Leukemia (2009), 1–10
© 2009 Macmillan Publishers Limited All rights reserved 0887-6924/09 $32.00
www.nature.com/leu

ORIGINAL ARTICLE

# Heat stress triggers apoptosis by impairing NF-κB survival signaling in malignant B cells

G Belardo[1,2], R Piva[3] and MG Santoro[1,2]

[1]Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, Italy; [2]Institute of Neurobiology and Molecular Medicine, Consiglio Nazionale delle Ricerche, via Fosso del Cavaliere 100, Rome, Italy and [3]Department of Biomedical Sciences and Human Oncology, Center for Experimental Research and Medical Studies (CERMS), University of Turin, Turin, Italy

**Nuclear factor-κB (NF-κB) is involved in multiple aspects of oncogenesis and controls cancer cell survival by promoting anti-apoptotic gene expression. The constitutive activation of NF-κB in several types of cancers, including hematological malignancies, has been implicated in the resistance to chemo- and radiation therapy. We have previously reported that cytokine- or virus-induced NF-κB activation is inhibited by chemical and physical inducers of the heat shock response (HSR). In this study we show that heat stress inhibits constitutive NF-κB DNA-binding activity in different types of B-cell malignancies, including multiple myeloma, activated B-cell-like (ABC) type of diffuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma presenting aberrant NF-κB regulation. Heat-induced NF-κB inhibition leads to rapid downregulation of the anti-apoptotic protein cellular inhibitor-of-apoptosis protein 2 (cIAP-2), followed by activation of caspase-3 and cleavage of the caspase-3 substrate poly(adenosine diphosphate ribose)-polymerase (PARP), causing massive apoptosis under conditions that do not affect viability in cells not presenting NF-κB aberrations. NF-κB inhibition by the proteasome inhibitor bortezomib and by short-hairpin RNA (shRNA) interference results in increased sensitivity of HS-Sultan B-cell lymphoma to hyperthermic stress. Altogether, the results indicate that aggressive B-cell malignancies presenting constitutive NF-κB activity are sensitive to heat-induced apoptosis, and suggest that aberrant NF-κB regulation may be a marker of heat stress sensitivity in cancer cells.**

*Leukemia* advance online publication, 19 November 2009; doi:10.1038/leu.2009.227

**Keywords:** apoptosis; cIAP-2; heat shock factor; heat stress; NF-κB

## Introduction

Nuclear factor-κB (NF-κB) transcription factors comprise a family of critical regulators of the innate and adaptive immune response that have an important role in promoting inflammation,[1] in the regulation of virus replication[2] and in the control of cell proliferation and survival.[3] NF-κB normally exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (RelA) subunits, bound to inhibitory proteins of the IκB family (IκBs), and is induced in response to a variety of pathogenic stimuli, including ultraviolet radiation, exposure to proinflammatory cytokines or mitogens, and to bacterial or viral infection.[1–3] Induction usually requires the activation of the IκB kinase (IKK) complex, which is composed of two catalytic subunits (IKKα and IKKβ) and the IKKγ/NEMO regulatory subunit. In the classical pathway, the activation of IKKβ causes the phosphorylation of IκBs at sites that trigger their polyubiquitination and degradation by the 26S proteasome complex. An alternative pathway responds to the engagement of receptors for cytokines, such as lymphotoxin-β or CD40, through the involvement of IKKα homodimers.[4] Both pathways ultimately elicit the degradation of the NF-κB inhibitory peptides, resulting in nuclear translocation of NF-κB dimers and their binding to DNA at specific κB sites, rapidly inducing a variety of genes encoding, among others, cell adhesion molecules, inflammatory and chemotactic cytokines, cytokine receptors and enzymes that produce inflammatory mediators.[1]

