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Inhibition of casein kinase 2 enhances the death ligand- and natural kiler cell-induced hepatocellular carcinoma cell death

H.-R. Kim,* K. Kim,† K.-H. Lee,‡ S. J. Kim* and J. Kim*

*Department of Microbiology and Brain Korea 21 Project for Medical Sciences, †Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, and ‡Department of Microbiology, Yonsei University Wonju College of Medicine, Wonju, Kangwon, Korea

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Correspondence: J. Kim, Department of Microbiology and Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemoon-gu, Seoul 120-752, Korea.
E-mail: jkim63@yumc.yonsei.ac.kr

Summary

Recent studies have shown that the inhibition of casein kinase 2 (CK2) sensitizes many cancer cells to Fas ligand- and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. However, it has not been demonstrated directly whether CK2 inhibition can also enhance the cytotoxicity of natural killer (NK) cells, which actually use the death ligands to kill cancer cells *in vivo*. To address whether NK cell-mediated cancer cell death is affected by the inhibition of CK2, we first checked whether the death ligand-induced apoptosis of hepatocellular carcinoma cells (HCCs) and HeLa were affected by CK2 inhibition. We then investigated the effect of CK2 inhibition on NK cytotoxicity against HCCs and HeLa cells and its mechanistic features. Inhibition of CK2 by emodin increased the apoptotic cell death of HepG2, Hep3B and HeLa when the cancer cell lines were treated with a soluble form of recombinant TRAIL or an agonistic antibody of Fas. This phenomenon appeared to be correlated with the expression level of death receptors on the cancer cell surface. More interestingly, the inhibition of CK2 also greatly increased the NK cell-mediated cancer cell killing. The NK cytotoxicity against the cancer cells increased about twofold when the target cells were pretreated with a specific CK2 inhibitor, emodin or 4,5,6,7-tetrabromobenzotriazole. Furthermore, the increase of the NK cytotoxicity against cancer cells by CK2 inhibition was granule-independent and mediated possibly by the death ligands on the NK cell surface. This suggests that CK2 inhibitors could be used to enhance the cytotoxicity of NK cells and consequently increase host tumour immunity.

Keywords: apoptosis, CK2 inhibitor, CK2, death ligands/ receptors, NK cells, NK cytotoxicity

Introduction

Natural killer (NK) cells are a distinct subset of large granular lymphocytes, which possess the ability to kill certain primary tumour cells, tumour cell lines, virus-infected cells and transplanted allogenic cells. NK cells are able to lyse target cells spontaneously without the need for specific antigen recognition or prior sensitization [1,2]. In particular, NK cells are believed to play important roles in tumour immunity [3]. It is well known that NK cells kill many leukaemia cells effectively [2,4], and more recent studies have demonstrated that NK cells can destroy many solid tissue-derived malignant cells, such as melanoma, breast cancer, lung cancer, gastric cancer, colon cancer, renal cancer, ovarian cancer and hepatoma cell lines [5–8].

Two major pathways are involved in the killing mechanism of NK cells, just as in the case of cytotoxic T cells. One pathway involves a polarized secretion of preformed perforin and granzymes by NK cell granule exocytosis, which leads to rapid caspase activation in target cells, as well as a caspase-independent death pathway [4,9–12]. Perforin can induce necrosis of target cells and granzymes can induce apoptosis of target cells [9,10,13]. In another pathway, death receptors on target cells are cross-linked by NK cells' death ligands and these interactions trigger a relatively well-studied apoptotic death pathway [5,6,14,15]. The non-secretory/apoptotic pathway is mediated by the Fas ligand (FasL)/Fas, tumour necrosis factor (TNF)/TNF receptor and TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor interactions, which

trigger a cell death pathway involving caspase activation [5,6,8,14–18].

Death ligands expressed on killer cells induce apoptosis of target cells *in vivo*, but it is well known that soluble forms of TRAIL or agonistic anti-Fas antibodies can also induce the apoptotic cell death of certain cancer cells [19,20]. However, many cancer cells are resistant to the soluble forms of TRAIL- and/or FasL-induced apoptosis, although most of them express death receptors on the surface [21,22]. In particular, most hepatocellular carcinoma cells (HCCs) are resistant to death receptor-mediated apoptosis when the cell surface death receptor is cross-linked *in vitro* with either agonistic antibodies or soluble forms of death ligand proteins [22–28].

Casein kinase 2 (CK2) is a serine/threonine protein kinase [29,30] which plays a key role in cell cycle control, cellular differentiation and proliferation [31–33]. CK2 also participates in the regulation of apoptosis by phosphorylating some apoptosis-related factors [21,34–36]. Interestingly, CK2 has been found to be up-regulated in many cancer cells [37–39]. Increased expression of CK2 protects cells from Fas- and drug-induced apoptosis, suggesting that CK2 may have a general anti-apoptotic function [36,40]. Recent studies have shown that the inhibition of CK2 sensitizes many cancer cells, such as rhabdomyosarcoma, colon carcinoma, breast cancer, cervical cancer and gastric cancer to FasL- and TRAIL-induced apoptosis [21,36,41–43]. However, it has not been demonstrated directly whether CK2 inhibition can also enhance the cytotoxicity of killer cells, which actually use the death ligands to kill cancer cells *in vivo*.

In a previous report, we demonstrated that NK cells can effectively kill HCCs using the granule-dependent necrotic pathway and the death ligand-dependent apoptotic pathway [8]. In this study, we addressed whether NK cell-mediated cancer cell death is affected by the inhibition of CK2 in target cells. We first checked whether the death ligand-induced apoptosis of HCCs and HeLa were affected by CK2 inhibition. Then, we investigated the effect of CK2 inhibition on NK cytotoxicity against HCCs and HeLa cells and its mechanistic features.

Materials and methods

Natural killer cell preparation

Natural killer cells were purified from the whole blood of healthy volunteers by negative selection using the RosetteSep™ NK enrichment antibody cocktail (StemCell Technologies Inc., Vancouver, Canada), as described previously [8]. Briefly, 1 ml of whole blood was mixed with 50 µl of RosetteSep™ NK enrichment cocktail and incubated for 20 min at room temperature. The blood sample was then diluted with the same volume of phosphate-buffered saline (PBS; pH 7·4) containing 2% of fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA). The diluted sample was layered on the top of Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 min at room temperature. The NK cell layer was collected and the enriched NK cells were washed three times with PBS containing 2% FBS. Purified NK cells were >80% $CD56^+$ $CD16^+$/$CD3^-$ (Beckman Coulter, Fullerton, CA, USA) and were maintained for 2 weeks in RPMI-1640 media containing 10% FBS and 100 units/ml of recombinant interleukin-2 (Endogen, Woburn, MA, USA).

Cell lines and culture

Hepatocellular carcinoma cell lines HepG2 [American Type Culture Collection (ATCC) HB 8065] and Hep3B (ATCC HB 8064) were used as target cell lines and maintained in modified Eagle's medium (MEM) containing 10% FBS (Gibco BRL). HeLa was also cultured in Dulbecco's MEM containing 10% FBS and used as target cells for cytotoxicity assay.

Antibodies and reagents

Anti-Fas antibody (clone CH11), recombinant TRAIL (rTRAIL), emodin and 4,5,6,7-tetrabrombenzotriazole (TBB) were purchased from Medical & Biological Laboratories (Nagoya, Japan), Serotec (Oxford, UK) and Calbiochem (San Diego, CA, USA) respectively. Anti-caspase-3 and anti-poly ADP-ribose polymerase (PARP) antibodies were purchased from Cell Signalling Technology (Boston, MA, USA).

Flow cytometric analysis

Cell surface receptors and ligands were quantified by flow cytometric analysis. NK cells and tumour cells were washed twice with ice-cold PBS containing 0·05% bovine serum albumin (BSA). Cells were incubated with phycoerythrin-labelled anti-DR4, anti-DR5, anti-Fas, anti-FasL and anti-TRAIL antibody (Biolegend, San Diego, CA, USA) for 30 min at 4°C. After two washes with 0·05% BSA–PBS, cells were analysed using a fluorescence activated cell sorter (FACScalibur) flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

The JAM test (DNA fragmentation assay)

Natural killer cell-mediated apoptotic target cell death was measured using a [^{3}H]-thymidine release assay, the JAM test [44]. For labelling, 2×10^4 cells were incubated with 20 µCi of [^{3}H]-thymidine (37 MBq/ml; Nen, Boston, MA, USA) for 20 h at 37°C in a 96-well microtitre plate. Cells were then washed three times with culture media without FBS. NK cells and [^{3}H]-thymidine-labelled target cells were mixed at the indicated effector : tarhet (E : T) ratio. After 2 h of incubation in the presence or absence of the CK2 inhibitor, the cells and their medium were aspirated onto glass fibre filters (size 90×120 mm) using a semi-automated 96-well harvester

(Tomtec, Hamden, CT, USA). The filters (Wallac Oy, Turku, Finland) were washed, dried and sealed with melt-on scintillator sheets (Wallac) and radioactivity was measured with a beta counter (Wallac). rTRAIL- and CH11-induced cancer cell death was measured similarly using the JAM test. For this assay, target cells were treated with rTRAIL or CH11 for 6 h in the presence or absence of the CK2 inhibitors. Percentage of apoptotic cell death was calculated by the following formula: % DNA fragmentation = [1 − (experimental value/control value)] × 100. The control value was determined by incubating target cells in culture medium alone.

Lactate dehydrogenase assay

Recombinant TRAIL- and CH11-induced cancer cell death was also assessed by lactate dehydrogenase (LDH) assay using the CytoTox 96 assay kit (Promega, Madison, WI, USA). Target cells, 1×10^4, were prepared in a 96-well microtitre plate and treated as indicated in the figure legends. For the LDH-positive control, 10 μl of lysis solution (10×) were added to all wells to lyse cells, and the target cells were incubated for 45 min. An aliquot of 50 μl was taken from each well and transferred to a fresh 96-well flat-bottomed (enzymetic assay) plate. To each well of the plate, 50 μl of substrate mix was added, and the plate was incubated for 30 min in the dark. After 30 min, 50 μl of stop solution was added to each well and the absorbance was recorded at 490 nm within 1 h using an enzyme-linked immunosorbent assay reader.

^{51}Chromium release assay

Natural killer cell-mediated target cell killing was assessed using a standard ^{51}Cr release assay, as described previously [8]. For labelling, 3×10^3 cells were incubated with 10 μCi of ^{51}Chromium (^{51}Cr) (Nen) for 60 min at 37°C in 96-well microtitre plates, then washed three times with culture media without 10% FBS. ^{51}Cr-labelled target cells and NK cells were mixed at the indicated E : T ratio. After 4 h of co-culture with NK cells, cell-free supernatant was collected and radioactivity was measured with a gamma counter. Percentage of specific ^{51}Cr release was calculated by the following formula:% cytotoxicity = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100. For control experiments, target cells were incubated either in culture medium alone to determine spontaneous release or in a mixture of 2% Triton X-100 to define maximum ^{51}Cr release. Data are presented as the mean of at least three independent experiments.

Western blot

Target cells were lysed with a lysis buffer (10 mM Tris-HCL, pH 7·4 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 15 μg/ml leupeptin, 2 mM NaF, 2 mM $NaVO_4$), and the lysates were separated on sodium dodecyl sulphide-polyacrylamide gels. The protein bands were transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA). The membranes were blocked with 5% BSA in PBS containing 0·1% Tween-20 (PBST) for 2 h, incubated with proper antibodies for 4 h, and washed with PBST. The membranes were then incubated with peroxidase-conjugated goat anti-mouse IgG (H + l) for 2 h, and washed with PBST. The blots were finally visualized by Supersignal WestDico chemiluminescent substrate (Pierce).

