymphocytic leukaemia patients who had not previously received chemotherapy (CLL-);

Figure 4 is a plot of tumour cell survival against VC concentration for cells from chronic lymphocytic leukaemia patients who had previously received chemotherapy (CLL+);

Figure 5 is a plot of tumour cell survival against VC concentration in previously treated (PT) and previously untreated (no PT) AML patients' cells when treated with VC and LDL-VC;

Figure 6 is a plot of LDL-VC therapeutic index (VC LC_{50}/LDL-VC LC_{50}) against VC LC_{50};

Figures 7 to 42 are a series of plots of tumour cell survival against VC concentration for each of 36 patients showing the differences between VC and LDL-VC;

Figures 7 to 15 relate to AML patients;

Figures 16 to 24 relate to NHL patients;

Figures 25 to 33 relate to CLL- patients;

Figures 34 to 42 relate to CLL+ patients; and

Figure 43 is a plot showing the cytotoxic effect of an LDL/taxol complex compared with taxol formulated in DMSO.

Example 1

Preparation of LDL/VC complex.

In preparing the LDL/VC complex, the general method set out in WO-A-8607540 was followed. 20 mg vincristine was dissolved in 2 ml water and 4 ml carbonate buffer (pH 9.6; containing 0.59 g sodium bicarbonate and 0.31 g sodium carbonate per ml water) added. The precipitated vincristine was dissolved in 4 ml methylene chloride and separated from the aqueous layer by centrifugation. The extraction was completed with 2 x 2 ml volumes methylene chloride. 3 g anhydrous sodium sulphate was added to remove excess water and the liquid filtered to remove solid material. The methylene chloride was evaporated under nitrogen to complete dryness.

The residual vincristine was dissolved in 4 ml methylene chloride and distributed between 4 flasks, each containing 20 mg LDL previously lyophilised from 5 ml aqueous solution containing 200 mg sucrose as protective agent. The methylene chloride was removed by evaporation under nitrogen.

The dry LDL/vincristine complex in each flask was dissolved in 5 ml PBS and filtered to remove insoluble vincristine and diluted to a concentration of 200 μg VC/ml.

Example 2

Effect of LDL/VC or VC sulphate on experimental cancer cells.

The Differential Staining Cytotoxicity (DiSC) assay was used to determine the sensitivity of fresh human tumour cells to LDL-vincristine (LDL-VC) and vincristine (VC).

Materials and Methods

Media RPMI 1640 medium was used to which was added 2 mM glutamine and 80 μg/ml gentamicin to make a "wash medium". "Complete medium" was made by adding 10% foetal bovine serum to RPMI 1640 medium, plus 2 mM glutamine and 80 μg/ml gentamicin.

Drugs LDL-VC from Example 1 was stored at 4°C for the duration of the experiment. Vincristine sulphate was dissolved in phosphate buffered saline (PBS) and diluted to 10 x the final concentration required in the test system (final concentration = 20 μg/ml = 21.67 μM) and

stored frozen (-70°C) until required. Drugs were tested at five four-fold dilutions (20, 5, 1.25 μg/ml etc) in duplicate.

Specimens Specimens from patients with chronic lymphocytic leukaemia (CLL), acute myeloid leukaemia (AML) and non-Hodgkin's lymphoma (NHL) were received after overnight transfer from UK hospitals. Blood specimens were collected into EDTA; bone marrow specimens into preservative-free heparin.

Test system Mononuclear cells were isolated by centrifugation of specimens over ficoll-hypaque. Cells were washed with wash medium before being incubated with drugs at 8 x 10^5/ml in 100 μl complete medium. Controls were also set up replacing drug with phosphate buffered saline (PBS) or the LDL vehicle. After incubation for 4 days at 37°C, cells were centrifuged onto microscope slides and stained. Loss of live tumour cells was counted as a percentage of the PBS controls, and the results of the duplicate slides averaged using the methods described by Bird et al, (Leuk. Res. **10**, (1986) 445-449) and Bosanquet (Lancet **331**, (1991) 711-714).

Data handling Tumour cell survivals (as a percent of control) have been plotted against VC concentration using the computer program Fig P (Biosoft, Cambridge). The program was also used to determine LC_{50} values (the VC concentration required to kill 50% of tumour cells) by fitting the data to curves with the general formula:

$$\text{Tumour cell survival (\%)} \quad \frac{100}{1+([VC]/LC_{50})^{-F}}$$

where [VC] is the VC concentration and F is a slope factor.

Correlation coefficients (r) and significance values (P) were also calculated using Fig P.

Results

In the tables, CLL patients who had received prior chemotherapy are designated CLL+ and those who had not are designated CLL-. Characteristics of the 36 specimens tested are presented in Table 1. Five of the AML specimens had received prior chemotherapy (no VC); 8 NHL specimens had received prior chemotherapy (6 prior VC); and 3 of the CLL+ had had VC.

Table 1. Characteristics of the 36 specimens tested.

Specimen	Source	Previous drugs
AML		
1760	marrow	None
1795	blood	Ida, AraC, VP16
1849	blood	Chl, Pred, AraC
1856	marrow	Dnr, AraC, Mzn
1862	marrow	AraC, Asp, Dnr, VP16, Mzn
1886	marrow	None
1887	blood	None
1897	blood	None
1899	blood	Mzn, Dnr, AraC, Tg
NHL		
1754	blood	None
1799	blood	Vc, Chl, Cy, Pred, Dex, Epi, Vb
1843	blood	Vc, Cy, Pred, Chl
1845	blood	Vc, Cy, Dox, Pred, Ifos, Mtx, AraC, VP16, Vb, Bleo
1860	blood	Vc, Cy, Mzn, Pred, Mep, AraC, Cp, VP16
1869	marrow	Chl, Pred
1877	blood	Chl, Pred, Flud
1893	blood	Vc, Dox, Cy, Pred, Chl, Mep
1906	blood	Vc, Chl, Cy, VP16, Flud, Dox

Table 1. (continued)

Specimen	Source	Previous drugs
CLL-		
1846	blood	None
1852	blood	None
1854	blood	None
1855	blood	None
1861	blood	None
1868	blood	None
1872	blood	None
1888	blood	None
1890	blood	None
CLL+		
1755	blood	Vc, Chl, Cy, Pred, Flud
1768	blood	Vc, Chl, Cy, Pred
1800	blood	Chl, Cy, Dox, Flud
1801	blood	Cy, Pred, Dox
1832	blood	Vc, Chl, Cy, Dox, Pred
1836	blood	Chl
1841	blood	Chl, Cy, Pred
1848	blood	Chl
1870	blood	Chl

Drug abbreviations: Vc, vincristine; Chl, chlorambucil; Cy, cyclophosphamide; Pred, prednisolone; Flud, fludarabine; Dox, doxorubicin; Ida, idarubicin; AraC, cytarabine; VP16, etoposide; Dex, dexamethasone; Epi, epirubicin; Vb, vinblastine; Mzn, mitoxantrone; Mep, methylprednisolone; Ifos, ifosfamide; Mtx, methotrexate; Dnr, daunorubicin; Tg, thioguanine; Cp, cisplatin, Bleo, bleomycin; Asp, asparaginase.

The raw results are presented in Table 2 and in Figures 7 to 42. In these Figures, best-fit curves calculated by computer are also displayed. Survival of the LDL controls was 100% in all the specimens. In almost all cases it can be seen that LDL-VC is more active than VC.

Table 2. Tumour cell survival values (%) at various vincristine concentrations (μg/ml).

	Vc					LDL-Vc				
Specimen	0.08	0.31	1.25	5.00	20.0	0.08	0.31	1.25	5.00	20.0
AML										
1760	35.5	28.5	31	22	14.5	26	16.5	10	8.5	27.5
1795	100	57.5	3	0	0	59	30	0	0	0.5
1849	61	51	6	3.5	1	54.5	20	6	2	1.5
1856	56	52.5	45.5	47.5	53	91	65	69	91.5	47
1862	61.5	27	6.5	1	1	37.5	14	6	2	1
1886	13.5	18.5	10.5	7	3	8	7	8	5	6
1887	27.5	9	2.5	2	0	12.5	8	3	2	0.5
1897	60.5	79	70.5	45	37.5	75	72.5	80.5	50	27.5
1899	100	34.5	36.5	26	23.5	46.5	36	34.5	24	1.5
NHL										
1754	100	88	38	21	3.5	100	93.5	34.5	35	9
1799	100	88.5	64.5	40	7	88.5	91	63.5	42.5	4
1843	100	31.5	9	4.5	0.5	23.5	14	6.5	6	0
1845	100	72	34	12.5	0	100	53	39	5.5	0.5
1860	87	27.5	19	9.5	0	29	25	13	8	2
1869	100	83.5	91.5	58.5	40.5	100	99.5	83.5	54.5	29.5
1877	79	43.5	44	17	1.5	52	43	40	12.5	1.5
1893	89.5	33.5	14.5	1	0.1	55.5	29.5	11	1.5	0
1906	100	28.5	4.5	1	0	37	6.5	7	1.5	0

Table 2. (continued)

	Vc					LDL-Vc				
Specimen	0.08	0.31	1.25	5.00	20.0	0.08	0.31	1.25	5.00	20.0
CLL-										
1846	86.5	16	8.5	15	4	30.5	12.5	13	17.5	4
1852	31.5	11	4	2	0.5	11	7.5	4	1.5	1
1854	69.5	15.5	4	1.5	0.5	25.5	6	2.5	1	0
1855	66.5	37	7	0.5	0	71.5	17	4.5	0	0
1861	79.5	71.5	51.5	32.5	7	66	69.5	41	18	6
1868	100	48	24.5	4.5	2	52.5	23	24.5	7	0
1872	47	15	21.5	10	2	27	16	20.5	12.5	2
1888	69.5	18	3.5	3	1	36	11	7	2.5	1
1890	78	18	6	5	1	16.5	10	10	3.5	0
CLL+										
1755	86.5	30	27.5	1	1	41	23.5	12.5	0	0.5
1768	70.5	53.5	14.5	8.5	12.5	87.5	62	27	20	11.5
1800	100	79.5	58.5	5	0.5	82.5	74	46	11	0.5
1801	95.5	75	34	5.5	4	70	36	17.5	17.5	0
1832	8.5	9.5	3	1	0	3	3.5	1	1	0
1836	92.5	36.5	11	8	3	44.5	13	14	10	2.5
1841	100	40	16	3.5	0.5	70.5	44.5	18	7.5	1
1848	7.5	0.5	0	0	0	0	1	0	0.5	0
1870	22.5	28	9	3.5	0	22.5	5	2.5	0.5	0

In Table 3 and Figures 1 to 4, the mean results (± SEM) for each disease group are presented, and in Figure 5 the AML results have been split into previously treated (n = 5) and previously untreated (n = 4). In these figures it can be seen that LDL/VC has a greater cytotoxic effect than VC with the greatest effect being in NHL and CLL specimens.

Table 3. Mean tumour cell survival values (%) at various vincristine concentrations (μg/ml).

Specimen type	Vc					LDL-Vc				
(No)	0.08	0.31	1.25	5.00	20.0	0.08	0.31	1.25	5.00	20.0
AML- (4)	34.2	33.8	28.6	19.0	13.8	30.4	26.0	25.4	16.4	15.4
AML+ (5)	75.7	44.5	19.5	15.6	15.7	57.7	33.0	23.1	23.9	10.3
AML (9)	57.3	39.7	23.6	17.1	14.8	45.6	29.9	24.1	20.6	12.6
NHL (9)	95.1	55.2	35.4	18.8	5.9	65.1	50.6	33.1	18.6	4.7
CLL- (9)	69.8	27.8	14.5	8.2	2.0	37.4	19.2	14.1	7.1	1.6
CLL+ (9)	65.0	39.3	19.4	4.1	2.5	46.9	29.2	15.5	7.6	1.9

These results are confirmed by analysis of the tumour cell survival results at 0.078 μg/ml VC by Student's 2-tailed t-test (Table 4). The results with the NHL specimens are most interesting. Six of these patients had already received VC and they were resistant to VC whilst LDL-VC produced significantly more kill of tumour cells *in vitro* at 0.078 μg/ml ($P<0.02$).

Table 4. Comparison of LDL-Vc to Vc by Student's 2-tailed t-test on the 0.078 μg/ml Vc tumour cell survival values.

Specimen type (No)	t	P
AML- (4)	0.06	>0.2
AML+ (5)	1.17	>0.2
AML (9)	1.32	>0.2
NHL (9)	3.02	<0.02
CLL- (9)	4.45	<0.01
CLL+ (9)	2.52	<0.05

In Table 5 the calculated LC_{50}s for VC and LDL-VC are presented as well as the therapeutic index of LDL-VC (VC LC_{50}/LDL-VC LC_{50}) for each specimen. Almost all specimens show a therapeutic index of >1, indicating greater efficacy of LDL-VC over VC. Thus, only 2/36 specimens (nos 1768 and 1856) show a poorer effect with LDL-VC. In Figure 6, LDL-VC therapeutic index is plotted against VC LC_{50} (n = 23). It can be seen that there is a trend towards greater therapeutic index for the sensitive specimens although this does not reach significance.

Table 5. Calculated LC_{50}s and therapeutic index of LDL-Vc.

Specimen	Vc (μg/ml)	LDL-Vc (μg/ml)	LDL-Vc Therapeutic index (Vc LC_{50}/LDL-Vc LC_{50})
AML			
1760	-*	-	>1
1795	0.353	0.117	3.02
1849	0.185	0.091	2.03
1856	3.881	-	<1
1862	0.121	0.043	2.81
1886	-	-	>1
1887	-	-	>1
1897	5.305	4.854	1.09
1899	0.556	0.0705	7.89
NHL			
1754	1.061	1.364	0.78
1799	2.635	2.710	0.97
1843	0.245	-	>1
1845	0.760	0.562	1.35
1860	0.212	0.0104	20.40
1869	10.784	6.814	1.58
1877	0.419	0.150	2.81
1893	0.234	0.106	2.21
1906	0.243	0.051	4.78

Table 5. (continued)

Specimen	Vc (μg/ml)	LDL-Vc (μg/ml)	LDL-Vc Therapeutic index (Vc LC_{50}/LDL-Vc LC_{50})
CLL-			
1846	0.163	-	>1
1852	0.033	-	>1
1854	0.124	0.030	4.13
1855	0.165	0.132	1.25
1861	1.197	0.585	2.05
1868	0.382	0.077	4.96
1872	0.045	-	>1
1888	0.128	0.040	3.18
1890	0.148	-	>1
CLL+			
1755	0.243	0.047	5.16
1768	0.270	0.563	0.48
1800	1.306	0.860	1.52
1801	0.731	0.189	3.87
1832	-	-	>1
1836	0.246	0.045	5.49
1841	0.282	0.227	1.24
1848	-	-	>1
1870	-	-	>1

*Specimens too sensitive or too resistant to Vc to calculate LC_{50}s. However, in every case except specimens 1768 and 1856, the cells were more sensitive to LDL-Vc than to Vc.

It is interesting to note that in many cases the VC curve is steeper than the LDL-VC. This suggests greater LDL-VC efficacy, particularly at the lowest VC concentration.

Thus, LDL-VC has shown greater activity *in vitro* than VC in almost all of the 36 specimens tested. This is particularly so for specimens from AML patients who had previously received chemotherapy and whose VC resistance was markedly higher than previously untreated patients. It was also true for specimens from NHL patients with enhanced cytotoxicity in four of the six patients previously treated with VC.

Example 3

Preparation of LDL/taxol complex.

In preparing the LDL/taxol complex the general method set out in WO-A-8607540 was followed. 5.5 mg taxol was dissolved in 2 mls methylene chloride and added to a flask containing 20 mg LDL previously lyophilised from 5 ml aqueous solution containing 200 mg sucrose as protective agent. The methylene chloride was removed by evaporation under nitrogen.

The drug LDL/taxol complex was dissolved in 10 mls PBS and passed through a 0.2 μm membrane filter. The filtered solution was passed through a gel filtration column to remove insoluble taxol and diluted in PBS to a concentration of 10 μg/ml taxol. It was refiltered through a 0.2 μm membrane filter.

Example 4

Effect of LDL/taxol or taxol on experimental cancer cells.

The methyl tetrazolium (MTT) assay was used to determine the sensitivity of a murine myelomonocytic leukaemia cell line (WEHI-3B) to LDL/taxol and taxol dissolved in DMSO.

Materials and Methods

Medium "Complete RPMI 1640" medium consists of RPMI 1640 medium supplemented with 10% foetal calf serum, 1μM sodium pyruvate, 50 iu/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine.

Drugs LDL/taxol from Example 3 was diluted to give final dilutions of 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.01, 0.001 μg/ml in "complete RPMI 1640" medium in 96 well plates. Taxol was dissolved in DMSO to provide a stock solution at a concentration of 20 μg/ml which was further diluted to give final dilutions in 96 well plates of 2, 1.6, 1.2, 0.8, 0.4, 0.2, 0.02, 0.002, 0.0002, μg/ml.

Cells WEHI-2B cells were harvested in the exponential growth phase and plated into 96 well plates at a concentration of 2.8×10^5 cells/ml "complete RPMI 1640" medium.

Drug sensitivity test Dilutions of the drug preparations were added to the cell suspensions in 96 well plates to give the final drug concentrations listed above.

The cells were incubated for 4 days at 37°C in an atmosphere containing 5% CO_2 and 95% air. The MTT assay was performed by replacing the old growth medium with fresh medium and adding MTT solution to a final concentration of 1 mg/ml. After 4 hours incubation at 37°C the growth medium was removed from the wells and formazan crystals from the MTT substrate were dissolved in DMSO. The absorbence of the resulting solution was read at 550 nm to allow the percentage cell survival to be calculated using values obtained from control cells incubated in the absence of drug.

Results

The percentage cell survival at each drug concentration is plotted in Figure 43 and shows that LDL/taxol preparations are more toxic than taxol dissolved in DMSO with IC_{50} values (concentration required to inhibit 50% cell growth) of 0.045 and 0.2 μg/ml respectively. Interestingly, DMSO itself has considerable toxicity whereas LDL on its own has no toxicity.

This Sheet Has Been Replaced

CLAIMS:

1. The use of a lipophilic cytotoxic substance and a low density lipoprotein (LDL), wherein the activity of the cytotoxic substance is greater than its activity when not in the presence of LDL, in the preparation of an agent for the treatment of a cancer.

2. The use as claimed in claim 1, wherein the cytotoxic activity of the substance is increased by at least 10%.

3. The use of a lipophilic cytotoxic substance and a low density lipoprotein (LDL) in the preparation of an agent for the treatment of a cancer which is resistant to the cytotoxic substance when administered without LDL.

4. The use as claimed in any one of claims 1 to 3, wherein the cytotoxic substance is complexed with the LDL.

5. The use as claimed in any one of claims 1 to 4, wherein the cytotoxic substance is vincristine, doxorubicin, AD32, AD312, chlorambucil, prednimustine, WB4291, aclacinomycin, nitrosoureas, taxol and its analogues and other lipophilic drugs.

6. The use as claimed in any one of claims 1 to 5 in which no cholesteryl esters are present with the cytotoxic substance in the LDL particles.

7. The use as claimed in any one of claims 1 to 5, wherein the LDL particles contain, in addition to the cytotoxic substance, cholesteryl esters.

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* C L M S *

8. The use as claimed in any one of claims 1 to 7, wherein the cancer is a leukaemia or lymphoma.

9. The use as claimed in any one of claims 1 to 8, wherein the agent is formulated for parenteral administration.

10. The use as claimed in claim 9, wherein the agent is formulated for intravenous administration.

11. A method for the treatment of cancer, the method comprising administering to a patient an effective amount of a cytotoxic substance formulated with LDL, wherein the effective amount of the cytotoxic substance is not greater than 80% of an effective amount of the cytotoxic substance when not formulated with LDL.

12. A method for the treatment of drug resistant tumours, the method comprising administering to a patient an effective amount of a cytotoxic substance formulated in LDL.

13. A method for the treatment of cancer, the method comprising administering to a patient an effective amount of a cytotoxic substance formulated in LDL, wherein the cytotoxic substance is insoluble in non-toxic solvents.