Nuclear factor-κB activation has been connected with multiple aspects of oncogenesis, including the control of cell migration, cell-cycle progression and differentiation, as well as apoptosis.[5] NF-κB generally acts as an anti-apoptotic factor, and suppresses cell death pathways by switching on genes that hinder pro-apoptotic signals, including members of the B-cell leukemia/lymphoma 2 (Bcl-2) family, tumor necrosis factor (TNF) receptor-associated factors 1 and 2, the FADD-like IL-1β-converting enzyme-inhibitory protein cFLIP, cellular inhibitor-of-apoptosis proteins 1 and 2 (cIAP-1 and cIAP-2) and X-chromosome-linked inhibitor-of-apoptosis protein (XIAP).[3] NF-κB-driven anti-apoptotic action confers a tremendous advantage in the clonal selection of neoplastic cells. In fact, sustained constitutive NF-κB activity has been found in several types of neoplastic cells, including various hematological malignancies.[6] In addition, activation of NF-κB in cancer cells by chemotherapy or by radiation has been shown to induce the multi-drug resistance response, which impinges on the ability of the therapy itself to induce cell death.[7] In this perspective, inhibition of NF-κB is expected to be therapeutic in those tumors in which NF-κB seems to have a unique survival role, such as multiple myeloma,[8] Hodgkin's and non-Hodgkin's B-cell lymphomas[9–11] and Burkitt's lymphoma.[12]

Our previous studies on the biological activity of cyclopentenone prostanoids led to the discovery of a cross-talk between NF-κB and heat shock factor type-1 (HSF1), a transcription factor with a major role in the control of the heat shock response (HSR).[13] In humans, the HSR is regulated by a family of three functionally different heat shock transcription factors.[14–16] HSF1, which mediates signaling of stress-induced stimuli, such as elevated temperatures, is generally found in the cytoplasm as an inert monomer lacking transcriptional activity. After exposure to heat shock and other types of stress, monomers oligomerize to a trimeric state, form stress-induced intranuclear granules and bind to heat shock element (HSE) sequences that are located upstream of heat shock-responsive genes, switching on

Correspondence: Dr MG Santoro, Department of Biology, University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, 00133, Italy.
E-mail: santoro@bio.uniroma2.it
Received 13 November 2008; revised 30 September 2009; accepted 2 October 2009

stress-induced transcription of heat shock proteins (HSP), including members of the HSP70 and HSP90 families, HSP27 and other proteins of the network.[17,18] HSPs act as molecular chaperones in assisting the correct folding, assembly, and intracellular transport of nascent proteins, as well as in circumventing stress-induced degradation and aggregation of misfolded proteins that could be toxic to the cell.[19,20] As HSP synthesis increases, HSP70 and other chaperones relocalize in the nucleus and bind to HSF1, leading to dissociation of trimers and attenuation of the response.[18,19]

We have previously shown that different chemical and physical inducers of the HSR, including cyclopentenone prostanoids, sodium arsenite, serine protease inhibitors as well as heat shock itself, prevent NF-κB activation triggered by cytokines, mitogens or viruses.[13,21,22] Starting from these observations, in this study we analyzed whether heat stress may also inhibit constitutive NF-κB activity, and examined its effect on the survival of B-cell neoplasms.

## Materials and methods

### *Cell culture, treatments and* in vitro *transduction*

Eleven human cell lines were used for this study: Burkitt's lymphoma HS-Sultan and BL-41, acute leukemia K562 cells and multiple myeloma U266 (obtained from the American Tissue Culture Collection, Manassas, VA, USA); KMM1 and RPMI 8226 multiple myeloma (kindly provided by Professor A Neri, Policlinico di Milano, Milan, Italy); activated B-cell-like (ABC) OCI-Ly10 and U2932, and germinal center B-cell-like (GCB) OCI-Ly7 and OCI-Ly19 diffuse large B-cell lymphoma (DLBCL; kindly provided by Dr F Bertoni, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland). In addition, a clone of drug-resistant JJN3 multiple myeloma cells (JJN3r), not presenting NF-κB aberrations,[23] was used. Cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% carbon dioxide. For heat treatments, cells ($5 \times 10^5$cells/ml) were subjected to heat shock at the indicated temperatures in a precision water bath-W14 (Grant Instruments, Cambridge, UK) at 24 h after plating. TNF-α, cycloheximide and dexamethasone were purchased from Sigma (St Louis, MO, USA) and bortezomib (Velcade) was from Millennium Pharmaceuticals (Cambridge, MA, USA). Construction of lentiviral vectors LV-shp65, containing the human p65-specific short-hairpin RNA (shRNA) oligonucleotide, and LV-shC, containing the mutated p65 target sequence, was described previously.[24] High-titer lentiviral vector stock was produced in 293T cells by calcium phosphate-mediated transfection of the modified transfer vector and the packaging vectors, pMDLg/pRRE, pRSV-Rev and pMD2.VSVG. Virus harvested at 60 h was concentrated using ultracentrifugation (50 000 × *g*). Virus titers were determined by measuring the amount of human immunodeficiency virus type 1 p24 antigen using enzyme-linked immunosorbent assay (PerkinElmer Life Science, Boston, MA, USA). HS-Sultan and K562 cells ($1 \times 10^5$) were transduced with 300 ng of lentiviral p24 in the presence of polybrene (8 μg/ml).

