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(54) Title: PROPHYLACTIC AND/OR THERAPEUTIC TREATMENT OF PROLIFERATIVE AND CONFORMATIONAL DISEASES

(57) Abstract: The present invention relates to the use of compounds modulating the pathways leading to cholesterol esterification for the preparation of a medicament for the treatment and/or prevention of proliferative and/or conformational diseases or of early ageing. The medicament further comprises a compound endowed with antiproliferative and/or anti-protein misfolding activity.
Prophylactic and/or therapeutic treatment of proliferative and conformational diseases

FIELD OF THE INVENTION

This invention relates to studies on cause-effect relationships between alterations of cholesterol homeostasis, the ageing process and the development of proliferative diseases (such as atherosclerosis and neoplasms) and/or conformational diseases (such as Alzheimer's disease (AD) and prion-related diseases) in humans and/or other mammals. The invention describes pharmacological interventions aimed at therapeutic or prophylactic treatments of individuals who are affected by, or are at risk of developing, the above mentioned proliferative and/or conformational diseases. Treatments are based on the use, alone, in proper sequence, or in combination, of the following drugs: i) modulators of the intracellular cholesterol trafficking and metabolism and/or ii) drugs endowed with antiproliferative and/or anti-protein misfolding activity, which may be properly encapsulated in vehicles, such as nanospheres, capable of slow release and/or regioselective delivery of active principles to target organs, including penetration of the blood-brain barrier included.

BACKGROUND OF THE INVENTION

Cholesterol trafficking and metabolism in peripheral cells

As summarized in Fig. 1, intracellular cholesterol derives from: i) endogenous neosynthesis (1) in the ergastoplasmic reticulum (ER) through the activity of hydroxyl-methyl-glutaryl-coenzime-A reductase (HMGCoA-R) and ii) circulating low density lipoproteins (LDL) (2), which are first internalised via LDL receptors (a) and then hydrolytically processed in lysosomes to generate free cholesterol (FC) through the activity of acid cholesterol ester hydrolase (aCEH) (b). Most of the newly synthesized FC participates to the physiological turnover of cholesterol in the rafts (c), and/or to the biogenesis of new membrane domains in ER and Golgi. If plasma membrane FC exceeds a threshold level, the excess FC is rapidly transported to the ER (d) by a P-glycoprotein (MDRI-Pgp3, firstly described for its ability to catalyse ATP-dependent efflux of cytotoxic agents from tumour cells) (1-3), encoded by the multidrug resistance (MDRI) gene. Then, the ER-resident enzyme acyl-coenzyme-A cholesterol-acyl-transferase (ACAT) (e) converts FC into cholesteryl-esters (CE), which
are stored in droplets in the cytoplasm (4-6). Although neosynthesized FC in the ER could be efficiently used for esterification by virtue of its proximity to ACAT, only a small percentage of it is directly esterified. Virtually all the esterified FC derives from the plasma membrane.

If cells require cholesterol, CE is re-hydrolyzed to FC by the enzyme neutral cholesterol-esters-hydrolase (nCEH) (f) and re-delivered to the plasma membrane by caveolin-1. If in excess, FC is eliminated from the cells through an efflux pathway spanning from the ER to the plasma membrane and involving caveolin-1, the ATP-Binding Cassette of the subfamily A, member 1 (ABC-Al) and plasma HDLs (g) (4-9).

**Role of cholesterol in lipid rafts**

Recent work has led to a new way of considering biological membranes, which are now viewed as a "mosaic of lipid domains", rather than as an "homogeneous fluid mosaic". It appears that cholesterol is not uniformly distributed within membranes, but is distributed into cholesterol-poor and cholesterol-rich domains. Among the latter, those containing saturated sphingolipids are referred to as lipid rafts (10), which float freely in plasma membranes carrying a few passenger proteins. When the passenger proteins are activated by a ligand, lipid rafts coalesce to form larger platforms where many different proteins converge in order to perform specific functions, such as signalling, processing or transport (10, 11).

Examples of raft passenger proteins are receptors for growth factors, signal transducing proteins (P21Ras), chemokine receptors, proteins of the MHC classes, antigen receptors, and various proteins with yet undefined functions, such as the amyloid precursor protein (APP) and the cellular prion protein (PrPc) involved in AD and Prion-related disorders, respectively. Interestingly, the amount of cholesterol associated with these domains exerts profound effects on the functions of the raft-resident proteins. For instance, perduing low levels of cholesterol in lipid rafts lead to a continuous stimulus for cell growth promotion (12), or induce APP and cellular prion protein PrPc to undergo pathologic processes leading to the generation of their corresponding pathogenic forms: the amyloidogenic A-beta peptide and the scrapie prion protein (PrPsc), respectively (13,14).

**Cholesterol trafficking and metabolism in proliferative diseases**

The initial studies of the authors on cholesterol trafficking and metabolism in proliferative diseases have been carried out in: i) rat acinar cell pancreatic carcinoma and ascite hepatoma (15,16); ii) tissue biopsies from human solid and haematologic neoplasms (17-
20); iii) cell lines derived from various human and animal tumours (21-23) and, iv) vessel tissues from healthy and atherosclerotic patients (24,25).

In these in vivo and in vitro models the authors have demonstrated that the rate of cell proliferation correlates: i) positively, with the levels of MDRI-Pgp and ACAT and related mRNAs, leading to FC esterification; and ii) negatively, with the levels of nCEH and caveolin-1 and relative mRNAs, leading to intracellular CE accumulation (Table 1).

Accordingly, when compared to healthy controls, subjects affected by the above mentioned proliferative diseases possess lower FC levels, significantly higher CE levels and lower HDL-cholesterol levels.

### TABLE 1: Correlations of intracellular free (FC) and esterified (CE) cholesterol with plasma HDL-cholesterol in proliferative diseases.

<table>
<thead>
<tr>
<th>Proliferative Diseases</th>
<th>FC</th>
<th>CE</th>
<th>HDL-Cholesterol</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat ascite hepatoma</td>
<td>µg/10^7 cells</td>
<td>mg/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 days after transplantation</td>
<td>39.9</td>
<td>9.0</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>7 days after transplantation</td>
<td>35.6</td>
<td>18.0</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td><strong>Human neoplasias</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult lung cancer</td>
<td>1.0</td>
<td>1.0</td>
<td>15-35</td>
<td>18</td>
</tr>
<tr>
<td>Controls</td>
<td>1.4</td>
<td>0.25</td>
<td>45-60</td>
<td></td>
</tr>
<tr>
<td>Adult gastrointestinal cancer</td>
<td>1.8</td>
<td>0.8</td>
<td>12-25</td>
<td>19</td>
</tr>
<tr>
<td>Controls</td>
<td>1.5</td>
<td>0.2</td>
<td>45-60</td>
<td></td>
</tr>
<tr>
<td>Child solid cancers</td>
<td></td>
<td></td>
<td>20-30</td>
<td>20</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>40-45</td>
<td></td>
</tr>
<tr>
<td>During remission</td>
<td></td>
<td></td>
<td>40-45</td>
<td></td>
</tr>
<tr>
<td>Adult hemat. neoplasias</td>
<td>µg/mg protein</td>
<td>mg/dl</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>10-25</td>
<td></td>
</tr>
<tr>
<td>Child hemat. Neoplasias</td>
<td></td>
<td></td>
<td>15-25</td>
<td>20</td>
</tr>
<tr>
<td>Child solid cancers</td>
<td></td>
<td></td>
<td>20-30</td>
<td></td>
</tr>
<tr>
<td>Leukemia cell lines</td>
<td>µg/mg protein</td>
<td>mg/dl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The authors have also found that drugs that specifically target the above mentioned steps of the cholesterol metabolism/trafficking inhibit cell growth in models of pathologic proliferative conditions (21,25,26).

5 **Cholesterol trafficking and metabolism in conformational diseases**

Among the increasing number of neurodegenerative pathologies classified as conformational diseases, AD and Prion-related disorders (also known as Transmissible Spongiform Encephalopathies, TSEs), can be considered prototypes of non-transmissible and transmissible cerebral amyloidoses, respectively (27-29).

Although there are obvious differences in the aetiology and pathogenesis of these diseases, intracellular CE accumulation is a common hallmark indicating a link between these pathogenetic processes and the alteration of cholesterol homeostasis.

**Unsolved Problems**

All the above diseases have reached serious proportions in terms of incidence. However, while in the case of atherosclerosis and tumors therapeutic strategies based on HMGCoA-R inhibitors and antiproliferative drugs, respectively, exist, no conclusive means of diagnosis, prevention or therapy are yet available for AD and prion-related disorders (30-32).