Fixation of NK cells

For blocking granule release, NK cells were incubated for 20 min with RPMI-1640 containing 0·5% paraformaldehyde and washed twice with PBS. The concentration of paraformaldehyde and the incubation time was minimized to avoid adverse effects as much as possible.

Results

Casein kinase 2 inhibitor augments rTRAIL-induced apoptosis of HCCs

In a previous report, we demonstrated that NK cells effectively killed HCCs using the granule-dependent necrotic pathway and the death ligand-dependent apoptotic pathway [8]. Interestingly, recent studies have shown that inhibition of CK2 sensitizes many cancer cells to TRAIL-induced apoptosis [21,36,41–43]. As a first step to investigate the effect of CK2 inhibition on the NK cytotoxicity against HCCs, we checked whether the death ligand-induced apoptosis of HCCs were affected by CK2 inhibition, as it has not yet been demonstrated. For this purpose, HepG2 and Hep3B cell lines were treated for 24 h with emodin, a CK2 inhibitor [45], in the presence and absence of rTRAIL (10 ng/μl). In order to observe the apoptotic target cell death, the extent of DNA fragmentation was measured using a [^{3}H]-thymidine release assay [44]. As shown in Fig. 1a, rTRAIL or emodin alone induced only limited apoptotic cell death of HepG2 or Hep3B. However, the HepG2 and Hep3B cells exhibited a significant amount of DNA fragmentation when the cells were co-treated with rTRAIL and emodin (Fig. 1a). Similar phenomena were observed when the target cell death was measured by LDH assay (Fig. 1b). Emodin or rTRAIL alone induced only limited cell death of HepG2 and Hep3B, but co-treatment of rTRAIL with emodin increases significantly the rTRAIL-induced cell death. Both HepG2 and Hep3B expressed DR5 (TRAIL receptor 2), but not DR4 (TRAIL receptor 1) (Fig. 1c). As a result, both HepG2 and Hep3B cells were sensitive to rTRAIL-induced apoptosis, and was enhanced in the presence of emodin (Fig. 1a and b).

We next investigated the dose-dependent effects of rTRAIL and emodin on the apoptosis of HCCs by using the

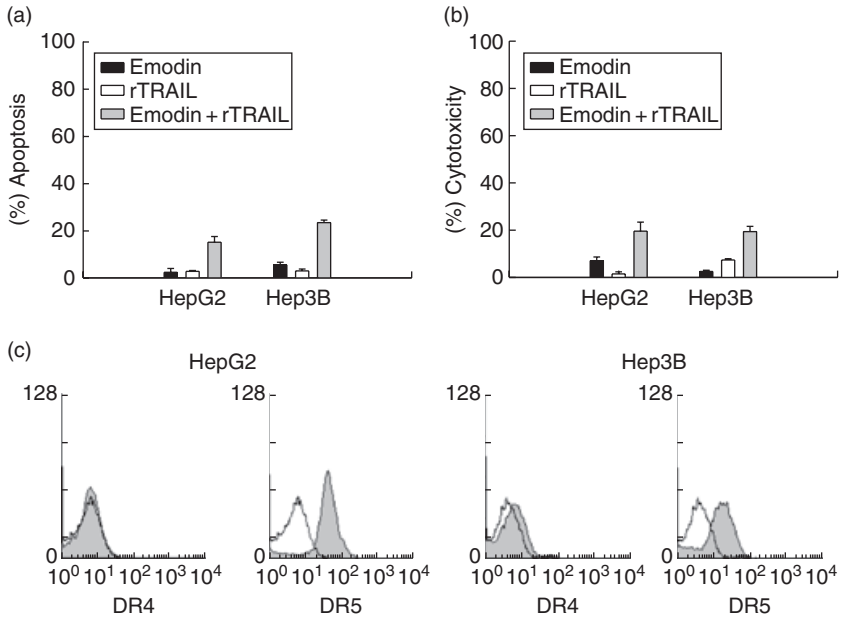

Fig. 1. Effects of casein kinase 2 inhibitor (emodin) on the recombinant tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL)-induced cancer cell death. (a) rTRAIL-induced apoptotic cell death of hepatocellular carcinoma cells assessed by the JAM test. HepG2 and Hep3B cells were labelled with [^{3}H]-thymidine for 1 day and treated with 10 μg/ml of emodin. After 1 day, 10 ng/ml of rTRAIL was used to treat the target cells for 6 h, and the radioactivity was measured by a β-counter. (b) rTRAIL-induced target cell death measured by the lactate dehydrogenase (LDH) assay. Target cells were seeded to 96-well microplates and treated with emodin. After 1 day, rTRAIL was used to treat target cells for 18 h, and 50 μl aliquots of the media were used for the LDH assay. The data are presented as a mean of at least three independent experiments (mean ± standard deviation). (c) DR4 and DR5 expressions on HepG2 and Hep3B were determined by flow cytometry (black line: isotype control, grey filled area: receptor expression).

JAM test. As shown in Fig. 2a, apoptotic cell death of HepG2 increased gradually as the amount of emodin increased from 2 to 20 μg/ml in the absence of rTRAIL (black bars, left panel). Furthermore, much greater apoptotic cell death of HepG2 was observed when the amount of emodin was varied from 2 to 20 μg/ml in the presence of a fixed amount of rTRAIL (10 ng/ml; white bars, left panel). The apoptotic cell death of HepG2 increased only slightly when the amount

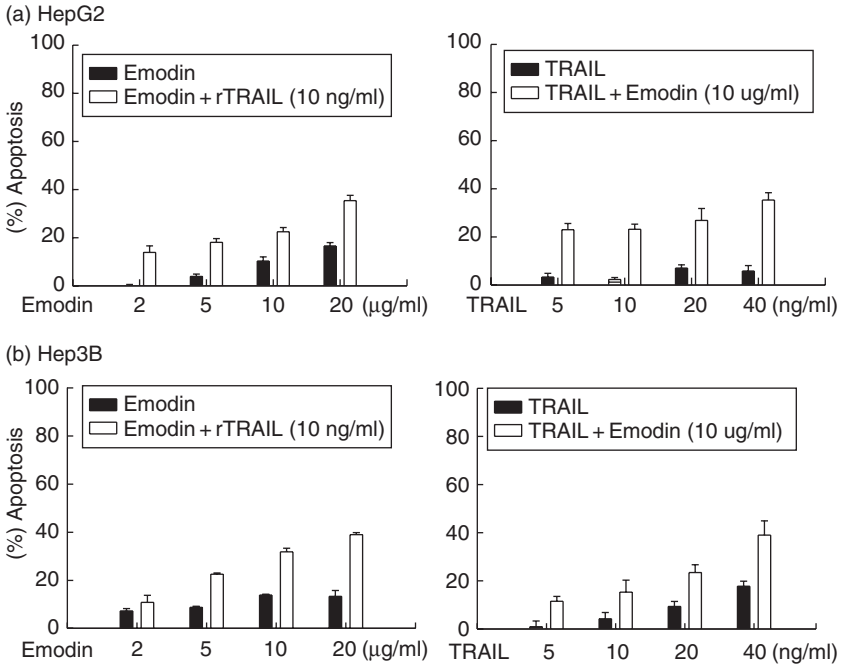

Fig. 2. Dose-dependent effect of recombinant tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL) and emodin. (a) HepG2 and (b) Hep3B target cells were used for the JAM test. Left panels show the dose-dependent effect of emodin on the rTRAIL-induced target cell death. rTRAIL at a concentration of 10 ng/ml was used to induce apoptotic cell death of target cells. Right panels show the dose-dependent effect of rTRAIL on the target cell death in the presence of 10 μg/ml emodin. The target cells were incubated with emodin for 1 day and treated with rTRAIL. After 6 h, the radioactivity of target cells was detected by a β-counter. The data are presented as a mean of at least three independent experiments (mean ± standard deviation).

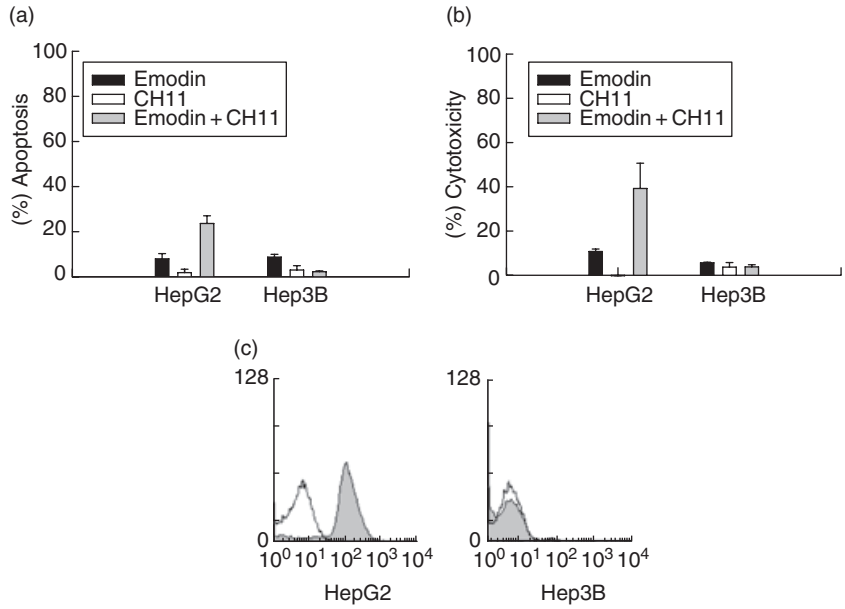

Fig. 3. Effects of casein kinase 2 inhibitor on the CH11-induced target cell death. CH11-induced apoptotic cell death of target cells was assessed by the JAM test. HepG2 and Hep3B cells were labelled with [^{3}H]-thymidine for 1 day and treated with 10 μg/ml of emodin. After 1 day, 250 ng/ml of CH11 was used to treat the target cells for 6 h, and the radioactivity was measured by a β-counter. (b) CH11-induced target cell death was measured by the lactate dehydrogenase (LDH) assay. Target cells were seeded in 96-well microplates and treated with 10 μg/ml of emodin. After 1 day, 250 ng/ml of CH11 was used to treat target cells for 18 h, and 50 μl aliquots of the media were used for the LDH assay. The data are presented as a mean of at least three independent experiments (mean ± standard deviation). (c) Fas expressions on HepG2 and Hep3B were determined by flow cytometry (black line: isotype control, grey filled area: Fas expression).

of rTRAIL increased from 5 to 40 ng/ml in the absence of emodin (black bars, right panel). However, the apoptotic cell death of HepG2 increased substantially as the amount of rTRAIL increased in the presence of the fixed amount of emodin (10 μg/ml; white bars, right panel). Similar dose-dependent effects of rTRAIL and emodin were observed when Hep3B cells were treated with emodin or rTRAIL (Fig. 2b, black bars), and when the cells were co-treated with rTRAIL and emodin (white bars, Fig. 2b).

Casein kinase 2 inhibition increased the FasL-induced apoptosis of HCCs

We next checked whether the FasL-induced apoptosis of HCCs were also affected by CK2 inhibition. For this purpose, HepG2 and Hep3B cell lines were treated for 24 h with emodin in the presence or absence of an agonistic monoclonal anti-Fas antibody (CH11) [46]. When the amount of cell death was measured by the JAM test, CH11 or emodin alone induced only limited apoptotic cell death of HepG2 and Hep3B (Fig. 3a). It is well known that HepG2 cells are resistant to FasL-induced apoptosis, although it expresses Fas [22–28]. However, the HepG2 cells exhibited a significant amount of DNA fragmentation when the cells were co-treated with CH11 and emodin (Fig. 3a, left panel). Unlike HepG2 cells, Hep3B cells did not express Fas (Fig. 3c). Consequently, simultaneous treatment of CH11 and emodin did not induce any significant apoptosis in Hep3B cells (Fig. 3a, right panel). Similar phenomena were observed when the target cell death was measured by LDH assay (Fig. 3b). CH11 or emodin alone induced only a limited amount of cell death in HepG2 and Hep3B; however, CH11 and emodin co-treatment increased the CH11-induced cell death significantly in HepG2 cells, but not in Hep3B cells.

Effect of CK2 inhibition on death ligand-induced HeLa cell death

We next investigated the effect of CK2 inhibition on death ligand-induced cell death of other tumour cells. A cervical cancer cell line, HeLa, was chosen for this purpose. HeLa cells strongly expressed Fas and DR5, death receptors for FasL and TRAIL respectively (Fig. 4a). As expected, CK2 inhibition appeared to augment the rTRAIL- and CH11-induced apoptotic cell death of HeLa in the JAM test (Fig. 4b). Interestingly, we found that HeLa cells were sensitive to emodin treatment. As shown in Fig. 4a, emodin alone induced more apoptotic cell death than rTRAIL or CH11 for unknown reason.

Effects of CK2 inhibition on NK cell-mediated apoptosis

We next investigated the effect of CK2 inhibition on NK cytotoxicity against three cancer cell lines, HepG2, Hep3B and

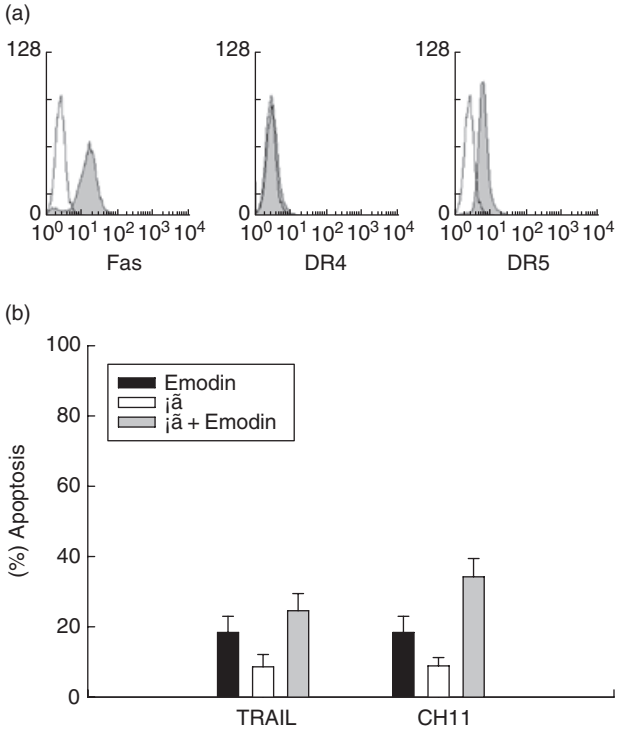

Fig. 4. Effects of emodin on the recombinant tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL)- and CH11-induced HeLa cell death. (a) Fas, DR4 and DR5 expressions on HeLa were determined by flow cytometry (black line: isotype control, grey filled area: receptors expression). (b) JAM test. HeLa cells were labelled with [^{3}H]-thymidine for 1 day and treated with 10 μg/ml of emodin. After 1 day, rTRAIL or CH11 was used to treat the target cells for 6 h. The radioactivity of HeLa cells was measured by a β-counter. The data are presented as a mean of at least three independent experiments (mean ± standard deviation). ▲: rTRAIL or CH11 alone.

HeLa. In order to observe the apoptotic target cell death mediated by NK cells, the extent of DNA fragmentation was measured by the JAM test, a [^{3}H]-thymidine release assay. When target cells were co-incubated with NK cells for 2 h, Hep3B and HeLa cells exhibited a significant amount of DNA fragmentation, whereas HepG2 cells showed a relatively small amount of DNA fragmentation (Fig. 5a, white bars). When the target cells were pretreated with emodin and co-incubated with NK cells, all three cancer cell lines exhibited a greatly increased amount of apoptotic cell death (grey bars). Emodin alone induced limited apoptotic cell death only in HepG2 and Hep3B, but for unknown reasons it induced a significant apoptotic cell death in HeLa (black bars).

Similar results were obtained when the NK cells and target cells were co-incubated in the presence of TBB, a more specific CK2 inhibitor [47]. TBB is one of the most efficient inhibitors for CK2, along with 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole [48]. As shown in Fig. 5b, when the target cells were pretreated with TBB and co-incubated with NK cells, all three cancer cell lines exhibited a greatly increased amount of apoptotic cell death (grey bars) as measured by the JAM test. We next investigated the effect of TBB on the necrotic target cell death mediated by NK cells using a 4 h ^{51}Cr release assay. The standard 4 h ^{51}Cr release assay is known to measure primarily the necrotic cell death induced by the cytotoxic granules of NK cells [7,8,24]. As shown in Fig. 5c and d, NK cells induced necrotic cell death when the target cells were co-incubated with NK cells (white bars). However, treatment of neither emodin nor TBB increased the NK cytotoxicity against the cancer cell lines (grey bars). These results indicated that the inhibition of CK2 of cancer cells augmented the NK cell-mediated apoptotic killing of target cells.

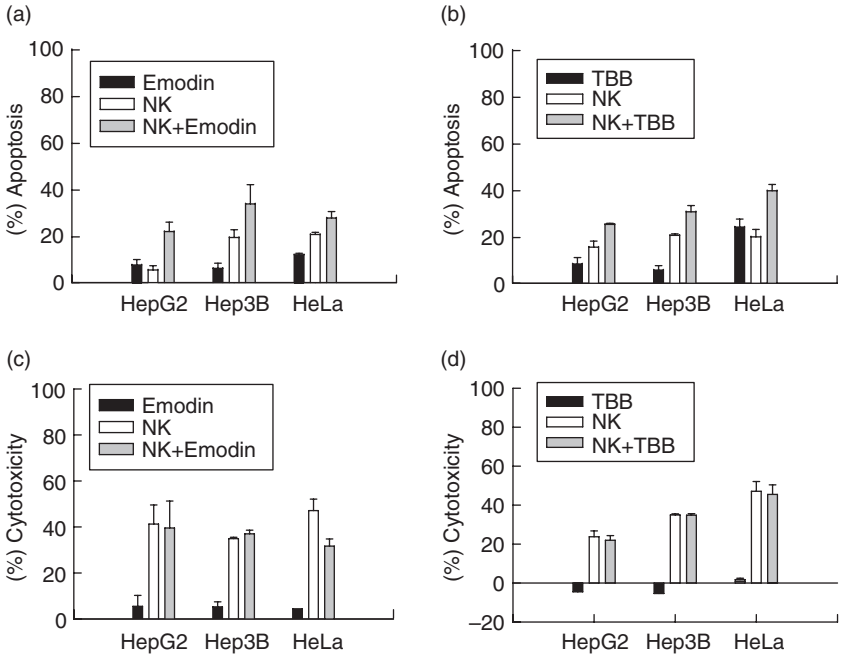

Fig. 5. Effects of casein kinase 2 inhibitors on natural killer (NK) cell-mediated tumour cell killing. NK cell-induced apoptotic target cell death was assessed by the JAM test. Target cells were labelled with [^{3}H]-thymidine for 1 day, and 10 μg/ml of emodin (a) and 5 μM TBB (b) were used to treat the target cells. After 1 day, target cells were co-cultured with NK cells with an effector : target (E : T) ratio of 3:1 for 2 h. The radioactivity of target cells was measured by a β-counter. The NK cytotoxicity against tumour cells was also determined by ^{51}Cr release assay. Target cells were incubated with emodin (c) and TBB (d) for 1 day. Then, the target cells were labelled with ^{51}Cr and incubated with NK at an E : T ratio of 3:1 for 4 h. The data are presented as a mean of at least three independent experiments (mean ± standard deviation).

Casein kinase 2 inhibition increases the NK cell-mediated cancer killing by a granule-independent process

In order to investigate whether or not the increase of NK cytotoxicity against cancer cells by CK2 inhibition is granule-dependent, NK cells were fixed mildly with 0·5% paraformaldehyde and the cytotoxicities against cancer cells in the presence or absence of CK2 inhibitor were compared. The mild fixation of NK cells or macrophages eliminates the granule release without affecting the structure and function of the cell surface receptors [7,8,49]. As reported previously [8], the fixed NK cells were almost as effective as the untreated NK cells in the JAM test against the HepG2, Hep3B and HeLa cells (Fig. 6, black bars). When the target cells were pretreated with emodin or TBB and co-incubated with NK cells, all three cancer cell lines exhibited almost the same amount of apoptotic cell death as in the cases of the untreated NK cells (Fig. 6, white bars and grey bars respectively). In order to confirm the increased apoptotic cell death induced by NK cells in the presence of TBB, caspase-3 and PARP cleavages were examined by immunoblotting (Fig. 6b). The Western blot analysis shown in Fig. 6b indicated that PARP was more cleaved in TBB-treated HepG2 cells when the cells were co-incubated with NK cells, although caspase-3 cleavage did not appear to be significantly changed. These results suggest that the increase of the NK-mediated apoptotic cancer cell death by CK2 inhibition is granule-independent and mediated possibly by the death ligands on the NK cell surface.

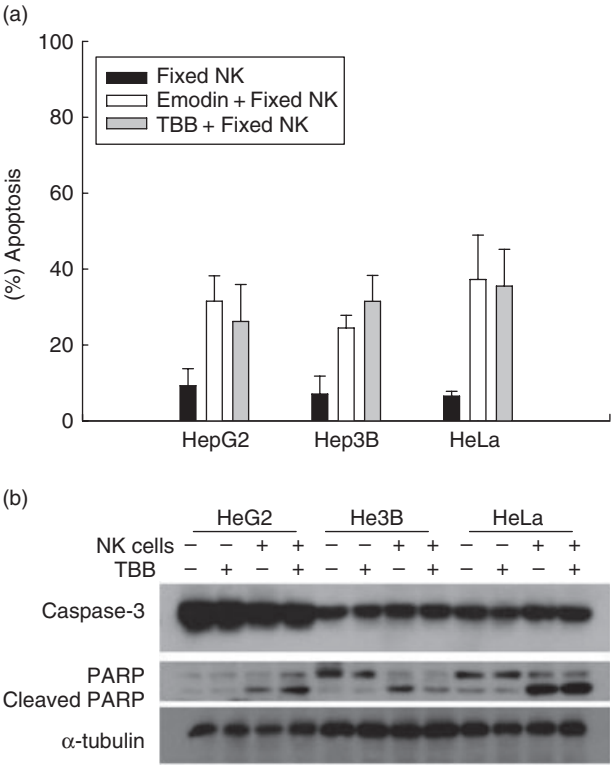

Fig. 6. Casein kinase 2 inhibition increased the natural killer (NK) cell-mediated cancer killing by a granule-independent apoptotic process. The fixed NK activity of target cell lysis was determined by JAM test. Target cells were labelled with [^{3}H]-thymidine for 1 day and pretreated with 10 μg/ml of emodin or 5 μM TBB. Primary NK cells were fixed by 0·5% paraformaldehyde for 20 min and then added to target cells at an E : T ratio of 3:1. Fixed NK cells and target cells were co-cultured for 2 h, and the radioactivity of target cells was measured by a β-counter. The data are presented as a mean of at least three independent experiments (mean ± standard deviation). (b) Immunoblot analysis of caspase3 and poly ADP-ribose polymerase (PARP) cleavage in the HepG2, Hep3B and HeLa cells. Target cells treated by 5 μM of TBB for 1 day were co-cultured with NK cells with E : T ratio of 3:1 for 2 h. The target cells were collected and lysed and Western blotting was performed with anti-caspase3 and anti-PARP antibodies.

Discussion

Recent studies have shown that increased expression of CK2 protects cancer cells from apoptotic cell death and the inhibition of CK2 sensitizes many cancer cells to death ligand-induced apoptosis [22,36,40–43]. In this study, we demonstrated that HCCs and HeLa cells could also be sensitized to death ligand-induced apoptosis by inhibition of CK2. Inhibition of CK2 by emodin increased the apoptotic cell death of HepG2, Hep3B and HeLa when the cancer cell lines were treated with a soluble form of rTRAIL or an antagonistic antibody of Fas. This phenomenon appeared to be correlated with the expression level of death receptors on the cancer cell surface. More interestingly, inhibition of CK2 also increased greatly the NK cell-mediated cancer cell killing. The NK cytotoxicity against the cancer cells increased about twofold when the target cells were pretreated with a CK2 inhibitor, emodin or TBB. Furthermore, the increase of the NK cytotoxicity against cancer cells by CK2 inhibition was granule-independent. These results suggest that NK cytotoxicity against cancer cells could be augmented *in vivo*, as well as *in vitro*, by inhibition of CK2 using a specific CK2 inhibitor.

Unlike normal hepatocytes, most HCCs are resistant to death receptor-mediated apoptosis when the cell surface death receptor is cross-linked *in vitro* with either agonistic antibodies or soluble forms of death ligand proteins [22–28]. The resistance of HCCs against death receptor-mediated apoptosis might be an essential step in escaping from host immune surveillance. Interestingly, however, our data showed that HCCs can be sensitized to death ligand-induced apoptosis by inhibiting the CK2 with emodin. CK2 inhibition by emodin appeared to increase rTRAIL-induced cell death significantly in HepG2 and Hep3B (Fig. 1), as well as increase the agonistic monoclonal anti-Fas antibody (CH11)-induced cell death in HepG2 (Fig. 3). The death ligand-induced apoptosis was generally proportional to the dose of the CK2 inhibitor (Fig. 2). Furthermore, death ligand-induced apoptotic cell death in the presence of CK2 inhibitor was also correlated with the death receptor

expression level on HCCs. These results suggest that CK2 also plays a critical role in the death receptor-mediated apoptosis of HCCs, as in other cancer cells.

It is well known that NK cells effectively kill many leukaemia cells and that the process is mediated primarily by perforin and granzymes [4,9–11]. Perforin induces the necrosis of the target cells and granzymes induce the apoptosis of the target cells [15–17]. More recent studies showed that NK cells can also effectively kill many solid tissue-derived tumour cells and that the non-secretory/apoptotic pathway plays a more important role when NK cells eliminate solid tumour cells [7,8]. The non-secretory/apoptotic pathway is mediated by FasL/Fas, TNF/TNF receptor and TRAIL/TRAIL receptor interactions [5,6,8,14–17], and the death receptor-mediated apoptotic pathway is also involved in the cytotoxicity of NK cells against many leukaemia cells [12,18]. Previously, we showed that NK cells can effectively destroy HCCs, like other solid tumours, and that the death receptor-mediated apoptotic pathway is involved in the NK cell-mediated HCC killing [8]. In this study, we demonstrated that the inhibition of CK2 by emodin or TBB augments NK cytotoxicity against HCCs and HeLa cells in the 2 h [^{3}H]-thymidine release assay (Fig. 5a and b), which measures primarily the extent of apoptotic target cell death [7,8,24]. Furthermore, we demonstrated that the mildly fixed NK cells are as effective as untreated NK cells in the cytotoxicity against target cells pretreated with emodin or TBB (Fig. 6a). Because the fixed NK cells cannot secrete the cytotoxic granules, it is highly likely that the increased NK cytotoxicity against cancer cells pretreated with the CK2 inhibitors might be mediated by interactions between death ligands on NK surface and death receptors on cancer cell surface. This is supported further by the fact that the CK2 inhibition of cancer cells did not increase the NK cytotoxicity against these target cells in the 4 h ^{51}Cr-release assay (Fig. 5c and d), which measures primarily necrotic cell death mediated by cytotoxic granules [7,8,24].

Like NK cells, cytotoxic T cells kill target cells by using the secretory cytotoxic granule-mediated pathway and the death receptor-mediated apoptotic pathway [18]. Because the death receptor-mediated apoptotic target cell killing mechanism of cytotoxic T cells is exactly the same as that of NK cells, it is tempting to speculate that the inhibition of CK2 could also enhance cytotoxic T cell-mediated target cell killing. This suggests that CK2 inhibitors could increase the host's innate and adaptive immunity against cancer by enhancing the cytotoxicity of NK cells and cytotoxic T cells which are armed with death ligands.

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References

1 Kiessling R, Klein E, Wigzell H. 'Natural' killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol 1975; **5**:112–17.
2 Trinchieri G. Biology of natural killer cells. Adv Immunol 1989; **47**:187–376.
3 Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007; **7**:329–39.
4 Shi L, Kraut RP, Aebersold R *et al.* A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J Exp Med 1992; **175**:553–66.
5 Kashii Y, Giorda R, Herberman RB *et al.* Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. J Immunol 1999; **163**:5358–66.
6 Lee RK, Spielman J, Zhao DY *et al.* Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. J Immunol 1996; **157**:1919–25.
7 Vujanovic NL, Nagashima S, Herberman RB *et al.* Nonsecretory apoptotic killing by human NK cells. J Immunol 1996; **157**:1117–26.
8 Kim HR, Park HJ, Park JH *et al.* Characteristics of the killing mechanism of human natural killer cells against hepatocellular carcinoma cell lines HepG2 and Hep3B. Cancer Immunol Immunother 2004; **53**:461–70.
9 Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity 1997; **6**:655–61.
10 Podack ER, Hengartner H, Lichtenheld MG, A. central role of perforin in cytolysis? Annu Rev Immunol 1991; **9**:129–57.
11 Yagita H, Nakata M, Kawasaki A *et al.* Role of perforin in lymphocyte-mediated cytolysis. Adv Immunol 1992; **51**:215–42.
12 Oshimi Y, Oshimi K, Miyazaki S. Necrosis and apoptosis associated with distinct Ca2+ response patterns in target cells attacked by human natural killer cells. J Physiol 1996; **495**:319–29.
13 Smyth MJ, Trapani JA. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today 1995; **16**:202–6.
14 Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med 1995; **181**:1235–8.
15 Screpanti V, Wallin RP, Ljunggren HG *et al.* A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. J Immunol 2001; **167**:2068–73.
16 Montel AH, Bochan MR, Hobbs JA *et al.* Fas involvement in cytotoxicity mediated by human NK cells. Cell Immunol 1995; **166**: 236–46.
17 Zamai L, Ahmad M, Bennett IM *et al.* Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. J Exp Med 1998; **188**:2375–80.
18 Oshimi Y, Oda S, Honda Y *et al.* Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. J Immunol 1996; **157**:2909–15.
19 Pitti RM, Marsters SA, Ruppert S *et al.* Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem 1996; **271**:12687–90.
20 Walczak H, Miller RE, Ariail K *et al.* Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat Med 1999; **5**:157–63.

21 Shin S, Lee Y, Kim W *et al.* Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. EMBO J 2005; **24**:3532–42.

22 Shin EC, Shin WC, Choi Y *et al.* Effect of interferon-gamma on the susceptibility to Fas (CD95/APO-1)-mediated cell death in human hepatoma cells. Cancer Immunol Immunother 2001; **50**:23–30.

23 Higaki K, Yano H, Kojiro M. Fas antigen expression and its relationship with apoptosis in human hepatocellular carcinoma and noncancerous tissues. Am J Pathol 1996; **149**:429–37.

24 Kim YS, Schwabe RF, Qian T *et al.* TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. Hepatology 2002; **36**:1498–508.

25 Nagao M, Nakajima Y, Hisanaga M *et al.* The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance *in vivo*? Hepatology 1999; **30**:413–21.

26 Natoli G, Ianni A, Costanzo A *et al.* Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene 1995; **11**:1157–64.

27 Shin EC, Seong YR, Kim CH *et al.* Human hepatocellular carcinoma cells resist to TRAIL-induced apoptosis, and the resistance is abolished by cisplatin. Exp Mol Med 2002; **34**:114–22.

28 Yamanaka T, Shiraki K, Sugimoto K *et al.* Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. Hepatology 2000; **32**:482–90.

29 Padmanabha R, Chen-Wu JL, Hanna DE *et al.* Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. Mol Cell Biol 1990; **10**:4089–99.

30 Kikkawa U, Mann SK, Firtel RA *et al.* Molecular cloning of casein kinase II alpha subunit from *Dictyostelium discoideum* and its expression in the life cycle. Mol Cell Biol 1992; **12**:5711–23.

31 Luscher B, Kuenzel EA, Krebs EG *et al.* Myc oncoproteins are phosphorylated by casein kinase II. EMBO J 1989; **8**:1111–19.

32 Allende JE, Allende CC. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB J 1995; **9**:313–23.

33 McElhinny JA, Trushin SA, Bren GD *et al.* Casein kinase II phosphorylates I kappa B alpha at S-283, S-289, S-293, and T-291 and is required for its degradation. Mol Cell Biol 1996; **16**:899–906.

34 Wang D, Westerheide SD, Hanson JL *et al.* Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J Biol Chem 2000; **275**:32592–7.

35 Li PF, Li J, Muller EC *et al.* Phosphorylation by protein kinase CK2: a signaling switch for the caspase-inhibiting protein ARC. Mol Cell 2002; **10**:247–58.

36 Desagher S, Osen-Sand A, Montessuit S *et al.* Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. Mol Cell 2001; **8**:601–11.

37 Munstermann U, Fritz G, Seitz G *et al.* Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. Eur J Biochem 1990; **189**:251–7.

38 Faust RA, Niehans G, Gapany M *et al.* Subcellular immunolocalization of protein kinase CK2 in normal and carcinoma cells. Int J Biochem Cell Biol 1999; **31**:941–9.

39 Landesman-Bollag E, Romieu-Mourez R, Song DH *et al.* Protein kinase CK2 in mammary gland tumorigenesis. Oncogene 2001; **20**:3247–57.

40 Guo C, Yu S, Davis AT *et al.* A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. J Biol Chem 2001; **276**:5992–9.

41 Izeradjene K, Douglas L, Delaney A *et al.* Influence of casein kinase II in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human rhabdomyosarcoma cells. Clin Cancer Res 2004; **10**:6650–60.

42 Izeradjene K, Douglas L, Delaney A *et al.* Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. Oncogene 2005; **24**:2050–8.

43 Ravi R, Bedi A. Sensitization of tumor cells to Apo2 ligand/TRAIL-induced apoptosis by inhibition of casein kinase II. Cancer Res 2002; **62**:4180–5.

44 Matzinger P. The JAM test. A simple assay for DNA fragmentation and cell death. J Immunol Methods 1991; **145**:185–92.

45 Yamada M, Katsuma S, Adachi T *et al.* Inhibition of protein kinase CK2 prevents the progression of glomerulonephritis. Proc Natl Acad Sci USA 2005; **102**:7736–41.

46 Itoh N, Yonehara S, Ishii A *et al.* The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991; **66**:233–43.

47 Sarno S, Reddy H, Meggio F *et al.* Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). FEBS Lett 2001;**496**:44–8.

48 Sarno S, Ruzzene M, Frascella P *et al.* Development and exploitation of CK2 inhibitors. Mol Cell Biochem 2005; **274**:69–76.

49 Kurt-Jones EA, Beller DI, Mizel SB *et al.* Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci USA 1985; **82**:1204–8.