### *Western blot analysis*

Equal amounts of protein (20 μg/sample) from whole-cell extracts prepared after lysis in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM $Na_3VO_4$ and 1 mM phenylmethyl-sulfonyl-fluoride and protease inhibitors) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and filters were incubated with the primary antibodies, followed by decoration with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (1:10 000; Amersham, Arlington Heights, IL, USA). Immunocomplex detection was performed using enhanced chemiluminescence system (SuperSignal-West, Pierce, Rockford, IL, USA), and images were captured using Luminescent-Image-Analyzer LAS-3000 (Fujifilm Corporation, Tokyo, Japan). Monoclonal antibodies to α-tubulin were purchased from Sigma, to HSP70 from Stressgen (Victoria, BC, Canada) and to Bcl-2 from Upstate (Millipore Corporation, MA, USA). Rabbit polyclonal antibodies to p65, poly(adenosine diphosphate ribose)polymerase (PARP), and to caspase-3 (procaspase and activated fragments p19/17) were purchased from Cell Signaling Technology (Beverly, MA, USA), and to cIAP-2 and XIAP from R&D Systems (Minneapolis, MN, USA).

### *Electrophoretic mobility shift assay (EMSA)*

Aliquots of total extracts (12 μg protein/sample) in 0.1% Triton X-100 lysis buffer were incubated with $^{32}$P-labeled κB[25] or HSE[26] DNA probes in binding buffer for 30 min as described.[26] DNA–protein complexes were analyzed using nondenaturing 4% polyacrylamide gel electrophoresis. To determine the specificity of NF-κB-DNA complexes, whole-cell extracts from HS-Sultan cells were preincubated with 200 ng of polyclonal antibodies specific for c-Rel, p65, p50 and p52 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for c-Jun (Santa Cruz Biotechnology), as an unrelated antibody for 1 h before electromobility supershift assay. Quantitative evaluation of NF-κB-κB and HSF-HSE complex formation was determined using Typhoon-8600 imager (Molecular Dynamics Phoshor-Imager, MDP) (Amersham Biosciences, Piscataway, NJ, USA) and ImageQuant software (Amersham Biosciences) (MDP analysis). For control of equal loading, NF-κB and HSF values were normalized to the level of the nonspecific protein–DNA complex in the same lane.

### *Analysis of apoptosis*

For annexin-V staining, cells were suspended in staining buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) with 5 μl annexin V-fluorescein isothiocyanate or annexin V-allophycocyanin conjugate for the analysis in p65-interfered cells (Becton-Dickinson, San Jose, CA, USA). Alternatively, cells were stained with the mitochondrion-permeable, voltage-sensitive dye tetramethylrodamine methyl ester (Molecular Probes, Eugene, OR, USA) for the analysis of apoptotic DLBCL cells.[23] After 15 min of incubation in the dark, cells were analyzed by flow cytometry (FACS) using FACScan (Becton-Dickinson) and evaluated using CellQuest Program (Becton-Dickinson).

### *Reverse transcription-PCR*

Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and processed as described.[27] Primers for HSP70 were 5′-CAGGTGATCAA-C GACGGAGACA-3′ (sense) and 5′-GTCGAT-CGTCAGGATGG ACACG-3′ (antisense), and for Bcl-2 were 5′-CACCTGTGGTCC ACCTGAC-3′ (sense) and 5′- TTGACGCTCTCCACACACAT-3′ (antisense). Primers for cIAP-2 and glyceraldehyde 3-phosphate dehydrogenase were described previously.[27]

## Results and Discussion

### *Hyperthermic stress inhibits constitutive NF-κB activity in HS-Sultan B-cell lymphoma*

Burkitt's lymphoma is an aggressive B-cell tumor, which often presents aberrations in NF-κB regulation, whose hallmark is the activation of the *c-myc* oncogene through a reciprocal translocation that juxtaposes the *c-myc* gene on chromosome-8 to one of the immunoglobulins loci.[28] It has been shown that NF-κB activity is required for *c-myc* expression under the control of the immunoglobulin heavy chain enhancer, suggesting that interference with NF-κB function may represent a successful approach to Burkitt's lymphoma treatment.[28] To analyze the effect of heat stress on constitutive NF-κB DNA-binding activity, we used as a model the HS-Sultan Burkitt's lymphoma cell line, which was previously shown to present aberrant NF-κB regulation.[8,24] HS-Sultan cells were either kept at 37 °C or subjected to hyperthermic treatment (HT) at 41, 43 or 45 °C for 45 min. After different periods of recovery at 37 °C, whole-cell extracts were analyzed for HSF and NF-κB DNA-binding activity using EMSA (Figure 1). Exposure to 43 and 45 °C strongly activated HSF (10- and 12- to 15-fold, respectively), whereas treatment at 41 °C caused only a modest activation of HSF (50% above control), as demonstrated by MDP analysis (Figure 1a, bottom). As expected according to the transient nature of HSF1 activation,[29] both the intensity and the length of HSF activation were dependent on the temperature used. After exposure to 41 and 43 °C, HSF DNA-binding activity returned to control level after 2 h recovery at 37 °C, whereas after treatment at 45 °C HSF activation persisted at elevated levels for at least 3 h.

As expected, HS-Sultan cells were found to show constitutively high NF-κB DNA-binding activity. The major NF-κB band in HS-Sultan cells was shown to consist of the p65/RelA subunit.[24] HT was found to cause an inhibition of constitutive NF-κB activity, which was inversely correlated with HSF activation in HS-Sultan cells. NF-κB activity was not significantly altered in HS-Sultan cells heat-shocked at 41 °C; HT at 43 °C resulted in a transient inhibition of NF-κB, whereas HT at 45 °C resulted in a marked and persistent inhibition of constitutive NF-κB DNA-binding activity (Figure 1b). The levels of the RelA/p65 NF-κB subunit were not altered under these conditions (data not shown).

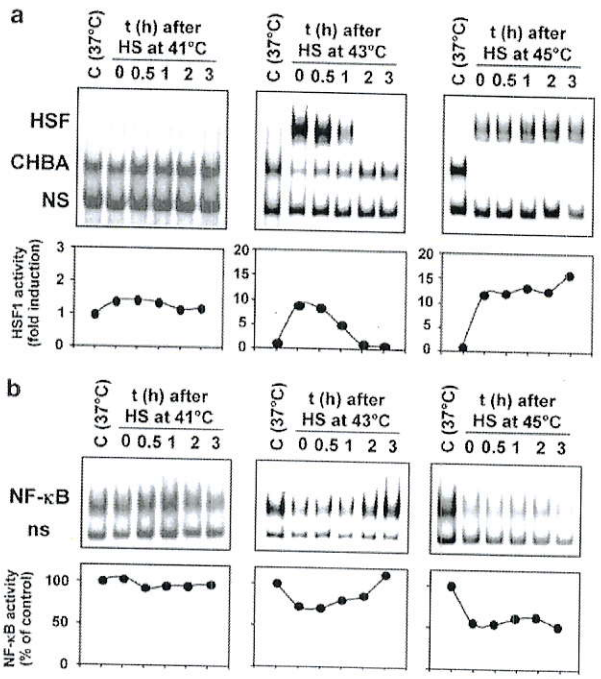


**Figure 1** