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(54) Title: TREATMENT OF CANCER

(57) Abstract: The present invention includes the use of at least one agent which induces endoplasmic reticulum (ER) stress together with at least one agent which gives rise to inhibition of protein disulfide isomerase (PDI) or GRP78 function for the preparation of a medicament, more particularly a medicament for the treatment of cancer, and to pharmaceutical compositions comprising an effective amount of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of PDI or GRP78 function, and which together provide a therapeutic effect.

TREATMENT OF CANCER

The present invention relates to the use of agents for the treatment of cancer.

Agents useful for the treatment of cancer act by a variety of pathways, for example some compounds intercalate between strands of DNA thereby preventing replication, some compounds inhibit microtubule assembly thereby preventing cell division and some compounds interfere with the blood supply to a tumour.

Cancer cells acquire survival mechanisms which allow them to circumvent DNA damage, such as inactivation of the P53 gene family[1]. In normal cells, death can be induced as a result of a stress-signalling pathway activated by accumulation of misfolded or mutated proteins in the endoplasmic reticulum (ER)[2]. Chromosomal rearrangements during carcinogenesis can result in the accumulation of aberrant proteins in the ER[3], and therefore to survive the consequences of genomic instability, an increased homeostatic response to ER stress may be important in cancer cells.

Understanding ER stress has led to a greater understanding of the mechanisms of cell death and therefore potentially better drugs for treating/preventing a wide range of conditions/diseases.

Maniratanachote *et al*[4] investigated further the *in vitro* apoptotic cell death of various hepatic cell types reported by Bae and Song[5] as a result of exposure to the antidiabetic drug troglitazone (TRO).

Maniratanachote *et al*[4] were interested in the involvement of proteins whose up or down regulation correlated with the TRO induced toxic effects. Analysis of a protein spot separated by electrophoresis revealed two protein chaperones, immunoglobulin heavy chain binding protein (BiP or Grp78), and protein disulphide isomerase-related protein (PDIrp or ERp72). The findings suggest that TRO targets the ER and causes the overexpression of BiP, a chaperone in response to cytotoxicity i.e. BiP is overexpressed by the cell in an attempt to survive.

A second paper by Hetz *et al*[6] investigates the relationship between prion replication and induction of ER stress. Prion diseases are characterised by extensive neuronal apoptosis and accumulation of misfolded prion protein (PrP^{SC})[7]. Hetz identified a correlation between upregulation of the ER chaperone of the glucose-regulated protein Grp58 (also known as ERp57) and the accumulation of PrP^{SC}.

Hetz concluded that targeting GRP58 may have applications for developing novel strategies for treatment and early diagnosis of prion disease.

It has been found that certain chemotherapeutic agents induce apoptosis in cancer cells. Unlike most retinoids, fenretinide (N-(4-hydroxyphenyl)retinamide) induces apoptosis. Fenretinide has an increasingly important profile as a cancer preventative and chemotherapeutic agent and normal, non-cancerous cells are unaffected by it[8]. Therefore the inventors carried out further studies to characterise the mechanism of cancer cell death induced by fenretinide.

It was found that apoptosis was accompanied by the induction of the ER stress-associated transcription factor GADD153/CHOP[9]. This suggests that the activation of ER stress responses is a consequence of treating cells with fenretinde.

Certain types of tumours, in particular cutaneous melanoma, become 'bullet proof' against chemotherapeutic drugs due to an intrinsic resistance to cell death.

Thus, it is an object of the present invention to provide a chemotherapeutic regime which enhances the cell-killing effects of ER stress-inducing agents by manipulating ER stress responses in order to induce apoptosis.

According to a first aspect of the present invention there is provided the use of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of protein disulfide isomerase or GRP78 function, for the preparation of a medicament.

According to a second aspect of the present invention there is provided the use of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of protein disufide isomerase or GRP78 function, for the preparation of a medicament for the treatment of cancer.

It is to be understood that any agent giving rise to any degree of inhibition of protein disulfide isomerase and/or inhibition of GRP78 function is suitable for use in the present invention. Inhibition may be by any direct and/or indirect mechanism. Thus, the inhibition may comprise a reduction in PDI and/or GRP78 expression.

The inventors have studied the ER stress response in cancer cells, in particular neuroblastoma and melanoma cells. When cells are subject to ER stress the production of a number of ER stress protein chaperones is induced in cancer cells. These proteins include ERp57, ERdj5, GRP78. Knockdown of these proteins *in vitro* results in an increase in the apoptotic response to ER stress inducing agents such as fenretinide.

The *in vitro* results were achieved using siRNA and were not expected to be suitable for *in vivo* knockdown of ER stress protein chaperones, since the use of RNA *in vivo* is in its infancy. Furthermore, there are problems with target specificity and therefore off-target effects. The use of RNA *in vivo* also provokes an interferon response thereby adversely affecting the immune system.

The inventors reasoned that a common property of ER stress-induced chaperones is that they are functionally and structurally related to the enzyme protein disulphide isomerase (PDI), and developed the novel idea that PDI inhibitors might have the same effect as siRNA-mediated knockdown of ERp57 or ERdj5 in increasing the cell death response to ER stress-inducing agents such as fenretinide.

It was then found that agents which give rise to inhibition of protein disulfide isomerase and/or GRP78 function in combination with agents which induce ER stress do in fact increase apopotosis in cancer cells.

These results suggest that agents which give rise to inhibition of PDI and/or GRP78 function make cancer cells more susceptible to ER stress and thus apoptosis occurs more readily.

The combination of the present invention is particularly suitable for the treatment of melanoma and neuroblastoma.

When treating melanoma and neuroblastoma, the agent giving rise to inhibition of PDI inhibits the activity of ERdj5 and ERp57.

Whilst any agent which induces ER stress is suitable for use with the present invention, fenretinide and velcade are two particularly preferred examples. Other known ER stress inducers include thapsigargin, Brefeldin A, tunicamycin, and AIF-4[20, 21].

Whilst any agent which gives rise to inhibition of PDI is suitable for use with the present invention, the antibiotic bacitracin and derivatives thereof are particularly preferred. Bacitracin is a known PDI inhibitor[22, 23]. Another known inhibitor of PDI function is scrambled RNase[22].

It will also be apparent that inhibitory antibodies, or active fragments thereof, may usefully be employed as inhibitors of PDI[22]. Inhibitory antibodies may be prepared by any of the methods well known to the skilled person.

Additionally or alternatively, agents which inhibit the expression of PDI are also suitable for use in the present invention.

With regard to inhibtion of GRP78 function, again, any inhibitory antibodies, or active fragments thereof, may be used.

Inhibitors of GRP78 expression may also be used in the present invention. WO 2006/101073 discloses a compound capable of inhibiting the induction of the

expression of GRP78 and named "prunustatin". Prunustatin has a structure similar to that of a known compound SW-163A and can be produced by *Streptomyces Violaceoniger* strain 4521-SVS3 or the like. This compound may be employed in the present invention. WO 03/044209 discloses a tetronic acid derivative versipelostatin, which inhibits GRP78 expression, is isolated from a metabolite of *Streptomyces versipellis* 4083-SVS6 strain. This compound may also be used in the present invention.

It is known that GRP78 can bind caspases thereby preventing activation of apoptosis. This caspase-binding function is dependent on the ATP-binding domain of GRP78[25] and abrogated by increases in dATP[26]. Thus, an agent which gives rise to inhibition of GRP78 function may comprise an agent which interferes with caspase binding by GRP78, for instance a purine nucleoside analogue, for example 2cdA[24]. Purine nucleoside analogues increase intracellular dATP.

According to a third aspect of the present invention there is provided the use of bacitracin for the preparation of a medicament for the treatment of cancer.

In vitro studies using a variety of cancer cell lines have shown that the percentage of apoptosis at least doubles when fenretinide and bacitracin are used together compared to fenretinide alone. Bacitracin on its own had no effect on these cancer cells. In fact, the percentage of apoptosis seen for the combination of agents is significantly greater than the sum of the percentage of apoptosis seen when each agent is used independently. This demonstrates that the two agents act synergistically.

According to a fourth aspect of the present invention there is provided a method for enhancing the efficacy of drugs which induce the apoptosis of tumour cells via ER stress comprising the steps of: administering a combination of at least one agent which induces ER stress and at least one agent which gives rise to inhibition of protein disulfide isomerase or GRP78 function.

The compounds may be administered simultaneously or sequentially depending upon the treatment regime.

Whilst it may be possible for agents to be administered as raw chemicals, it is preferable to present the compounds in a pharmaceutical composition.

Thus, according to a further aspect of the present invention there is provided a pharmaceutical composition comprising an effective amount of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of protein disulfide isomerase or GRP78 function, which together provide a therapeutic effect.

Such pharmaceutical compositions for medical use will be formulated in accordance with any of the methods well known in the art of pharmacy for administration in any convenient manner. The compounds will usually be admixed with at least one other ingredient providing a compatible pharmaceutically acceptable additive, carrier, diluent or excipient, and may be presented in unit dosage form.

The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The possible formulations include those suitable for oral, rectal, topical and parenteral (including subcutaneous intramuscular and intravenous) administration or for administration to the lung or other absorptive site such as the nasal passages.

All methods of formulation in making up such pharmaceutical compositions will generally include the step of bringing one and/or all of the agents into association with a carrier which constitutes one or more accessory ingredients. Usually, the formulations are prepared by uniformly and intimately bringing all of the agents into association with a liquid carrier or with a finely divided solid carrier or with both and then, if necessary, shaping the product into desired formulations.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tables or lozenges, each containing a predetermined amount of the compounds described herein; as a powder or granules; or a suspension in an aqueous liquid or non-aqueous liquid such as a syrup, an elixir, an emulsion or a draught. The compounds described herein may also be presented as bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tables may be prepared by compressing, in a suitable machine, the compounds described herein in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Moulded tables may be made by moulding, in a suitable machine, a mixture of the powdered compound of formula I with any suitable carrier. A syrup may be made by adding the compounds described herein to a concentrated, aqueous solution of a sugar, for example sucrose, to which may be added any desired accessory ingredients. Such accessory ingredient(s) may include flavourings, an agent to retard crystallisation of the sugar or an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol.

Formulations for rectal administration may be presented as a suppository with a usual carrier such as cocoa butter.

Formulations suitable for parental administration conveniently comprise a sterile aqueous preparation of the compounds described herein, which is preferably isotonic with the blood of the recipient.

In addition to the aforementioned ingredients, formulations of this invention, for example ointments, creams and such like, may include one or more accessory ingredients, for example a diluent, buffer, flavouring agent, binder, surface active agent, thickener, lubricant and/or a preservative (including an antioxidant) or other pharmaceutically inert excipient.

The agents of the present invention may also be made up for administration in liposomal formulations, which can be prepared by methods well-known in the art.

Therefore, the invention also includes the use of the agents hereinbefore defined for the manufacture of medicaments or pharmaceutical compositions for treating cancer, wherein the agents themselves provide an effective antitumour agent.

The present invention will now be described further by way of example only. The following examples and description of experiments serve further to illustrate the present invention.

As mentioned previously, the inventors have discovered that apoptosis by fenretinide is accompanied by the induction of the ER stress-associated transcription factor GADD153/CHOP. This suggests that the activation of ER stress responses is a consequence of treating cells with fenretinide, a previously unreported phenomenon.

This hypothesis was tested by screening a 24K microarray for the induction of ER-stress genes in SH-SY5Y neuroblastoma cells after 6 hours treatment with fenretinide. In additon to GADD153, four other genes associated with ER stress were induced greater than 2-fold: ERdj5 (an ER-resident protein containing DnaJ and thioredoxin domains)[10]; ERp57 (GRP58; an ER-resident protein-disulfide isomerase)[11]; and calreticulum and calnexin (both ER-resident chaperones)[12].

The induction of these genes in SH-SY5Y cells in response to fenretinide was confirmed by Western blotting and reverse-transcriptase-polymerase chain reaction (RT-PCR) in independent experiments (Figs. 1A and 1B).

In order to confirm the findings, the response of SH-SY5Y cells to thapsigargin, a well-characterised inducer of ER stress[13], and fenretinide were compared.

These experiments were also performed using a different neuroectodermal tumour cell line, SK-Mel-110 human melanoma cells[10]. Thapsigargin and fenretinide induced cell death in both cell lines. However, as has been reported for other melanoma lines with respect to fenretinide sensitivity[15], SK-Mel-110 cells were more resistant than SH-SY5Y cells and needed 5-fold higher concentrations of either agent to induce comparable levels of cell death.

Table 1 shows a comparison of the results.

Treatment agent	Mean % apoptosis after 24 hour treatment (± 95% confidence)	
	SH-SY5Y cells	SK-Mel-110 cells
Control (ethanol)	4(±2.4)	4(±2.4)
3 μM fenretinide	20.5(±5.7)	-
15 μM fenretinide	-	22(±5)
1.5 μM thapsigargin	31(±5)	-
7.5 μM thapsigargin	-	24(±6.2)
	NB: ANOVA, $F_{2,6}$=162.6, P<0.0001	NB: $F_{2,6}$=97.1, P<0.0001

NB: all pair wise comparisons after Bonferroni correction, P≤0.0013

Table 1

The induction of eIF2α phosphorylation and splicing of Xbp-1 mRNA are the events used to define ER stress. In SH-SY5Y and SK-Mel-110 cell, phosphorylation of eIF2α was induced by thapsigargin (1.5 μM and 7.5 μM, respectively) and within 15 minutes of treatment with fenretinide (3 μM and 15 μM, respectively) (Fig. 1C). Under similar conditions for both cell lines the splicing of Xbp-1 mRNA was induced within 6 hours of thapsigargin treatment and within 6-18 hours after treatment with fenretinide (Fig. 1D). These data indicate that fenretinide induces ER stress in SH-SY5Y and SK-Mel-110 neuroectodermal tumour cells.

Previous studies have shown that the specific 12-lipoxygenase inhibitor baicalein blocks ROS induction and apoptosis in response to fenretinide in SH-SY5Y cells[9]. Baicalein also blocked the induction of GRP78 and ERp57 protein and mRNA for ERp57 and ERdj5 in response to fenretinide in SH-SY5Y SK-Mel-110 cells (Fig. 1E and Fig. 1F), suggesting that fenretinide induces ER stress in these cells via the 12-lipoxygenase-dependent induction of ROS.

In order to determine whether the apoptotic response to fenretinide would be increased by knockdown of ER stress chaperones ERp57 and ERdj5, SH-SY5Y and SK-Mel-110 cells were treated with fenretinide after siRNA-mediated knockdown of expression of each gene. RT-PCR for ERp57 and ERdj5, confirmed that fenretinide-induced increase in mRNA levels was blocked by siRNAs for ERp57 and ERdj5, respectively, but not by the control siRNA (Fig. 2). After transfection with the ERp57 siRNA, that basal levels of ERp57 remained similar to control cells (Fig. 2) and similar results were obtained by Western blotting (data not shown); in contrast, one of the two ERdj5siRNAs used was efficient at knocking down ERdj5mRNA below the basal levels in control cells (Fig. 2). Under these conditions of ERp57 or ERdj5 knockdown, apoptosis of both cell lines in response to fenretinide was increased by at least 2-fold (Fig. 2).

These results suggest that inhibiting or down-regulating ER stress responses may enhance the cell-killing effects of ER stress-inducing agents such as fenretinide. ERp57 is a thiol-oxidoreductase chaperone of the PDI family and can be physically associated with calnexin and calreticulin, but in addition to the DnaJ and thioredoxin domains it is a chaperone that co-localises with PDIs, has a PDI-like domain and interacts with GRP78. Since effective ER stress responses may protect cancer stem cells from chemotherapeutic drug-induced apoptosis, the ER resident proteins ERdj5 and ERp57 may be targets for the development of novel chemotherapeutic strategies.

Having established that down-regulating components of homeostatic stress responses increased cell death in response to ER stress inducers, this suggested that this

approach may lead to new methods for enhancing the efficacy of drugs which induce the apoptosis of tumour cells via ER stress.

Various experiments were conducted to confirm this. The results are shown in the attached drawings.

The present invention will now be described further by way of example only and with reference to the accompanying drawings, in which:

Figure 1 shows various Western blots exemplifying the induction of ER-stress genes in response to fenretinide or thapsigargin in SH-SY5Y and SK-Mel-110;

Figure 2 shows various bar graphs exemplifying the siRNA-mediated knockdown of ERdj5 or ERp57 increased apoptosis in response to fenretinide in SH-SY5Y and SK-Mel-110 cells;

Figure 3 shows a flow cytometry histogram illustrating the cell cycle of SK-Mel-110 melanoma cells;

Figure 4 shows a flow cytometry histogram illustrating the cell cycle of SK-mel-110 melanoma cells following treatment with fenretinide;

Figure 5 shows various bar graphs illustrating the relationship between fenretinide and apoptosis in various melanoma cell-types;

Figure 6 shows various bar graphs illustrating the effect of a combination of fenretinide and bacitracin on apoptosis in various melanoma cell types;

Figure 7 shows a graph illustrating the induction of apoptosis in human A375 melanoma cells using bacitracin, velcade, or bacitracin plus velcade at a fixed dose of 15,000:1;

Figure 8 shows bar graphs illustrating the effect of an anti-GRP78 antibody on fenretinide- and velcade-induced apoptosis of CHL-1 cells;

Figure 9 shows bar graphs illustrating the effect of an anti-GRP78 antibody on fenretinide- and velcade-induced apoptosis of WM266-4 cells;

Figure 10 shows bar graphs illustrating the apoptotic effect on CHL-1 cells and WM266-4 cells of a combination of fenretinide and different concentrations of the purine nucleoside inhibitor 2cdA;

Figure 11 shows bar graphs illustrating the apoptotic effect on CHL-1 cells and WM266-4 cells of a combination of fenretinide with bacitracin, 2cdA, or bacitracin plus 2cdA; and

Figure 12 shows bar graphs illustrating the combined effect of bacitracin, anti-GRP78 antibody and 2cdA on fenretinide-induced apoptosis of CHL-1 and WM266-4 cells.

Figure 1

The induction of ER-stress genes in response to fenretinide or thapsigargin in SH-SY5Y and MK-Mel-110 cells. Fenretinide (Janssen-Cilag Ltd., Basserdorf, Switzerland) or thapsigargin (Sigma Chemical Co.) were added in ethanol or DMSO, respectively, to SH-SY5Y and SK-MEL110 cells[16] with an equal volume of vehicle used to treat control cells. ER stress genes induced by fenretinide in SH-SY5Y cells treated with or without 3μM Fenretinide for 6 h were identified from microarray analysis: cells were lysed using Trizol (Invitrogen Life Technologies, Carlsbad, CA) and total RNA extracted according to the manufacturer's instructions. Poly(A)+RNA was isolated by oligo-dT latex bead chromatography (Qiagen Inc, Valencia, CA USA). Reverse transcriptase, primed with poly(dT), was used to synthesize Cy3- and Cy5-labelled cDNA using the Cyscribe cDNA labelling kit (Amersham Biosciences UK Ltd, Amersham, UK). Labelled cDNA probes were hybridised to glass slide

micro-arrays containing 24,000 genes (Stanford University, USA) and detected using a Packard MicroArray scanner (Packard BioScience, Billerica, MA) with Quant Array sotfware. Experiments were performed in triplicate and expression confirmed by Western blotting and RT-PCR in separate experiments. A, Western blots of total protein, separated by electrophoresis through 12% SDS-PAGE gels (30 μg per track) and blotted onto nitrocellulose, extracted from SH-SY5Y cells (left-hand column) and SK-MEL-110 cells (right-hand column) treated with thapsigargin (Thap; 1.5 μM or 7.5 μM for SH-SY5Y or SK-Mel-110 cells, respectively) or Fenretinide (FenR; 3 μM or 15 μM, respectively) for 0, 6, 18 or 24 h. Blots were probed with antibodies to GADD 153 (Santa Cruz iotechnology Inc, Santa Cruz, CA, USA, diluted 1:1000), ERp57 (Stressgen, Victoria, BC, Canada, diluted 1@5000), calreticullinn (Stressgen, dilutee 1:2000), calnexin (Santa Cruz diluted 1:1000), GRP78 (Santa Cruz, diluted 1:1000) and, as a loading control, β-tubulin (Sigma diluted 1@5000)[9]. For detection, secondary peroxidase-conjugated antibodies (Jackson ImmunoResearch Inc, West Grove, PA) were diluted 1:5000 and visualized using the ECL Plus system (Amersham Biosciences UK)[17]. B, An antibody to ERdj5 was not available so RT-PCS was used to quantify ERdj5 and GAPDH (as a loading control) in RNA extracted from cells treated with thapsigargin or fenretinide. Note that the order of thapsigargin and fenretinide-treated samples is reversed compared with A. Cells were lysed and total RNA extracted with Trizol. A poly(dT) primer (1 mM) was used to generate cDNA from 2μg of RNA and human ERdj5 sequence was amplified with the primer pair GCCATTTTAGTGGGCACAGATCAGG and CAGCCAGCCAATACCAGCAGCA. Amplification of human GAPDH sequence was with the primer pairs GATATCGCCGCGCTCGTCGTCG and AGGTAGTCAGTCAGGTCCCGGC. For PCR reactions the number of cycles used for each primer pair was adjusted so that amplification remained within an approximately linear range. C, Western blots (as in A) probed using antibodies for eIF2α (Cell Signaling, diluted 1:1000) and phosphorylated eIF2α (P-eIF2α; Cell Signaling, diluted 1:1000). For this experiment, cells were treated with fenretinide for 0.25, 0.5, 1, 2 or 4 h, or thapsigargin for 4 h. Controls were at 0h, induction of XBP-1 splicing in response to fenretinide or thapsigargin. The human XBP-I sequence was amplifed by RT-PCR with thte primer pair AAACAGAGTAGCAGCTCAGACTGC

and CCTTCTGGGTAGACCTCTGGGAG[18]. E and F, Expression of the ERp57 and GRP78 protein (E) and ERp57 and ERdj5 mRNA (F) in response to 3 or 15μM fenretinide (SH-SY5Y and SK-Mel-100 cells, respectively) for 22 h was not blocked by 2 μM baicalein added 2 h before the fenretinide. Western blots were probed for ERp57, GRP78 and β-tubulin as in A; RT-PCR amplification of ERdj5 was as in B and the human ERp57 sequence was amplified with the primer pair ACGTGCTAGAACTCACGGA and ACTGAAGCTGGCCTGCCTG.

Figure 2

SiRNA-mediated knockdown of ERdj5 or ERp57 increased apoptosis in response to fenretinide in SH-SY5Y and SK-Mel-110 cells. For both ERdj5 and ERp57, two different siRNAs were evaluated against a scrambled siRNA control, and at doses of 20nM and 40 nM for each siRNA in both cell lines. A and B, Apoptosis of SH-SY5Y cells (A) or SK-Mel-110 cells (B) after transfection with 20 nM or 30 nM scrambled control siRNA (siRNA control), ERdj5-2 siRNA or ERdj5-3 siRNA and subsequent treatment with (grey bars) or without (black bars) 3 μM fenretinide. RT=PCR results to verify EDdj5 knockdown, evaluated against a GAPDH loading control by RT-PCT as in Fig. 1, are shown in the panel below the graphs (C, control; F, fenretinide-treated). C and D, Apoptosis of SH-SY5Y cells (C) or SK-Mel-110 cells (D) after transfection with 20 nM or 40 nM scrambled control siRNA (siRNA control), ERp57-1 siRNA or ERp57-2 siRNA and subsequent treatment with (grey bars) or without (black bars) 3μM fenretinide. On all graphs, error bars are 95% confidence intervals. For both cell lines, the reduction in expression of ERp57 was also evident on Western blots (data not shown). The siRNA knockdown experiments were done by plating 0.25 x 10^6 cells in 6 well plates overnight and transfection for 24 h with 29 nM or 40 nM (final concentrations) siRNA using lipofectamine in Optimem culture medium (Invitrogen)[16]. After incubation overnight at 37°C, fetal bovine serum was added to 10% and cells incubated with or without 3 μM fenretinide for a further 24 hrs. Four replicates were used per condition, three for analysis of apoptosis and one for RT-PCR to verify knockdown. SiRNAs, supplied desalted and annealed by Eurogentec (Southampton, UK) were: ERdj5-2 GGAGGAGAUUGUUUGACUU; ERdj5-3 AGACAAGCUUUCAAGAAAU; ERp57-1 GGACAAGACUGUGGCAUAU;

ERp57-2 GAAGCUAAAUCCAAAGAAA; Scrambled non-silencing control UUCUCCGAACGUGUCACGU. Apoptosis was evaluated by flow cytometry of propidium iodide (PI)-stained cells[19]. Statistical analysis of apoptosis in response to fenretinide in the presence of the conrol siRNA, knockdown siRNA-1 or knockdown siRNA-2 was by 2-way ANOVA on siRNA type and siRNA dose (20 or 40 nM). For both cell lines with ERdj5 or ERp57 there was a significant effect of the siRNA ($F_{2,12}$>177,P<0.00001), and the ERdj5 or ERp57 siRNAs significantly increased apoptosis relative to the control siRNA ($F_{1,12}$>210,P<0.00001). There was also a significant effect of siRNA dose for all except the SK-Mel-110 cells with the ERp57 siRNAs ($F_{1,12}$>14.5,P<0.0025, except $F_{1,12}$=4.6,P=0.054).

Figure 3

It is clear from Fig. 3 that untreated SK-mel-110 cells undergo little apoptosis. The amount of apoptosis seen (Fig. 3a) is commensurate with apoptosis which occurs naturally in the lifetime of cells.

Figure 4

It is clear from Fig. 4 that when SK-mel-110 cells are treated with fenretinide a significant degree of apoptosis occurs.

Therefore, fenretinide brings about apoptosis in SK-mel-110 cells.

Figure 5

It is clear from Fig. 5 that the degree of apoptosis observed following treatment with fenretinide is dose dependent. This is true for all types of melanoma cells tested.

Figure 6

It is clear from Fig. 6 that bacitracin is of minimal toxicity to the melanoma cells, with a similar degree of apoptosis being observed to that of the control. That is, that which occurs in the normal life cycle of cells.

Treating the cells with fenretinide produces a marked increase in the degree of apoptosis observed compared to treatment with bacitracin.

However, when cells are treated with a combination of bacitracin and fenretinide a significant increase in the degree of apoptosis is observed. The degree of apoptosis is greater than the degree of apoptosis seen when the cells were treated with bacitracin or fenretinide alone. Therefore, this suggests that bacitracin and fenretinide act synergistically to enhance the degree of apoptosis observed.

Figure 7

It can be seen from the results in Fig. 7 that there is a synergistic induction of apoptosis when bacitracin plus velcade are administered at a fixed dose ratio of 15,000:1 to human A375 melanoma cells.

Figure 8

The results in Fig. 8 show the effect of an anti-GRP78 antibody (Santa Cruz Biotechnology) on CHL-1 cells. The data confirm that the PDI inhibitor bacitracin increases fenretinide- or velcade-induced apoptosis. The data further demonstrate that the anti-GRP78 antibody increases fenretinide- or velcade-induced apoptosis.

The inventors noted that the particular combinations of bacitracin, anti-GRP78 antibody and ER stress-inducing agents resulted in an apoptotic response at levels equivalent to or less than apoptosis induced with either ER stress agent alone. The reasons for this are unclear at this stage.

Figure 9

The results in Fig. 9 confirm the observations seen with CHL-1 cells (see Fig. 8) in WM266-4 cells. Thus, the anti-GRP78 antibody increased fenretinide- or velcade-induced apoptosis of WM266-4 cells.

Figure 10

The data in Fig. 10 show the results of a dose response assay using the purine nucleoside analogue 2cdA. This purine nucleoside analogue inhibits the caspase-binding properties of GRP78. CHL-1 cells or WM266-4 cells were treated with increasing concentrations of 2cdA in the presence or absence of fenretinide. It can be seen from Fig. 10 that concentrations of 2cdA of 1μm and above increased fenretinide-induced apoptosis.

Figure 11

The data in Fig. 11 show the apoptotic effect on CHL-1 cells and WM266-4 cells of a combination of fenretinide with bacitracin, 2cdA, or bacitracin plus 2cdA. Bacitracin and 2cdA were both shown to increase fenretinide-induced apoptosis. Furthermore, apoptosis was further increased by the combination of fenretinide with bacitracin and 2cdA.

Figure 12

The results in Fig. 12 show the effect treating CHL-1 or WM266-4 cells with fenretinide and various combinations of bacitracin, polyclonal anti-GRP78 antibody and 2cdA. These data confirm that 2cdA increases fenretinide-induced apoptosis, an effect further increased by the addition of bacitracin. In the WM266-4 cells, the combination of anti-GRP78 antibody and 2cdA increased fenretinide-induced apoptosis beyond the effect seen with 2cdA and no antibody. However, this effect was not observed when the combination of anti-GRP78 antibody, 2cdA and fenretinide was used to treat CHL-1 cells. Furthermore, the combination of antibody, 2cdA, bacitracin and fenretinide did not increase apoptosis beyond the level achieved using 2cdA, bacitracin and fenretinide. These results indicate that the combination of a PDI inhibitor and a purine nucleoside analogue is effective for increasing ER stress-induced apoptosis of melanoma cells.

It is of course to be understood that the invention is not intended to be restricted to the details of the above embodiments which are described by way of example only.

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CLAIMS

1. The use of at least one agent which induces endoplasmic reticulum (ER) stress together with at least one agent which gives rise to inhibition of protein disulfide isomerase (PDI) or GRP78 function for the preparation of a medicament.

2. The use of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of PDI or GRP78 function for the preparation of a medicament for the treatment of cancer.

3. The use according to claim 2, wherein said ER stress inducing agent and said agent which gives rise to inhibition of PDI or GRP78 function are used for the treatment of melanoma.

4. The use according to claim 2, wherein said ER stress inducing agent and said agent which gives rise to inhibition of PDI or GRP78 function are used for the treatment of neuroblastoma.

5. The use according to any one of the preceding claims, wherein the ER stress inducing agent comprises an agent selected from fenretinide, velcade, or combinations thereof.

6. The use according to any one of claims 1 to 5, wherein the agent which gives rise to inhibtion of PDI comprises bacitracin.

7. The use according to any one of claims 1 to 6, wherein the agent which gives rise to inhibition of PDI inhibits the activity of ERdj5.

8. The use according to any one of claims 1 to 6, wherein the agent which gives rise to inhibition of PDI inhibits the activity of ERp57.

9. The use according to any one of claims 1 to 8, wherein the agent which gives rise to inhibition of PDI or GRP78 function comprises an inhibitory antibody or an active fragment thereof.

10. The use of bacitracin for the preparation of a medicament for the treatment of cancer.

11. A method for enhancing the efficacy of drugs which induce the apoptosis of tumour cells via ER stress comprising the steps of: administering a combination of at least one agent which induces ER stress and at least one agent which gives rise to inhibition of PDI or GRP78 function.

12. A pharmaceutical composition comprising an effective amount of at least one agent which induces ER stress together with at least one agent which gives rise to inhibition of PDI or GRP78 function, and which together provide a therapeutic effect.

13. A pharmaceutical composition according to claim 12, wherein the ER stress inducing agent comprises fenretinide.

14. A pharmaceutical composition according to claim 12 or claim 13, wherein the agent which gives rise to inhibition of PDI comprises bacitracin.

15. A pharmaceutical composition according to any one of claims 12 to 14, wherein the composition is used for the treatment of melanoma.

16. A pharmaceutical composition according to any one of claims 12 to 14, wherein the composition is used for the treatment of neuroblastoma.

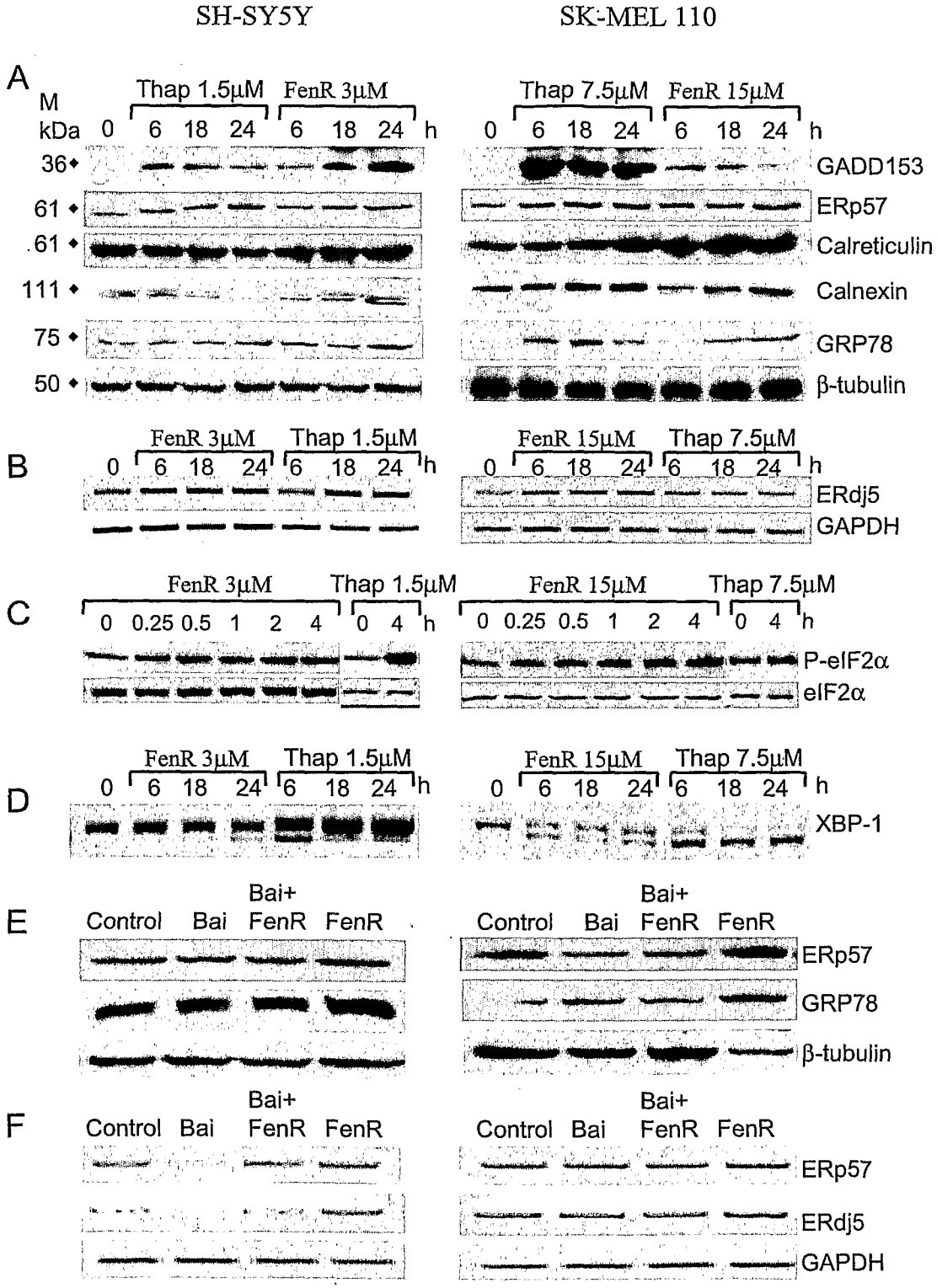

SH-SY5Y
SK-MEL 110
A
M
kDa
Thap 1.5μM
FenR 3μM
Thap 7.5μM
FenR 15μM
0 6 18 24 6 18 24 h
36
61
61
111
75
50
GADD153
ERp57
Calreticulin
Calnexin
GRP78
β-tubulin
B
FenR 3μM
Thap 1.5μM
FenR 15μM
Thap 7.5μM
ERdj5
GAPDH
C
0 0.25 0.5 1 2 4 0 4 h
P-eIF2α
eIF2α
D
XBP-1
E
Control
Bai
Bai+ FenR
FenR
ERp57
GRP78
β-tubulin
F
ERp57
ERdj5
GAPDH

FIG. 1

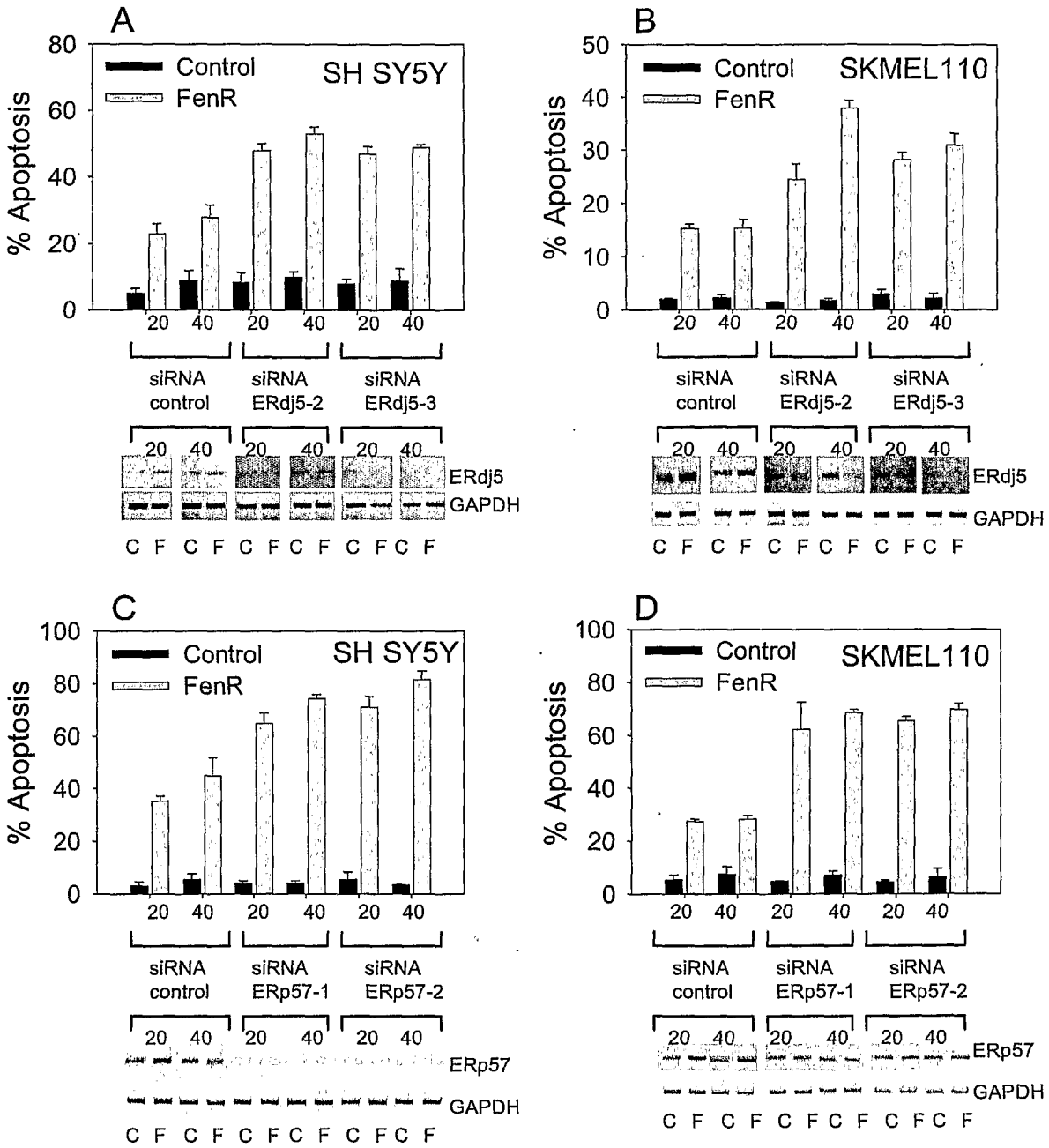

A
% Apoptosis
Control
FenR
SH SY5Y
siRNA control
siRNA ERdj5-2
siRNA ERdj5-3
ERdj5
GAPDH
B
SKMEL110
C
siRNA ERp57-1
siRNA ERp57-2
ERp57
D

FIG. 2

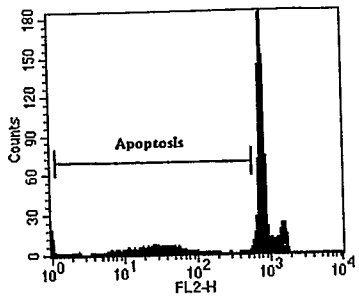


FIG. 3

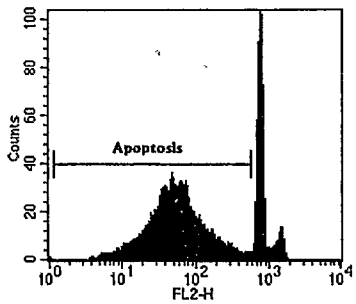


FIG. 4

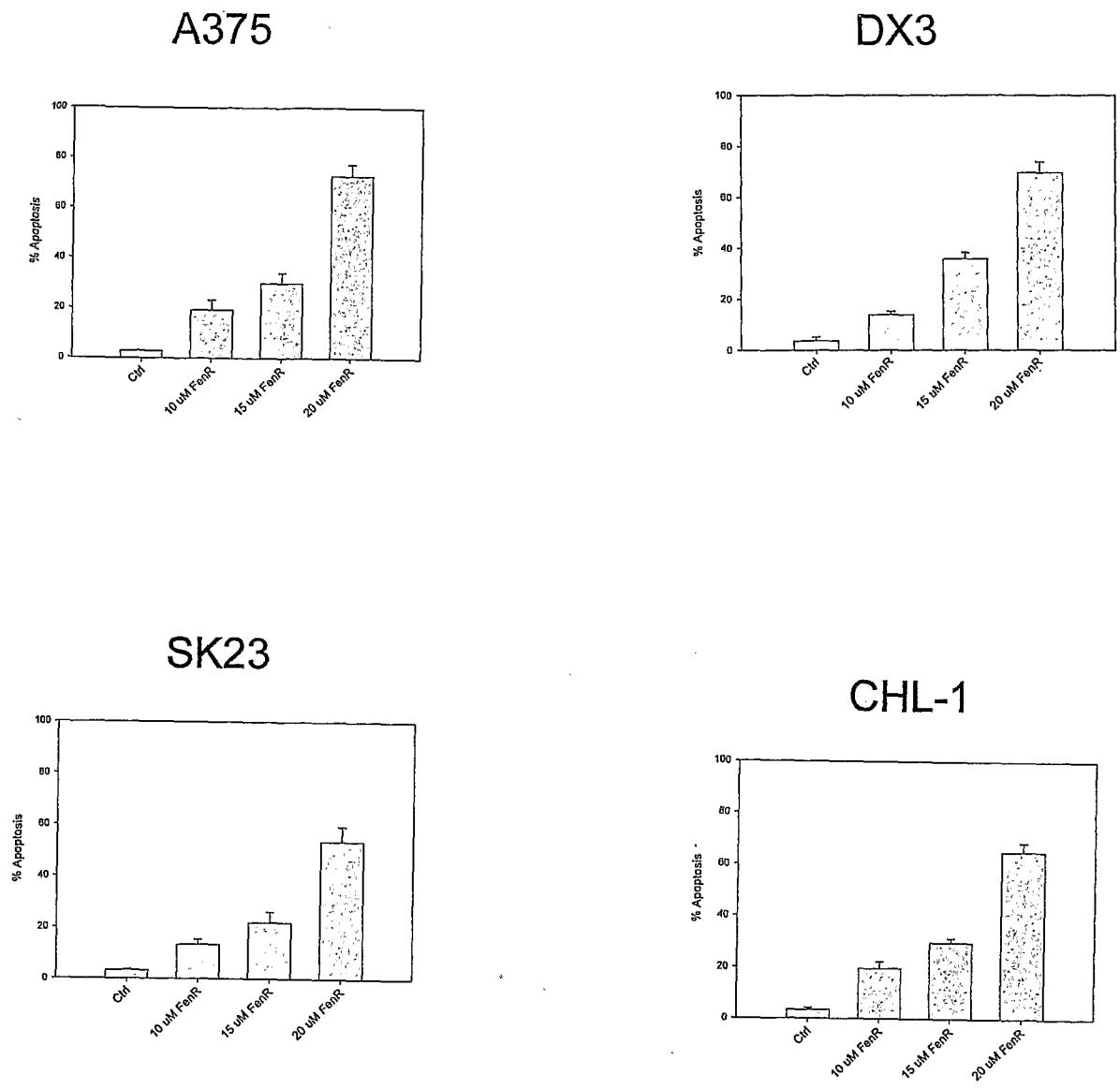

A375
DX3
SK23
CHL-1
% Apoptosis
0
20
40
60
80
100
Ctrl
10 uM FenR
15 uM FenR
20 uM FenR

FIG. 5

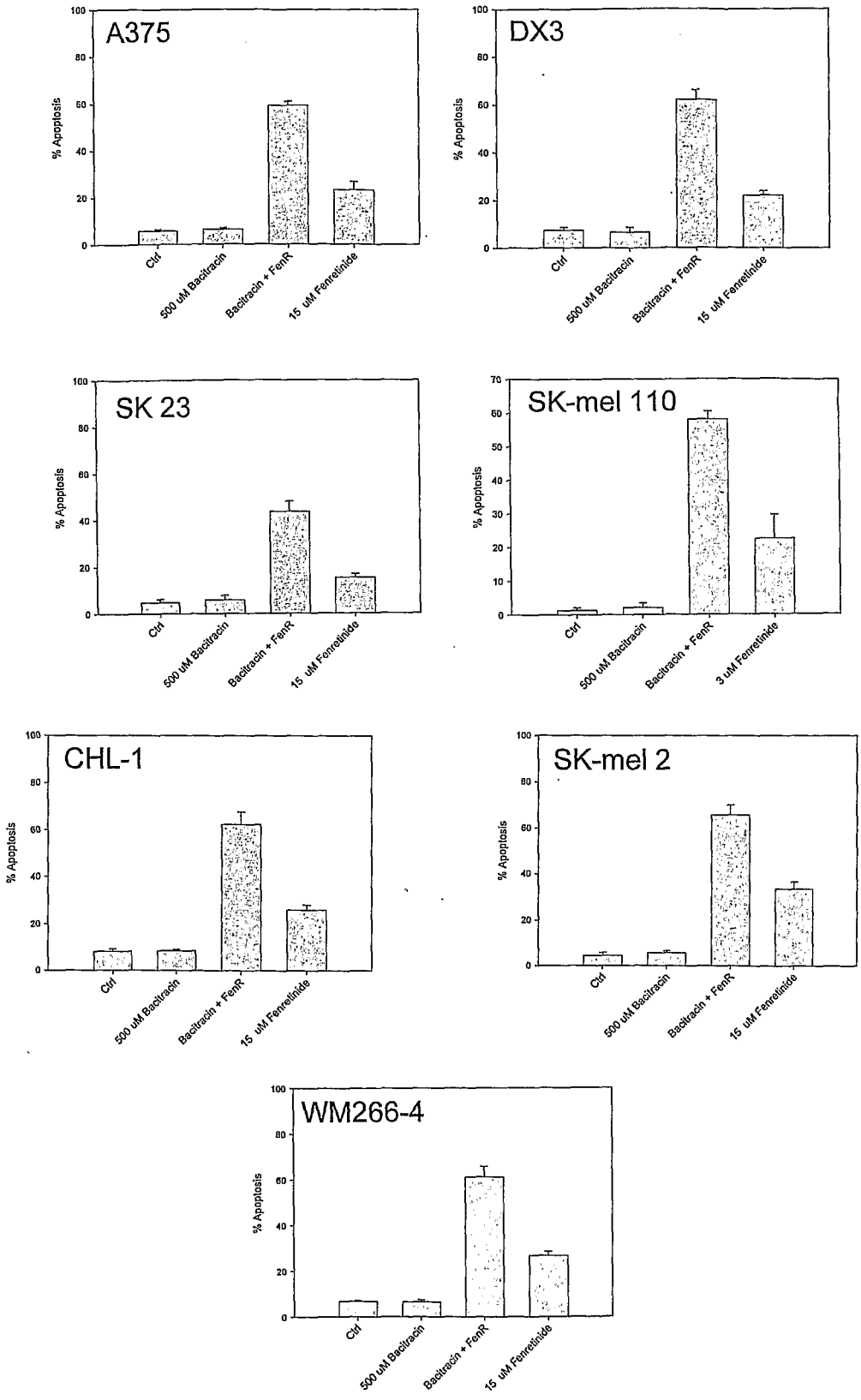

A375
DX3
SK 23
SK-mel 110
CHL-1
SK-mel 2
WM266-4
% Apoptosis
Ctrl
500 uM Bacitracin
Bacitracin + FenR
15 uM Fenretinide
3 uM Fenretinide

FIG.6

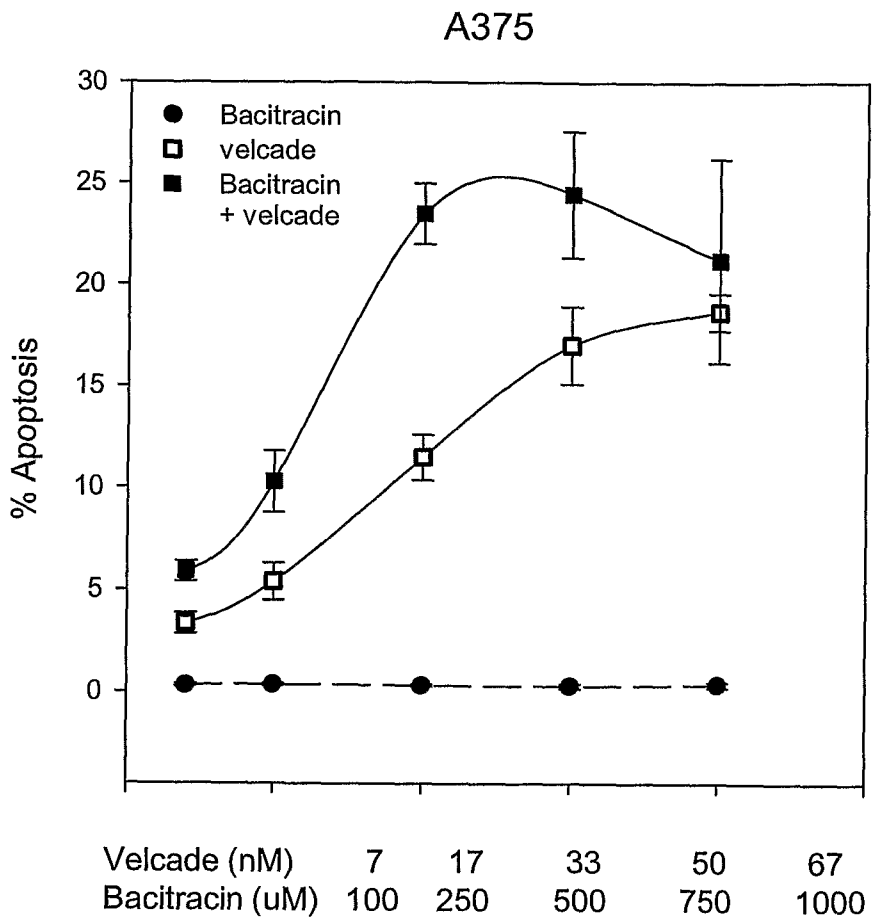

A375
% Apoptosis
30
25
20
15
10
5
0
Bacitracin
velcade
Bacitracin
+ velcade
Velcade (nM) 7 17 33 50 67
Bacitracin (uM) 100 250 500 750 1000

FIG. 7

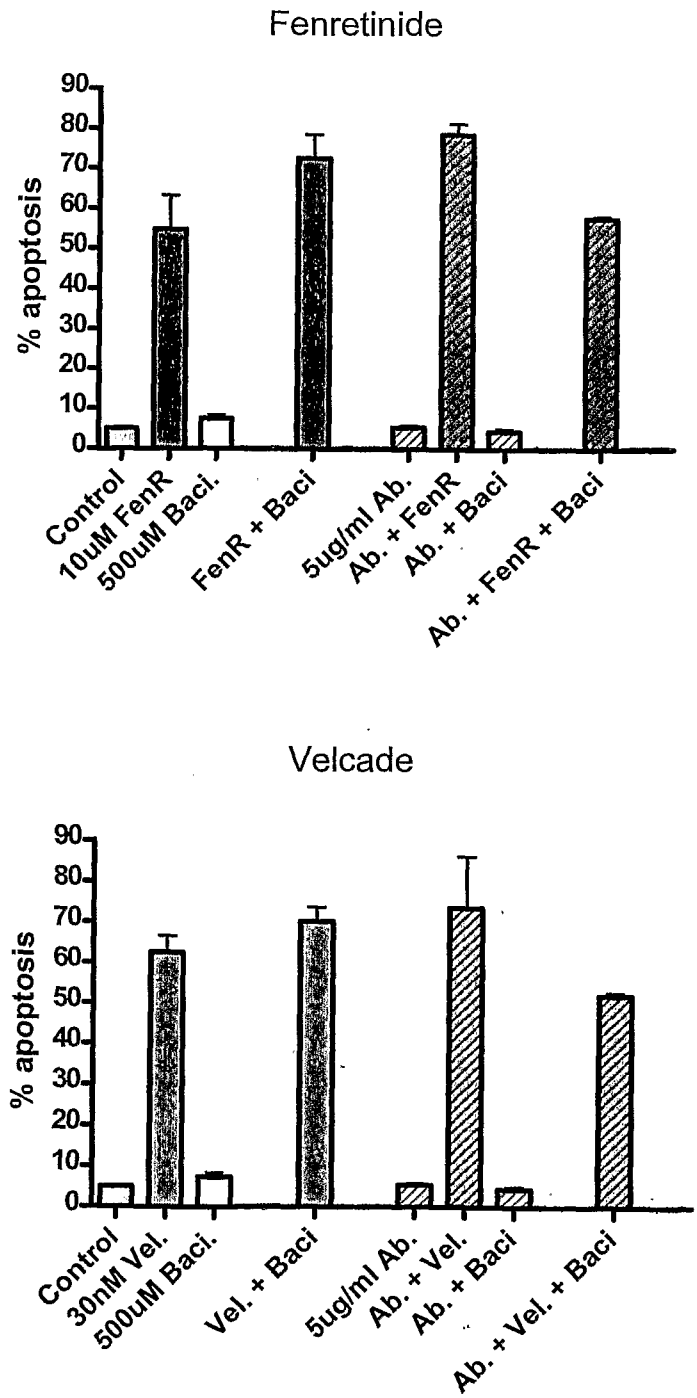

Fenretinide
% apoptosis
90
80
70
60
50
40
30
20
10
0
Control
10uM FenR
500uM Baci.
FenR + Baci
5ug/ml Ab.
Ab. + FenR
Ab. + Baci
Ab. + FenR + Baci
Velcade
% apoptosis
90
80
70
60
50
40
30
20
10
0
Control
30nM Vel.
500uM Baci.
Vel. + Baci
5ug/ml Ab.
Ab. + Vel.
Ab. + Baci
Ab. + Vel. + Baci

FIG. 8

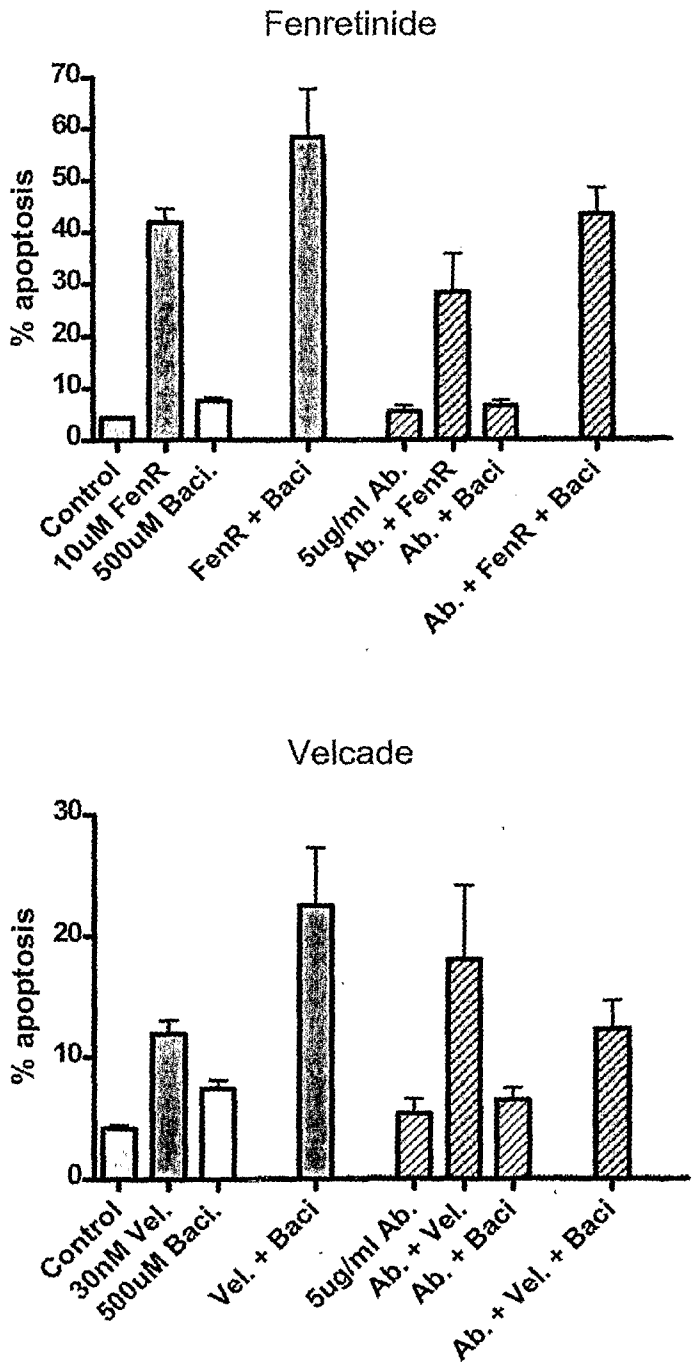
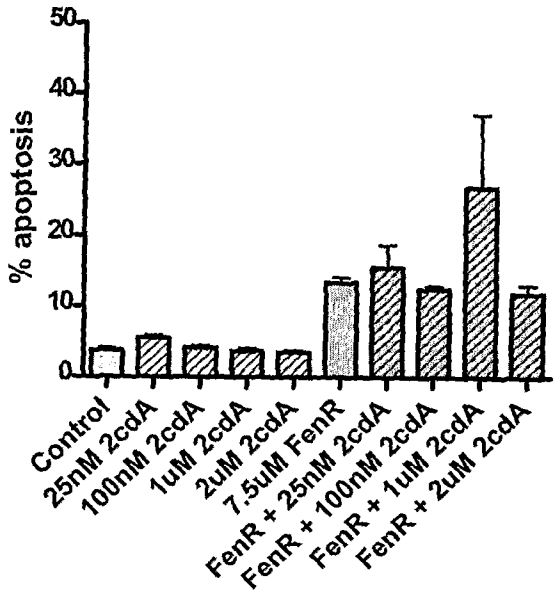

Fenretinide
% apoptosis
70
60
50
40
30
20
10
0
Control
10uM FenR
500uM Baci.
FenR + Baci
5ug/ml Ab.
Ab. + FenR
Ab. + Baci
Ab. + FenR + Baci
Velcade
% apoptosis
30
20
10
0
Control
30nM Vel.
500uM Baci.
Vel. + Baci
5ug/ml Ab.
Ab. + Vel.
Ab. + Baci
Ab. + Vel. + Baci

FIG. 9

CHL-1



WM266-4

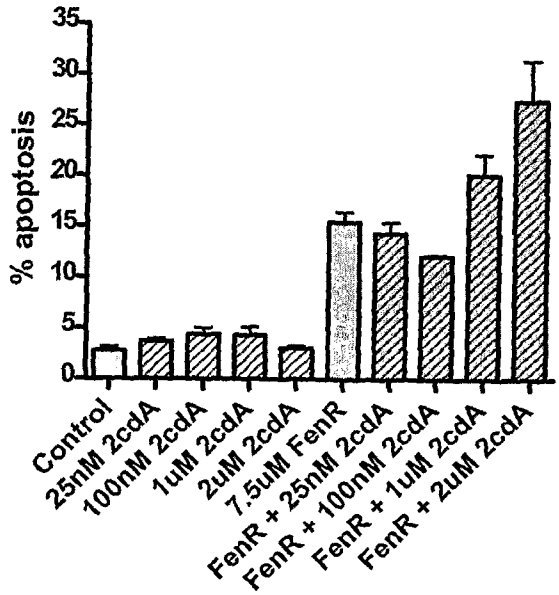


FIG. 10

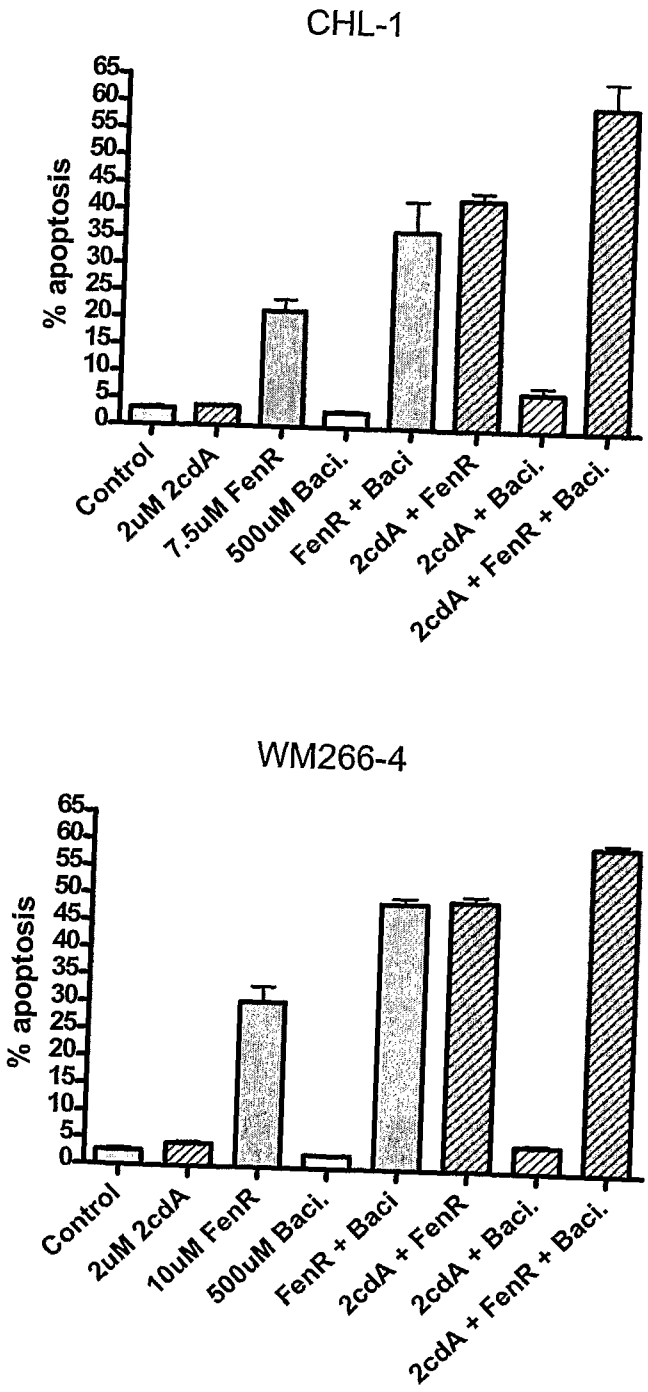

CHL-1
% apoptosis
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Control
2uM 2cdA
7.5uM FenR
500uM Baci.
FenR + Baci
2cdA + FenR
2cdA + Baci.
2cdA + FenR + Baci.
WM266-4
% apoptosis
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Control
2uM 2cdA
10uM FenR
500uM Baci.
FenR + Baci
2cdA + FenR
2cdA + Baci.
2cdA + FenR + Baci.

FIG. 11

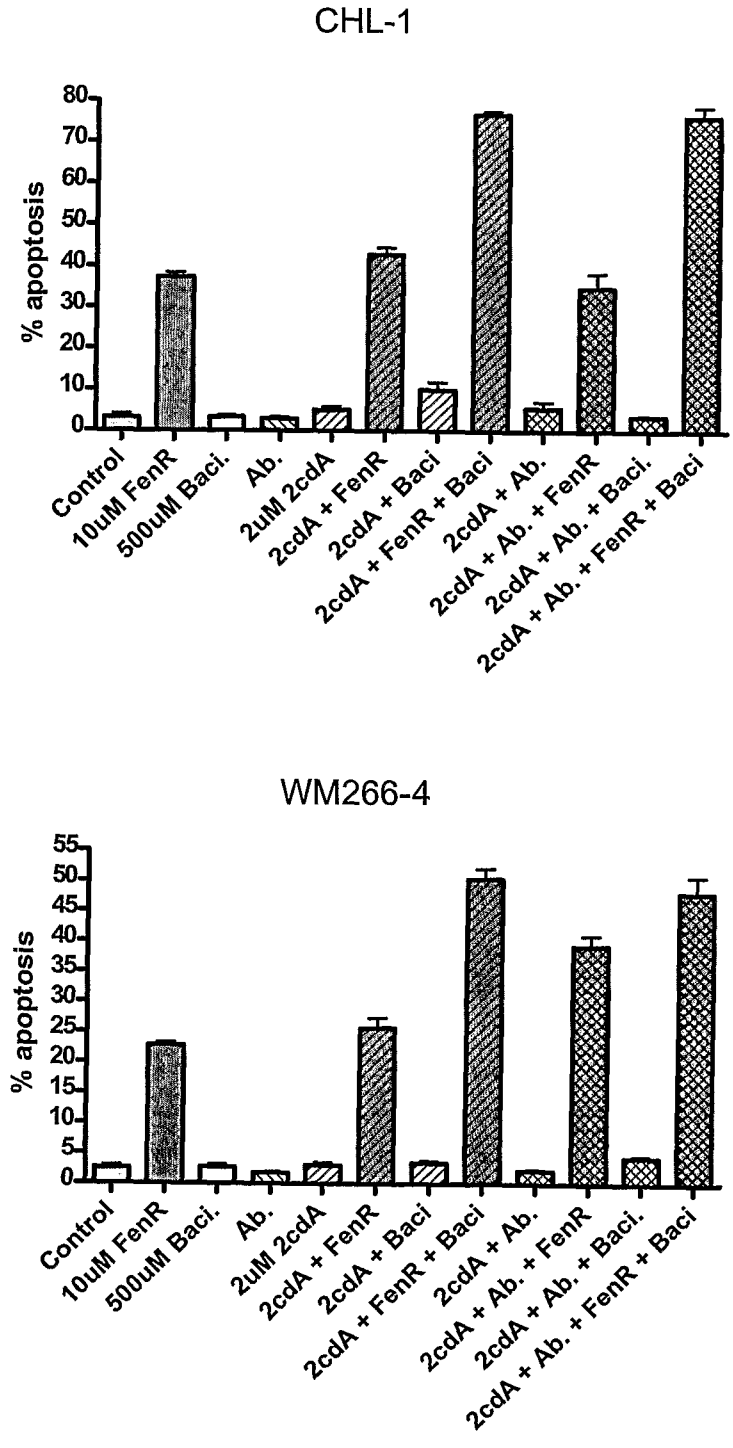

CHL-1
% apoptosis
80
70
60
50
40
30
20
10
0
Control
10uM FenR
500uM Baci.
Ab.
2uM 2cdA
2cdA + FenR
2cdA + Baci
2cdA + FenR + Baci
2cdA + Ab.
2cdA + Ab. + FenR
2cdA + Ab. + Baci.
2cdA + Ab. + FenR + Baci
WM266-4
% apoptosis
55
50
45
40
35
30
25
20
15
10
5
0
Control
10uM FenR
500uM Baci.
Ab.
2uM 2cdA
2cdA + FenR
2cdA + Baci
2cdA + FenR + Baci
2cdA + Ab.
2cdA + Ab. + FenR
2cdA + Ab. + Baci.
2cdA + Ab. + FenR + Baci

FIG. 12