As for the specific treatment of AD, drugs targeted at the following enzyme activities are under investigation: 1) inhibitors of beta' and gamma secretases (Pharma Companies developing drugs for AD include: Amgen, Bristol-Meyer Squibbs, Elan Pharmaceuticals, Scios Inc., Glaxo Smith Kline), 2) inhibitors of acetilcholinesterase, (i.e. memantine), which address the cognitive impairment by enhancing the cholinergic transmission, 3) inhibitors of HMGCoA-R (i.e. statins, such as squalestatin and lovastatin), which inhibit
the endogenous synthesis of cholesterol. All three classes of drugs, however, display either major side effects or inability to penetrate the blood-brain barrier (BBB) (33-35).

As far as prion-related disorders are concerned, potential therapeutic strategies rely on:
a) inhibitors of the structural beta-transition and polymerization of proteins (misfolding), b) compounds affecting fibril aggregation, and c) compounds able to revert conformational changes. Several classes of inhibitors have been described in animal models of prion diseases (i.e. congo red, tetracyclines, polyamions, branched polyamines, polyphenols, antipsychotic, antidepressants, analgesics and statins) (31). Some of these, (e.g. congo red and statins) have been proven to be active against both the generation of the AD amyloidogenic A-beta peptide and the accumulation of the scrapie prion protein (PrPsc) in cell models of AD and TSEs, respectively.

When tested in animal models of TSEs, some of the above compounds (pentosan, heparan sulphate, amphotericin B, iodo-deoxy-doxorubicin, tetracycline, phthalocyanin sulphate) have been found to prolong the incubation period in prion-inoculated rodents. However, the protective effect has been observed only when the compounds were administered around the time of prion inoculation, while they have been found inactive when administered at the time of neurological symptom development, thus underlining the clinical limitation of a monotherapy targeting only the misfolding/aggregation steps. Moreover, since some of most active compounds (polysulphonated glycosides, iodo-deoxy-doxorubicin, tetracycline, cyclic-tetrapyrroles) showed activity only when given intra-cerebrum (i.e.), the ability of drugs to penetrate the BBB represents a further challenge to treatment. In fact, the non-polar character of the BBB prevents large, charged molecules (namely proteins and drugs) from penetrating from the systemic circulation into the central nervous system (CNS), thus preventing access to the injured sites where they are needed.

The polysulphonated glycosides pentosan sulphate and heparan sulphate, the acridine compound quinacrine and the phenothiazine compound, chlorpromazine, have been recently evaluated in controlled trials of Creutzfeldt-Jacob Disease (CJD) patients, resulting in transient improvement of some clinical symptoms (36). However, there is a need for improving the therapeutic and prophylactic potential against neurodegenerative diseases. Therefore, besides synthesizing new compounds against known targets, it is crucial: i) to identify new co-factors involved in pathogenetic steps susceptible to be
exploited as selective targets; ii) to develop formulations allowing the proper slow delivery and bioavailability in the CNS required for the effectiveness of the drugs.

Increasing evidence suggests that modifications in cholesterol homeostasis may be involved in the conformational changes underlying the formation of amyloid plaques in AD and TSEs (10, 11). Therefore, the authors propose that drugs interfering with or modulating cholesterol trafficking and/or metabolism are used to slow down the ageing process, to decrease the risk of developing proliferative diseases and non-transmissible conformational diseases, such as AD, and to decrease the susceptibility to prion-related disorders, such as TSEs.

In addition, the authors propose a method for assessing in vitro efficacy of new treatments or for prognosis of therapeutic strategy by monitoring cholesterol trafficking/metabolism parameters in PBMCs or fibroblasts of disease patients or of individual at risk. The present invention is expected to lead to several contributions related to proliferative diseases and/or conformational diseases in mammals. Among them are: 1) identification of a cell phenotype predisposing to pathologic conditions; 2) definition of optimal drug combinations and/or drug formulations for preventive/therapeutic treatments; 3) identification of new, more safe / effective treatments.

DESCRIPTION OF THE INVENTION

It is the object of this invention a method for an effective treatment and/or prophylaxis of early ageing, proliferative and/or conformational diseases by using modulators of the pathways leading to cholesterol esterification, alone or in combination with small molecules endowed with antiproliferative and/or anti-protein misfolding activity, which should be encapsulated in nanosphere or other drug delivery systems capable to penetrate the BBB and to slowly release the drugs.

The present invention proliferative and conformational diseases comprise but are not limited to the diseases indicated in Table 2.

<table>
<thead>
<tr>
<th>TABLE 2: Examples of proliferative and conformational diseases.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferative diseases</strong></td>
</tr>
<tr>
<td>Atherosclerosis</td>
</tr>
<tr>
<td><strong>Restenosis after angioplasty</strong></td>
</tr>
<tr>
<td>Hematological neoplasms</td>
</tr>
<tr>
<td>Hodgkin and non-Hodgkin lymphomas</td>
</tr>
</tbody>
</table>
Therefore it is an object of the invention the use of at least one compound modulating the pathways leading to cholesterol esterification and/or exerting an anti-proliferative activity for the preparation of a medicament for the treatment and/or prophylaxis of proliferative and/or conformational diseases or of early ageing. Preferably, the compound is selected from the group of inhibitors of: cholesterol esterification, MDRI-Pgp and ACAT. More preferably, the compound is selected from the group of: progesterone, everolimus, pioglitazone, avasimibe, verapamil, cyclosporin A, Sandoz 58-035. Preferably, the medicament further comprises at least one compound endowed with antiproliferative and/or anti-protein misfolding activity. More preferably the compound endowed with antiproliferative and/or anti-protein misfolding activity belongs to the group as listed in Tables 3, 4A and 4B. In the present invention the proliferative disease is selected from the group of: atherosclerosis, restenosis after angioplasty, hematologic...
neoplasms, solid tumors. Preferably the hematologic neoplasms are selected from the group of: Hodgkin and non-Hodgkin lymphomas, acute and chronic leukemias, eritroleukemias, mielomas or policytemias. More preferably, the solid tumors are selected from the group of: brain, headneck, nasopharyngeal, breast, ling, gastrointestinal, colon, kidney or liver tumor. Preferably the conformational disease is selected from the group of: priori-related disorders, Alzheimer's disease, Parkinson's disease, Huntington disease, amyotrophic lateral sclerosis or spinocerebellar degenerations. More preferably, the prion-related disorders are selected from the group of: Creutzfeldt-Jacob disease, new variant Creutzfeldt-Jacob disease, Gerstmann-Straussler Sheinker syndome, fatal familial insomnia, bovine spongiform encephalopathy, scrapie, chronic wasting disease, feline spongiform encephalopathy.

A further object of the invention is a pharmaceutical composition comprising an effective amount of at least one compound modulating the pathways leading to cholesterol esterification, an effective amount of at least one compound endowed with antiproliferative and/or anti-protein misfolding activity, and suitable diluents and/or adjuvants and/or excipients. Preferably the compound modulating the pathways leading to cholesterol esterification is selected from the group of inhibitors of: cholesterol esterification, MDRI-Pgp and ACAT. More preferably the Compound modulating the pathways leading to cholesterol esterification is selected from the group of: progesterone, everolimus, pioglitazone, avasimibe, verapamil, cyclosporin A, Sandoz 58-035. Even more preferably, the compound endowed with antiproliferative and/or anti-protein misfolding activity belongs to the group as listed in Table 3, 4A and 4B. Still preferably the compounds are encapsulated in nanospheres.

According to the present invention, pharmaceutical compositions are provided wherein the cholesterol metabolism regulators and the anti-proliferation and/or anti-protein misfolding agents are present in ratios based on a fraction of their respective 50% effective dose (ED\textsubscript{50}) values, which ratios are from about 1:1 to about 500:1 and from about 1:500 to about 1:1. The amount of the compounds-of the invention to be used, and the frequency of exposure of a given subject, can be readily determined by one skilled in the art and will vary according to the cell phenotype of the subject, the compounds used and the effect sought. In determining optimum concentrations, appropriate in vitro assays may be performed using the various compounds in the nanomolar to micromolar range.
Since proliferative or conformational diseases are chronic disorders whose treatment would require continuous administration of drugs, the present invention includes dosage forms suitable for long-term treatments addressing specific requirements:

1) extended release of the active principle, in order to extend the administration times,

2) easy administration route, which is intended either per os or intradermic, in order to enhance the patient compliance,

3) high regioselectivity, in order to reduce undesired effects deriving from the systemic administration of drugs,

4) ability to cross the BBB for neurodegenerative disorders.

One of the systems that can be suitable for this aim uses biocompatible, biodegradable polymers to encapsulate drugs in micrometer-or nanometer-scale spheres. Appropriately named "microspheres" (or "nanospheres," depending on their size), these particles degrade in the body over an extended time period that can range from days to months (37). In addition to being easy to formulate and administer, microspheres are very stable and have a shelf life far exceeding that of drugs administered per os. To obtain microspheres, both hydrophilic and lipophilic matrices can be employed together with different reticulating agents in order to modulate the releasing speed of the loaded drug. Once the target site of the drug is individuated, the most efficient polymer matrix can be functionalised through chemical synthetic processes in order to optimise the therapeutic system from both the kinetic and region specificity points of view. For this purpose, a convenient preparation method can be set up in order to obtain microspheres with both uniform size and so as to be suitable to achieve the highest loading efficiency. The polymers used in this invention can be natural and/or synthetic and biodegradable ones; among the latter, chitosan can be used. This polymer can be obtained from the hydrolysis of the N-acetyl group of chitin, a natural and quite common polysaccharide. Chitosan exhibits interesting pharmaceutical properties due to its biodegradability, bioavailability and good bioadhesive properties.

The characterization of the formulations can be achieved by using several techniques, such as transmission electronic microscopy (TEM), light scattering, gel permeation and UV spectroscopy. Quantitative analysis of the encapsulating efficiency can be achieved by means of HPLC.

In AD and prion-diseases (such as TSE) therapy, a controlled release system would have several advantages. A primary advantage lies in the polymers' malleability. For instance, a microsphere sample encapsulating a therapeutic drug could readily be formulated with a
low surface charge and a diameter of as little as 200 nm. Both characteristics would facilitate crossing through the BBB and enhance entry into target cells, increasing the efficacy of treatments. In addition, the sustained release characteristics of such systems allow them to accommodate chronic treatment times (38-40).

In the present invention, compounds modulating the pathways leading to cholesterol esterification and/or having antiproliferative activity and compounds endowed with anti-protein misfolding activity comprise but are not limited to compounds indicated in Table 3 and Table 4A and 4B.

**TABLE 3:** Compounds modulating the pathways leading to cholesterol esterification and/or endowed with antiproliferative activity.

<table>
<thead>
<tr>
<th>DRUG/COMPOUND</th>
<th>BRAND NAME</th>
<th>MANUFACTURER</th>
<th>CLINICAL USE / BIOLOGICAL EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROGESTERONE</td>
<td>Crinone; Gestone; Progestasert; Others</td>
<td>SOLVAY; UPJOHN; BRISTOL-MEYER; SQUIBB</td>
<td>Contraception; Prev. endometrial cancer; Uterine bleeding</td>
</tr>
<tr>
<td>EVEROLIMUS</td>
<td>Certican®</td>
<td>NOVARTIS PHARMA</td>
<td>Heart transplant complications; Immunosuppressive; Antiproliferative</td>
</tr>
<tr>
<td>PIOGLITAZONE</td>
<td>Actos®</td>
<td>TAKEDA PHARMACEUTICALS AMERICA; ELI LILLY</td>
<td>Non Insulin-dependent diabetes mellitus (NIDDM). Insulin-sensitizing agent</td>
</tr>
<tr>
<td>AVASIMIBE</td>
<td>Phase III clinical trial</td>
<td>PFIZER</td>
<td>Anti-atherosclerotic; Hypercholesterolemia</td>
</tr>
<tr>
<td>VERAPAMIL</td>
<td>Calan; Verelan; Isoptin</td>
<td>KNOLL-PHARMACEUTICAL COMPANY</td>
<td>High blood pressure; Angina</td>
</tr>
<tr>
<td>CYCLOSPORIN A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SANDOZ 58-035</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 4A.** Classes of compounds endowed with anti-protein misfolding activity

<table>
<thead>
<tr>
<th>Anti-protein misfolding class/compound</th>
<th>Known effect/use</th>
<th>Affected step: misfolding/ aggregation</th>
<th>Effect in animal models</th>
<th>BBB penetration</th>
<th>Disadvantages/ comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLYSULPHONATED GLYCOSIDES:</td>
<td></td>
<td>-PrPSc formation in cells</td>
<td>-Reduced spleen infectivity of...</td>
<td>NO</td>
<td>Prophylactic use only</td>
</tr>
<tr>
<td>-dextran sulphate</td>
<td>Antiviral</td>
<td>-binding to PrP</td>
<td>-Delayed clinical onset of...</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-pentosan sulphate</td>
<td></td>
<td>-endocytic pathway of PrPC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-heparan sulphate</td>
<td>Anticoagulant</td>
<td>-competition with endogenous GAGs</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Toxicity  
Human trials  
Human
| **SULPHONATED DYES**<br> -congo red<br> -sirius red<br> -trypan blue | Hystopathology, amyloid staining | -inhibit beta-conversion (APP & Scrapie)<br> -binding to beta-sheets<br> -PrPSc overstabilization | Delayed clinical onset | POOR | Prophylactic use only<br> Toxicity |
| SURA.MIN | Antiparasitic (systemic use in humans) | - | Increased incubation period | | Toxicity |
| **POLYENE ANTIBIOTICS**<br> -amphotericin B (AmphB)<br> -MSS209 (AmphB deriv) | Antifungal<br> Antiparasitic<br> Antiviral | -PrPSc formation in cells<br> -binding to membrane cholesterol<br> -rafts & endocytic pathway of PrPC | Delayed clinical onset | YES | Prophylactic & therapeutic use<br> Toxicity<br> Strain specific |
| **ANTHRACYCLIN ANTIBIOTICS**<br> -ido-deoxy-doxorubicin (IDX) | Antineoplastic | -PrPSc formation in cells<br> -binding to amyloid fibrils | Delayed clinical onset only after drug/scrapie preincubation | VERY POOR | Toxicity |
| **TETRACYCLINE ANTIBIOTICS**<br> - | Antibacterial | -Binding to specific PrPSc aa sequences<br> -Pkp-resistance of preformed PrPSc<br> -peptide aggregates | Delayed clinical onset following drug/scrapie preincubation | NO | Prophylactic use only |
| **CYCLIC TETRAKYROLES**<br> -deuteroporphyrins (DP)<br> -phthalocyanin sulphates (PcTSs) | | Conformational change induction after protein binding | Increased survival time | NO | Prophylactic use only |
| **LYSOSOMOTROPIC DRUGS** | | -PrPSc destabilization in lysosomes<br> -prion rod disaggregation<br> -increased PK susceptibility | Prolonged incubation period | NO | Strain specific<br> Toxicity |
| **BRANCHED POLYAMINES**<br> -polystyreneimine (PEI)<br> -polymidoamine (PAMAM) | Antiprotozoal; Antirheumatic; Intraneural sclerosing | -PrPSc formation in cells<br> -PrPSc destabilization in lysosomes | Prolonged incubation period | YES | Human trials in CJD patients: (transient improvement of some clinical symptoms) |
| **ACRIDINES**<br> -quinacrine (bis-acridines) | | -PrPSc formation in cells<br> -PrPSc destabilization in lysosomes | Prolonged incubation period | YES | Human trials in CJD patients: (transient improvement of some clinical symptoms) |
| **PHENOTHIAZINES**<br> -chlorpromazine | Antipsycotic; Antiemetic | Destabilization of PrPSc in lysosomes | Prolonged incubation period | YES | Human trials in CJD patients: (transient improvement of some clinical symptoms) |
Table 4B. Main classes of antiproliferative agents currently used in cancer chemotherapy.

<table>
<thead>
<tr>
<th>ANTIOXIDANTS</th>
<th>Antioxidant</th>
<th>PrPSc accumulation</th>
<th>None</th>
<th>YES</th>
</tr>
</thead>
<tbody>
<tr>
<td>-tannic acid</td>
<td>Experimental use only</td>
<td>-Binding to specific Abeta-amyloid or PrPSc sequences</td>
<td>Up to 95% inhibition of scrapie infectivity after drug/scrapie preincubation</td>
<td>NO</td>
</tr>
<tr>
<td>-curcumin</td>
<td></td>
<td>-reversion of conformational changes</td>
<td>Potentially immunogenic</td>
<td>NO</td>
</tr>
</tbody>
</table>

| STATINS            | Anti-hypercholesterol agents | Reduced generation/accumulation of Abeta-amyloid and PrPSc in cells | Not determined | NO | Human drugs |
|--------------------|-------------------------------|-------------------------------------------------------------------|---------------|-----------|
| -lovastatin        |                               |                                                                   |               |           |
| -squalstatin       |                               |                                                                   |               |           |

Table 4B. Main classes of antiproliferative agents currently used in cancer chemotherapy.

<table>
<thead>
<tr>
<th>Antineoplastic agents</th>
<th>Main use in the clinic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkylation Agents</strong></td>
<td></td>
</tr>
<tr>
<td>Ciclofosfamide</td>
<td>chronic lymphocytic leukemia, lymphomas, solid tumors</td>
</tr>
<tr>
<td>Clorambucil</td>
<td>chronic lymphocytic leukemia, Hodgkin’s and non Hodgkin lymphomas, ovari cancer.</td>
</tr>
<tr>
<td><strong>Cytotoxic Antibiotics</strong></td>
<td></td>
</tr>
<tr>
<td>aclarubicine, daunorubicine, doxorubicine, epirubicine, idarubicine, mitoxantrone</td>
<td>acute leukemia, lymphomas, solid tumors.</td>
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<tr>
<td><strong>Antimetabolites</strong></td>
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<tr>
<td>Metotrexate</td>
<td>acute lymphoblastic leukemia, coriocarcinoma, non Hodgkin lymphoma, solid tumors.</td>
</tr>
<tr>
<td>Citarabine</td>
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<tr>
<td>Fludarabine</td>
<td>acute B cell lymphocytic leukemia</td>
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<tr>
<td><strong>Vinca Alkaloids</strong></td>
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</tr>
<tr>
<td>vinblastine, vincristine, vindesine, vinorelbine</td>
<td>acute leukemias, lymphomas, breast and lung cancers</td>
</tr>
<tr>
<td><strong>Other Antitumor Drugs</strong></td>
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<tr>
<td>Etoposide</td>
<td>small cell lung cancer, lymphomas, testis cancer</td>
</tr>
<tr>
<td>dacarbazine, temozolomide</td>
<td>metastatic melanoma, sarcomas</td>
</tr>
<tr>
<td>platin derivatives (carboplatin, cisplatin, oxaliplatin)</td>
<td>ovary cancer, lung cancer, metastatic seminoma and teratoma, cancers of the upper gastro-intestinal tract</td>
</tr>
<tr>
<td>taxans (paclitaxel, docetaxel)</td>
<td>advanced ovary cancer, metastatic breast cancer, non small cell lung cancer</td>
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<td>sodic porfimer</td>
<td>non small cell lung cancer</td>
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</tr>
<tr>
<td>topoisomerase I</td>
<td>metastatic cancers of colon and ovary</td>
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</tbody>
</table>
The invention will be now described by non limiting examples referring to the following figures:

**Figure 1:** Intracellular cholesterol homeostasis. Intracellular cholesterol derives from i) endogenous neosynthesis in the ergastoplasmic reticulum (ER) through the HMGCoA-reductase (1); ii) circulating low density lipoproteins (LDL) (2), which are first internalised via LDL receptors (a) and then hydrolytically processed in lysosomes to generate free cholesterol (FC) through the acid cholesterol ester hydrolase (aCEH) (b). Most of the newly synthesized FC, or the LDL-bound FC released in the lysosomes, rapidly emerges at cell surface caveolae, from where it may be used for cellular functions (c). If plasma membrane FC exceeds a threshold level, the excess FC is rapidly transported to the ER (d) by a P-glycoprotein (MDRI-Pgp) encoded by the multidrug resistance (MDRI) gene. Then, the ER-resident enzyme acyl-coenzyme A-cholesterol-acyl-transferase (ACAT) (e) converts FC into cytoplasmic cholesteryl-ester (CE) droplets for storage. If cells require cholesterol, CE is re-hydrolyzed to FC by the enzyme neutral cholesterol-esters-hydrolase (nCEH) (f) and reported to plasma membrane by caveolin-1. If in excess, FC is eliminated from the cells through an efflux pathway spanning from the ER to the plasma membrane and involving caveolin-1, the ABCA1 receptor, and plasma HDLs (g).

**Figure 2 and 2bis:** Alterations of cholesterol homeostasis in pathologic conditions. If alterations in cholesterol homeostasis occur, there is an increased cholesterol synthesis and uptake, and more cholesterol is transported to the ER by the MDRI-Pgp. This leads to activation of the ACAT enzyme which, in turn, esterifies cholesterol. Cholesterol esters (CE) are then accumulated in the cytoplasm as lipid droplets, and foam-like cells are formed (Schemes 2 A, B and C). The accumulation of cholesterol in the form of esters reduces the cholesterol pool to be transported by caveolin-1 to the plasma membrane (rafts) for excretion, with a consequent decrease in cholesterol efflux via ABCA-1 receptors leading to a decrease of HDL-cholesterol levels in plasma (Schemes 2 A, B, and C). Lower levels of FC into the rafts may lead to a continuous activation of raft-protein functions in signalling, processing and transport, thus triggering a variety of pathologic processes including atherosclerosis (Fig 2, Scheme 2A), tumors (Fig. 2, Scheme 2B), AD and TSE diseases (Fig. 2Bis, Scheme 2C). Treatment with modulators of cholesterol
metabolism/trafficking could restore the normal raft-protein functions by re-establishing
the intracellular cholesterol homeostasis (Fig. 2Bis, Scheme 2D).

**Figure 3: Neutral lipid content and mRNA expression levels of genes involved in cholesterol metabolism and trafficking in PBMCs from patients with Cronic Lymphocytic Leukaemia (CLL).** A) PBMCs from a CLL patient and from an healthy individual (control) stained for neutral lipid content by the ORO method (indicated by the arrows, see Materials and Methods) at the indicated times after PHA-stimulation. B) mRNA level of the indicated genes in PBMCs from CLL patients (PI and P2) and from healthy individuals (C1 and C2) by RT-PCR (see Materials and Methods). Beta-actin was used as an internal standard.

**Figure 4: Neutral lipids content in PBMCs from patients with atherosclerotic plaques.** PBMCs from atherosclerotic patients (patient 1 to 4) and from healthy individuals (controls 1 and 2) stained for neutral lipid content by the ORO method at the indicated times after PHA-stimulation.

**Figure 5: Neutral lipid ORO staining of skin fibroblasts and PBMCs from controls and Alzheimer's disease patients.** A) Skin fibroblasts from an Alzheimer's patient (A2) and from an healthy individual (A1) stained for neutral lipid by the ORO method; B) PBMCs from four different Alzheimer's patients (B5-B8) and from four healthy subjects (B1-B4) stained for neutral lipid content by the ORO method; C) PBMCs from a diabetic Alzheimer's patient before (C1) and after one-month of in vivo administration of Pioglitazone (C2), stained for neutral lipid content by the ORO method.

**Figure 6: Neutral lipid content in PBMCs from healthy and scrapie-infected sheep with genotypes known to affect scrapie susceptibility.** PBMCs from sheep with the scrapie-resistant ARR/ARR genotype (A1, A2), from sheep with the scrapie-susceptible ARQ/ARQ genotype non disease-infected (ARQ/ARQ-, panels A3, A4) and PBMCs from a scrapie-infected sheep with the scrapie-susceptible genotype (ARQ/ARQ+, panels A5-A7), were stained for neutral lipid content by the ORO method at O(A1, A3, A5) and 24 hours (A2, A4, A6) after PHA stimulation. PHA-stimulated PBMCs from the scrapie-infected, scrapie-susceptible genotype (ARQ/ARQ+) sheep were also treated with everolimus (EVE, 50 nM) for 24 hours (A7). Sheep PBMC samples were obtained and cultured as described in Material and Methods. A) representative images of ORO-stained PBMCs from an ARR/ARR genotype sheep, a non infected scrapie-susceptible genotype
sheep (ARQ/ARQ-) and a scrapie-infected scrapie-susceptible genotype sheep (ARQ/ARQ+).

B) amount of red stain per cell; values are the mean of ten different sheep of each genotype. Quantification of the intensity of the lipid-bound red color was determined by densitometric analysis through the Scion Image software (NIH). Means ± SE for triplicate determinations are presented. Statistical significance was determined by nonparametric Mann-Whitney test. P <0.05 was the criterion for significance. AU experiments were repeated at least twice. *P<0.05 vs ARR/ARR.

Figure 7: Neutral lipid content in serum-stimulated skin fibroblasts from sheep with the scrapie-resistant ARR/ARR genotype, untreated and treated in vitro with the modulators of cholesterol homeostasis everolimus, pioglitazone, progesterone and Sandoz 58035. Skin fibroblasts from sheep with the ARR/ARR scrapie-resistant genotype were serum-stimulated in the absence (AI-A3) or presence of everolimus (EVE, 50 nM, B1-B3), pioglitazone (PIO, 40 µM, C1-C3), progesterone (PG, 20 µM, D1-D3), Sandoz 58035 (SaH, 40 µM, E1-E3). At 24, 48 and 72 hours after FCS stimulation, cells were stained for neutral lipid content by the ORO method. Sheep skin fibroblasts were obtained and cultured as described in Material and Methods.

A) representative images of ORO-stained skin fibroblasts from an ARR/ARR sheep.

B) amount of red stain per cell; values are the mean of seven ARR/ARR sheep.

Quantification of the intensity of the lipid bound red color was determined by densitometric analysis through the Scion Image software (NIH). Means ± SE for triplicate determinations are presented. Statistical significance was determined by nonparametric Mann-Whitney test. P <0.05 was the criterion for significance. All experiments were repeated at least twice. *P<0.05, 72 hours, everolimus vs untreated ARR/ARR.

Figure 8: Neutral lipid content in serum-stimulated skin fibroblasts from non-infected sheep with the scrapie-susceptible ARQ/ARQ genotype (ARQ/ARQ-), untreated and treated in vitro with the modulators of cholesterol homeostasis everolimus, pioglitazone, progesterone and Sandoz -58035. Skin fibroblasts from non-disease infected sheep with the scrapie-susceptible ARQ/ARQ genotype (ARQ/ARQ-) were serum stimulated in the absence (AI-A3) or presence of everolimus (EVE, 50 nM, B1-B3), pioglitazone (PIO, 40 µM, C1-C3), progesterone (PG, 20 µM, D1-D3), Sandoz 58035 (SaH, 40 µM, E1-E3). At 24, 48 and 72 hours after FCS stimulation, cells were stained for neutral lipid content by the ORO method.
A) representative images of ORO-stained skin fibroblasts from an ARQ/ARQ sheep.
B) amount of red stain per cell; values are the mean of nine ARQ/ARQ sheep. Quantification of the intensity of lipid bound red color was determined by densitometric analysis through the Scion Image software (NIH). Means ± SE for triplicate determinations are presented. Statistical significance was determined by nonparametric Mann-Whitney test. P <0.05 was the criterion for significance. All experiments were repeated at least twice. *P<0.05, 72 hours, all drugs vs ARQ/ARQ- untreated.

Figure 9: Neutral lipid content in sejpijn stimulated skin fibroblasts from scrapie-infected ARQ/ARQ sheep, untreated and treated in vitro with the modulators of cholesterol homeostasis everolimus, pioglitazone, progesterone and sandoz 58035.

Skin fibroblasts from scrapie-infected sheep (ARQ/ARQ genotype) were serum-stimulated in the absence (A1-A3) or presence of everolimus (EVE, 50 nM, B1-B3), pioglitazone (PIO, 40 μM, C1-C3), progesterone (PG, 20 μM, D1-D3), Sandoz 58035 (SaH, 40 μM, E1-E3). At 24, 48 and 72 hours after FCS stimulation, cells were stained for neutral lipid content by the ORO method.

A) representative images of ORO stained skin fibroblasts from a scrapie-infected ARQ/ARQ sheep.
B) amount of red stain per cell; values are the mean of ten ARQ/ARQ sheep. Quantification of the intensity of lipid-bound red color was determined by densitometric analysis through the Scion Image software (NIH). Means ± SE for triplicate determinations are presented. Statistical significance was determined by nonparametric Mann-Whitney test. P <0.05 was the criterion for significance. AU experiments were repeated at least twice. *P<0.05, 48 and 72 hours, all drugs vs untreated)

Figure 10: Isoboles of the anti-prion activity of chlorpromazine in combinations with the cholesterol modulators everolimus (A), pioglitazone (B), progesterone (C), verapamil (D), sandoz 58035 (E) and cyclosporin A (F). Scrapie-infected mouse neuroblastoma cells were treated with different concentrations of the indicated cholesterol modulators for 8 hours prior to the addition of chlorpromazine. After 4 days from seeding, cells were lysed and processed for detection of the PK-resistant PrP by the dot blot method.

The amount of PK-resistant PrP was determined by densitometric analysis of dot blot autoradiograms through the Image J software (NTH). EC_{50}S were calculated for each inhibitor alone and in combination with each of the other drugs, and then plotted according to the isobole method (Suhnel, Antiv Res 1990, 13, 23-40) which identifies synergistic,
additive or antagonist interaction. EC_{50}'s are represented by the triangle symbol. Fractional Inhibitory Concentration indices (FIC) for quantification of synergism or antagonism were calculated for each drug combination according to the equation: FIC = (EC_{50A}^{comb} / EC_{50A}^{alone}) + (EC_{50B}^{comb} / EC_{50B}^{alone}), and are the numbers shown in parenthesis. FIC Indexes: ≤ 0.7, synergism; 0.8-1.2, additive; >1.3, antagonism.

**Figure 11. Isoboles of the anti-prion activity of quinacrine in combination with the cholesterol metabolism/trafficking modulators** everolimus, pioglitazone, progesterone and verapamil. Scrapie-infected MNB cells were treated with different concentrations of the indicated cholesterol modulators for 8 hours prior to the addition of quinacrine. After 4 days from seeding, the cells were lysed and processed for detection of the PK-resistant PrP by the dot blot method. The amount of PK-resistant PrP was determined by densitometric analysis of dot blot autoradiograms through the Image J software (NIH). EC_{50}'s were calculated for each inhibitor alone and in combination with each of the other drugs, and then plotted according to the isobole method (Suhnel, Antiv Res 1990, 13, 23-40) which identifies synergistic, additive, or antagonist type of interaction. EC_{50}'s are represented by the triangle symbol. FIC indices were calculated as above and are the numbers indicated in parenthesis. FIC Indexes: ≤ 0.7, synergism; 0.8-1.2, additive; >1.3, antagonism.

**Figure 12. Isoboles of the anti-prion activity of Dextran sulphate (DS500) and Tannic Acid in combination with the cholesterol metabolism/trafficking modulators** everolimus, pioglitazone, progesterone, verapamil, and sandoz 58035. Scrapie-infected MNB cells were treated with different concentrations of the indicated cholesterol modulators for 8 hours prior to the addition of the prion inhibitors. After 4 days from seeding, the cells were lysed and processed for detection of the PK-resistant PrP by the dot blot method. The amount of PK-resistant PrP was determined by densitometric analysis of dot blot autoradiograms through the Image J software (NIH). EC_{50}'s were calculated for each inhibitor alone and in combination with each of the other drugs, and then plotted according to the isobole method (Suhnel, Antiv Res 1990, 13, 23-40) which identifies synergistic, additive, or antagonist type of interaction. EC_{50}'s are represented by the triangle symbol. FIC Indices were calculated as above, and are the numbers indicated in parenthesis. FIC Indexes: ≤ 0.7, synergism; 0.8-1.2, additive; >1.3, antagonism.

**MATERIALS AND METHODS**

*Patients and Cells Source*
Individuals with clinical diagnosis, or at risk of AD. The clinical diagnoses of AD were made according to the NINCDS-ADRDA criteria. Additional measures included the MMSE and Dementia Severity Rating Scale (DSRS). Routine laboratory studies, including magnetic resonance imaging, were performed to exclude other causes of cognitive impairment. The Reisberg Global Deterioration Scale (GDS) was used to indicate the severity of the cognitive impairment of AD patients. Abnormal GDS levels start from level 3 and maximal deterioration grade corresponds to level 7. Elderly subjects with no cognitive decline were recruited in affiliated hospitals and served as healthy controls. PBMCs were collected from peripheral blood samples. The dermal biopsies were obtained from the upper forearm of the subjects by a 2-mm punch after local anesthesia with 2% xylocaine.

Eighteen patients with chronic lymphocytic leukemia (CLL) (aged 45-65 years) and twelve patients with acute lymphocytic leukemia (ALL) (aged 40-60 years) were recruited at diagnosis in local hospitals. Fifteen healthy age-matched subjects were also recruited as controls. Ten patients (7 with CLL and 3 with ALL) were randomly chosen to perform kinetic and molecular analyses. Informed written consent was obtained from all patients and healthy controls before initiating the study according to the policies of the hospitals Institutional Review Boards.

All subjects enrolled in the various studies did not receive any pharmacological treatment prior to blood or punch sampling.

Sheep

10 naturally scrapie-affected Sarda breed sheep with the scrapie-susceptible ARQ/ARQ genotypes and 10 experimentally scrapie-infected sheep with ARQ/ARQ genotype were used (all named as ARQ/ARQ+). All sheep were at the terminal clinical stage of the disease. The sheep were euthanized with a barbiturate followed by 4 ml of embutramide and mebenzonico-iodide (Hoechst Roussel Vet). Peripheral blood samples and skin biopsies were collected from the animals. The brains were collected and transverse sections taken for PrPSc detection by Western Blot. The same procedure was performed on 10 scrapie-resistant ARR/ARR genotype sheep and 10 ARQ/ARQ scrapie-free sheep (ARQ/ARQ+).

Isolation of PBMCs and Fibroblasts

Mononuclear cells were collected from peripheral blood of patients and controls and separated by Ficoll-Hypaque density gradient. After extensive washings, cells were
resuspended (IxIO\(^6\) cells/ml) in RPMI-1640 with 10% FCS and incubated overnight. For determinations, 2x10\(^5\) cells/ml nonadherent cells were incubated with phytohemagglutine (PHA, 10 μg/ml, cat. number L8902, SIGMA) at 37°C in RPMI-1640 supplemented with 10% FCS. Viability was evaluated after a time course by counting cells using trypan blue exclusion. Cells were harvested at different time points following incubation.

For fibroblasts isolation, biopsies were plated into 6 well plates for 2 hours. After adhesion for 2 hours, a few drops of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Lab NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and fungizone (Life Technologies, Inc.) covering each fragment, were added. The next day, the tissue fragments were covered with culture medium and maintained in a humidified incubator (37°C, 5% CO\(_2\)). The medium was changed every 2 days. After 5 to 6 days, fibroblasts begun to proliferate from the fragment margin (“halo of cells”) and create a monolayer. The outgrowing cells were morphologically consistent with fibroblasts by their characteristic spindle-shaped. After 4 weeks, fibroblasts were purified by repeated trypsinization (trypsin-EDTA-0.05%/0.02%) and passaging to achieve a homogenous population of spindle cells. Purified fibroblasts were washed twice with sterile PBS and centrifuged. IxIO\(^6\) cells were then seeded into 25 cm\(^2\) culture flask and grown to confluence. At this time cells were used for "in vitro" staining experiments, or resuspended into cryopreservation medium at a density of 1x10\(^7\) cells/ml. After swift freezing, fibroblasts were transferred into liquid nitrogen for long-term storage. Based on the need of determinations cryopreserved cells were removed from the liquid nitrogen tank and cultured as described above.

For determinations of CE content, skin fibroblasts were plated at a density of 5000 cell/cm\(^2\) in 6-well plates and then incubated for 48 hrs in MEM 199 containing 0.2% FCS to synchronise them in a quiescent state. Quiescent cells were stimulated to proliferate by adding FCS (10%) or, in alternative by adding a potent mitogen, such as β-FGF (β-fibroblast growth factor), and incubated for 12, 24, 48 and 72 hours in presence or in absence of different drugs.

AU determinations were made using fibroblasts at passages two - four.

**Lipid Staining**

For neutral lipid staining, isolated PBMC and skin fibroblasts were cultured as described above. At different times of incubation, the cells were washed three times with PBS and fixed by soaking in 10% formalin. The cells were treated with isopropyl alcohol (60%),
washed, and the nuclei and the intracellular neutral lipid droplets were then stained with Mayer's hematoxylin solution and Oil Red O (ORO, Sigma, O-0625), respectively. The detection of cytoplasmic CE by ORO staining is performed according to the following procedure: an aliquot of 1 x 10⁶ cells is transferred to a round bottom borosilicate tube, fixed with 10% formalin for 30 min and centrifuged at 500 rpm. The cell pellet is resuspended and stained with 1 ml of ORO (1:200 v/v in isopropyl alcohol) for 10 min at room temperature under continuous agitation. The excess ORO stain is discarded and the cells are counterstained with Mayer's Hematoxylin. An aliquot of stained cells (10 µl) is placed on a glass slide and inspected by light microscopy and photographed. The spectrophotometric quantification of lipid-bound ORO is performed according to the following procedure: the remaining cells are treated with 3 volumes of a mixture of chloroform/methanol (2:1 v/v) and vortexed to extract cell lipids. The tube is then centrifuged for 10 min at 500 rpm at room temperature to separate the chloroformic phase which is collected and transferred to a quartz cuvette. The optical absorbance of the chloroformic phase is then measured at 520 nm (the maximal optical absorbance of ORO). Absorbance values are plotted against a standard curve and expressed as mmol of ORO per number of cells.

Alternatively, the stained cells were examined and photographed under the light microscope. Lipid-bound ORO was quantified in intact cells by the Scion image analysis software (NIH Image 1.63 Analysis Software program).

Testing for PrPsc Inhibitory Activity in Cell Cultures

Approximately 20,000 mouse neuroblastoma cells (MNB-N2a) were infected with the RML or the 22L strains of mouse adapted scrapie (45, 46) and 100 µl of medium were added to each well of a Costar 3595 flat-bottom 96-well plate with a low evaporation lid (Corning Inc., Corning, N. Y.). The cells were allowed to settle overnight before test compounds were added. The stock solutions of test compounds were diluted in DMSO and then in phosphate-buffered saline (PBS) prior to being introduced to the cell medium. Five-microliter solutions were added to the cell medium. DMSO concentrations in the cell medium were never higher than 0.5% (vol/vol). After addition of compounds, cells were incubated for 4 days at 37°C in CO₂ incubator before being lysed. Prior to cell lysis, cell cultures were checked for cytotoxicity, bacterial contamination, and cell density compared to controls. After removal of the medium, 50 µl of lysis buffer was added to each well. Lysis buffer was composed of 0.5% (wt/vol) TritonX-100, 0.5% (wt/vol) sodium
deoxycholate, 5mM Tris-HCl (pH 7.4 at 4°C), 5mM EDTA, and 150mM NaCl. 5 min after the addition of the lysis buffer, 25 µl of proteinase K (PK, 0.1 mg/ml; Calbiochem) in Tris-buffered saline (TBS) was added to each well and incubated at 37°C for 50 min. A total of 225 µl of ImM Pefabloc® (Roche, Cat. Number 1 429 876) was added to each well to inhibit the PK activity. 250 µl of ImM Pefabloc® were also added to samples that were not PK treated.

High-throughput Measurement of PrPsc by a Dot Blot Procedure
A 96-well dot blot apparatus (Scheleicher & Schuell) was set up with a 4.5-µm-pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore), and each dot was rinsed with 500 µl of TBS. Under vacuum, the lysed and PK-treated samples were added to the apparatus over the PVDF membrane and rinsed with 500 µl of TBS. The PVDF membrane was removed and covered with 3M GdnSCN (Fluka) for 10 min at ambient temperature. GdnSCN was removed by five PBS rinses, and the membrane was blocked with 5% (wt/vol) milk - 0.05% (vol/vol) Tween 20 (Sigma) in TBS (TBST-milk) for 30 min. An appropriate dilution of monoclonal antibody 6B10, an immunoglobulin G2a antibody reactive against mouse, hamster, elk, and sheep PrP in immunoblotting assays and enzyme-linked immunosorbent assays, or 8 µg of purified anti-PrP mouse monoclonal antibody 6H4 (Prionics, cat. number 01-010) in 15 ml of TBST-milk was incubated with the membrane for 60 min. After TBST rinsing, a solution of -500 ng of an alkaline phosphatase-conjugated goat anti-mouse antibody (Zymed, cat. number 81-6522) in 15 ml of TBST-milk was added and incubated for 45 min. After additional TBST rinsing, the membrane was treated with a chemiofluorescence agent (Attophos, Amersham) for 10 min, allowed to dry, and then scanned with Storm Scanner (Molecular Dinamics). The intensity of the PrP signal from each well was quantified by using ImageQuant software (Molecular Dinamics). Each 96-well plate had six untreated control wells (negative controls) and six wells treated with the reference inhibitor of PrPsc accumulation, quinacrine (positive control).

Intracellular Lipid Content
For lipid cell content determinations, neutral lipids extracted from isolated cultured PBMCs and skin fibroblasts with cold acetone, are separated by thin layer chromatography (TLC). Free cholesterol (FC), cholesterol esters (CE), triglycerides (TG) and phospholipids (PH) mass are determined by enzymatic standard assay methods.

Plasma Lipid Testing
Heparinized plasma specimens are collected for lipid testing after an overnight fast and analyzed on the same day. Total cholesterol (TC), triglycerides (TG) and phospholipid (PL) levels are determined enzymatically (Boehringer Mannheim Diagnostics, Indianapolis, IN). High-density lipoprotein cholesterol (HDL-C) are determined after precipitation of the apolipoprotein B (Apo-B) containing particles by magnesium chloride and dextran sulfate.

**Drugs**


**EC50 and FIC calculation**

EC50s were calculated for each inhibitor alone and in combination with each of the other drugs, and then plotted according to the isobole method (Suhnel, Antiv Res 1990, 13, 23-40) which identifies synergistic, additive or antagonist interaction. EC50s are represented by the triangle symbol. Fractional Inhibitory Concentration indices (FIC) for quantification of synergism or antagonism were calculated for each drug combination according to the equation: FIC = (EC50A comb/ EC50A alone) + (EC50B comb/ EC50B alone), and are the numbers shown in parenthesis. FIC Indexes: ≤0.7, synergism; 0.8-1.2, additive; >1.3, antagonism.

**Statistics**

Statistical significance was determined by nonparametric Mann-Whitney test. P <0.05 was the criterion for significance.

**RESULTS**

**Alteration of cholesterol homeostasis in neoplastic and atherosclerotic cells**

Table 6 shows the lipid content in primary Acute and Chronic Lymphocytic Leukaemia (ALL and CLL, respectively) PBMC, and the lipid profiles in serum from patients with CLL (18 patients, ages 45-65 years), and ALL (12 patients, ages 40-60 years) at diagnosis. Age-matched healthy subjects (n = 15) were used as controls. The authors found that constitutive cholesterol ester levels are higher in leukemia cells than in controls. A strong decrease in the FC : CE molar ratio (1.1 in CLL and 0.85 in ALL, vs. 3.6 in controls) is
also observed in leukemia cells. No significant changes in other cellular lipid parameters are seen. HDL-C are significantly reduced (P < 0.05) in leukemia patients, compared to age-matched healthy controls. Total serum cholesterol (TC), LDL-C, TG, and PL levels are not significantly different between control subjects and tumor patients, although a trend toward hypocholesterolemia and hypertriglyceridemia is observed in the latter group (data not shown).

TABLE 6: Correlation between intracellular free and esterified cholesterol and plasma HDL cholesterol in proliferative diseases. Numbers in parenthesis are the ratio FC:CE.

<table>
<thead>
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<th>Proliferative Disease</th>
<th>FC</th>
<th>CE</th>
<th>HDL-Cholesterol</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>µg/10^6 cells</td>
<td>mg/dl</td>
<td></td>
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<tr>
<td>ALL</td>
<td>1.7</td>
<td>2.0 (0.85)</td>
<td>7-20</td>
</tr>
<tr>
<td>CLL</td>
<td>2.0</td>
<td>1.8 (1.1)</td>
<td>12-27</td>
</tr>
<tr>
<td>Controls</td>
<td>2.5</td>
<td>0.7 (3.6)</td>
<td>40-60</td>
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These results support the notion that changes in the lipid content, mainly in the levels of cellular cholesterol esters and plasma HDL-C, represent an identifiable profile for proliferative diseases.

To reinforce these results, the authors analyzed the cytoplasmic lipid content in normal and leukemic PBMCs, using the ORO staining method.

As shown in figure 3A at time 0 only leukemic PBMCs are positively stained (as indicated by the presence of red spots in cells shown with the arrows), while 24h and 48 h after PHA stimulation control cells also become positive. The intensity of the staining is proportional to the amount of cholesterol esters. The figure 3A shows that lipid accumulation is higher in leukemic cells than in control cells at all time points considered. The results shown are representative of 7 different patients and 7 control samples. Moreover, ACAT-I mRNA levels are higher, in leukemic patients PBMCs compared to healthy controls, whereas neutral cholesterol ester hydrolase (nCEH) and ABCAl mRNAs are lower in leukemic patients PBMCs compared to healthy controls (Fig. 3B). Similar results are obtained in mitogen-induced proliferating PBMCs from atherosclerotic patients compared with mononuclear cells from healthy control subjects (Fig. 4).
Alteration of cholesterol homeostasis in skin fibroblasts and PBMCs from AD patients

Our studies on intracellular cholesterol distribution reveal that, when compared to cells from healthy donors, peripheral cells from AD patients have an increased capacity to synthesize and accumulate CE, as determined by the ORO staining method. Both cultured skin fibroblasts from dermal biopsy (Fig. 5A, right) and isolated peripheral blood mononuclear cells (PBMCs) (Fig. 5B, right) from AD patients clearly show markedly higher contents of CE, as compared to control cells from healthy subjects (Fig. 5A and B left). In vivo administration (one-month treatment) to a diabetic AD patient of the insulin sensitizing drug pioglitazone, which also inhibits cholesterol esterification, readily leads to a reduction of CE accumulation in PBMCs (Fig. 5C; before treatment, left; after treatment, right). These in vivo results support the effects observed in vitro and confirm that compounds able to modulate cholesterol esterification are beneficial to patients affected by conformational or proliferative diseases.

Alteration of cholesterol homeostasis in PBMCs from scrapie-susceptible sheep

Similar observations can be made in PBMCs (Fig. 6) from scrapie-susceptible (ARQ/ARQ genotype) sheep, both uninfected and scrapie-infected, when compared to PBMCs from scrapie-resistant animals (ARR/ARR genotype). Intracellular CE levels are significantly higher (Fig. 6B) in the scrapie-susceptible genotype (ARQ/ARQ) animals (both uninfected and scrapie-infected), compared to the PBMCs of the scrapie-resistant (ARR/ARR genotype) sheep (Figs. 6A and B).

In agreement with the authors previous findings on the correlation between cell growth and CE accumulation (26), intracellular CE levels increase in the cells of all genotypes (Fig. 6B) after administration of a growth stimulus, such as PHA. Interestingly, after a 24 hour PHA-stimulation, CE levels in the cells of the scrapie-susceptible genotype increase up to 1.5 fold, whereas in the cells of the scrapie-resistant sheep a lower increase is observed.

When PBMCs from the scrapie-infected (ARQ/ARQ+) sheep are PHA-stimulated in the presence of the antiproliferative agent Everolimus (EVE, 50 nM), the CE content is reduced to the levels seen in the cells from the ARR/ARR animals.

Effect of modulators of cholesterol metabolism/trafficking on the intracellular accumulation of CE in sheep skin fibroblasts.

The authors then evaluated the ability of drugs such as progesterone and verapamil (targeting cholesterol transport from plasma membrane to ER by the MDRI-PgP), or pioglitazone, everolimus, and Sandoz 58035 (indirect regulators of intracellular cholesterol.
trafficking), to interfere with cholesterol esterification. To this end, skin fibroblasts from healthy ARR/ARR (Fig.7) and ARQ/ARQ (Fig.8) sheep, and from scrapie-infected ARQ/ARQ (Fig.9) animals, were cultured and stimulated with 10% serum in the absence or in the presence of everolimus (EVE, 50 nM), pioglitazone (PIO, 40 µM), progesterone (PG, 20 µM), Sandoz 58035 (SaH, 40-µM). At indicated times, cells were stained for neutral lipid and the contents of the latter were determined. Similarly to what happens in PBMCs, the following events take place also in skin fibroblasts: i) CE levels are higher in cells from sheep with the scrapie-susceptible genotype (ARQ/ARQ) infected or not by the disease compared to cells from sheep with the scrapie-resistant genotype (ARR/ARR) ; ii) following a growth stimulus, the CE increase is significantly greater (up to 2-fold) in cells with the ARQ/ARQ genotype; iii) in cells from the scrapie-infected ARQ/ARQ+ sheep, all cholesterol modulators effectively reduce the levels of intracellular CE. In cells from ARR/ARR genotype sheep, only everolimus inhibits CE accumulation at 72 hours. In non-infected ARQ/ARQ- cells, all drugs display inhibitory activity at 72 hours after serum stimulation, hi scrapie-infected ARQ/ARQ+ cells all drugs display inhibitory activity at 48 and 72 hours after serum stimulation.

**Anti-prion effect of modulators of cholesterol metabolism/trafficking alone and in combination with known prion inhibitors.**

Cholesterol modulators were tested in persistently scrapie-infected mouse neuroblastoma (MNB) cells to evaluate their ability to affect the protein misfolding process, as determined by the reduction of intracellular PK-resistant prion protein. Single treatments with progesterone, verapamil, pioglitazone, everolimus or Sandoz 58035 result in a selective anti-prion activity (EC_{50} in the range 1-40 µM), comparable to that obtained under the same experimental conditions with the known anti-prion compounds quinacrine, chlorpromazine, dextran sulphate (DX500), tannic acid, amphotericin B and congo red (Table 7). Everolimus is the most potent drug, with an EC_{50} of 1 µM, whereas pioglitazone and Sandoz 58035 are the least potent, with an EC_{50} of 40 µM.

**TABLE 7:** Comparative anti-prion activity of intracellular cholesterol modulators and of known prion inhibitors.

<table>
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<tr>
<th>Cholesterol modulator</th>
<th>PrP-Sc EC_{50} [µM]</th>
<th>Prion inhibitor</th>
<th>PrP-Sc EC_{50} [µM]</th>
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25
Combined treatments with one of the cholesterol modulators (progesterone, verapamil, pioglitazone, everolimus or Sandoz 58035) and one of the above anti-prion agents result in different types of interaction according to the prion inhibitor considered and to the cholesterol modulator concentration used. The most favourable anti-prion effect is observed when the cells are pre-treated for 8 hours with one of the above cholesterol modulators prior to the addition of the anti-protein misfolding agent. The types of inhibition range from strong synergistic to additive effects (Figs. 10-12). At each dose tested, all cholesterol modulators act synergistically with the anti-prion effect of chlorpromazine (Fig. 10) and quinacrine (Fig. 11) by reducing their EC₅₀ by 10- to 25-fold, and 2- to 10-fold, respectively. On the other hand, combinations of cholesterol modulators with DX500, tannic acid, and amphotericin B (not shown), result in additive anti-prion effects (Fig. 12).

Although further experiments are needed to unravel the molecular mechanism of the synergistic anti-prion effect observed in the combinations involving quinacrine and chlorpromazine, it seems likely that the known ability of both drugs to induce a redistribution of the intracellular cholesterol reinforces the inhibitory effect of the cholesterol modulators on the overall protein misfolding process via restoration of physiologic membrane levels of cholesterol.

In conclusion, the presence of high intracellular CE levels and, consequently, of low plasma membrane FC is indicative of a cell phenotype predisposing to several pathological conditions (Fig. 2A,B,C), including the formation/deposition of structurally aberrant proteins in conformational diseases (Fig. 2C). Recent studies showing that block of intracellular CE accumulation by ACAT inhibitors prevents generation of the beta-amyloid peptide in cell-based models of AD (41), as well as accumulation (by 88% - 99%) of cerebral amyloid plaques in a mouse model of AD (42), strongly support the authors's conclusions.
In addition, the authors had already shown that the above parameters also correlate with the ageing phenomenon (43). In fact, using the rat model, it had been proven that, as the age of animals increases, the levels of MDR1- Pgp and ACAT mRNAs significantly increase in several organs (liver, brain, hearth, kidney, arteries), while the levels of mRNA for nCEH and caveolin-1 expression significantly decrease, leading to intracellular CE accumulation and to decreased levels of plasma membrane FC, coupled with low levels of circulating HDL-cholesterol (43). The present results demonstrate that the above correlations can also be found in PBMCs and skin fibroblasts, thus allowing a prompt evaluation of the risk of developing the above age-related diseases.

REFERENCES

5. Lange, Y., Ye, J., and Chin, J. J Biol Chem 1997; 272, 17018-17022
19. Dessi S., Batetta B., Pulisci D., Spaho O., Anchisi C., Tessitore L., Costelli P.,
Baccino F.M., Aroasio E., Pani P. Cancer 1994; 73, 253-258.
20. Dessi S., Batetta B., Spano O., S-anna F., Tonello M., Giachino M., Tessitore L.,
RR, Amat di S.Filippo C., Vargiu L., Marceddu T., Sanna L., La Colla P., Dessi S.
22. Dessi, S., Batetta, B., Pani, A., Spano, O., Sanna, F., Putzolu, M., Bonatesta, R.,
Piras, S., and Pani, P. Biochem J 1997; 321, 603-608
23. Batetta, B., Pani, A., Putzolu, M., Sanna, F., Bonatesta, R.R., Piras, S., Spano, O.,
Mulas, M.F., and Dessi, S. Cell Prolif 1999; 32, 49-61
24. Batetta, B., Mulas, M.F., Petruzzo, P., Putzolu, M., Bonatesta, R.R., Sanna, F.,
Kluwer Academic Press publishers, 2004
30. Selkoe, DJ. Physiol Rev 2001; 81, 741-766
35. Pavlov OV, Bobryshev YuV, Balabanov YuV, Ashwell K. Neurotoxicol Teratol.
1995; 17, 31-9
21, 2097-101.
CLAIMS

1. Use of at least one compound modulating the pathways leading to cholesterol esterification and/or exerting an antiproliferative activity for the preparation of a medicament for the treatment and/or prophylaxis of proliferative and/or conformational diseases and/or of early ageing.

2. The use according to claim 1 wherein the compound is selected from the group of inhibitors of: cholesterol esterification, MDRI-Pgp and ACAT.

3. The use according to any previous claims wherein the compound is selected from the group of: progesterone, everolimus, pioglitazone, avasimibe, verapamil, cyclosporin A, Sandoz 58-035.

4. Use of the compound according to any previous claims wherein the medicament further comprises at least one compound endowed with antiproliferative and/or anti-protein misfolding activity.

5. The use according to claim 4 wherein the compound endowed with antiproliferative and/or anti-protein misfolding activity belongs to the group as listed in Tables 3, 4A and 4B.

6. The use according to any previous claims wherein the proliferative disease is selected from the group of: atherosclerosis, restenosis after angioplastic, hematologic neoplasms, solid tumors.

7. The use according to claim 6 wherein the hematologic neoplasms are selected from the group of: Hodgkin and non-Hodgkin lymphomas, acute and chronic leukemias, eritroleukemias, mielomas or polycytemias.

8. The use according to claim 6 wherein the solid tumors are selected from the group of: brain, headneck, nasopharyngeal, breast, ling, gastrointestinal, colon, kidney or liver tumor.
9. The use according to claims 1 to 5 wherein the conformational disease is selected from the group of: prion-related disorders, Alzheimer's disease, Parkinson's disease, Huntington disease, amyotrophic lateral sclerosis or spinocerebellar degenerations.

10. The use according to claim 9 wherein the prion-related disorders are selected from the group of: Creutzfeldt-Jacob disease, new variant Creutzfeldt-Jacob disease, Gerstmann-Straussler Sheinker syndrome, fatal familial insomnia, bovine spongiform encephalopathy, scrapie, chronic wasting disease, feline spongiform encephalopathy.

11. A pharmaceutical composition comprising an effective amount of at least one compound modulating the pathways leading to cholesterol esterification, an effective amount of at least one compound endowed with antiproliferative and/or anti-protein misfolding activity, and suitable diluents and/or adjuvants and/or excipients.

12. The pharmaceutical composition according to claim 11 wherein the compound modulating the pathways leading to cholesterol esterification is selected from the group of inhibitors of: cholesterol esterification, MDR1-Pgp and ACAT.

13. The pharmaceutical composition according to claim 11 or 12 wherein the compound modulating the pathways leading to cholesterol esterification is selected from the group of: progesterone, everolimus, pioglitazone, avasimibe, verapamil, cyclosporin A, Sandoz 58-035.

14. The pharmaceutical composition according to claims 11 to 13 wherein the compound endowed with antiproliferative and/or anti-protein misfolding activity belongs to the group as listed in Table 3, 4A and 4B.

15. The pharmaceutical composition according to claims 11 to 14 wherein the compounds are encapsulated in nanospheres.
Fig. 1
Fig. 2
Fig. 2bis
Fig. 3
Fig. 4

Time after PHA stimulation

Control 1
Control 2
Patient 1
Patient 2
Patient 3
Patient 4
Fig. 6
Fig. 7
Fig. 8
Fig. 10
Fig. 11
Fig. 12
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/IT2007/000109

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Date of mailing of the international search report
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Venturini, Francesca
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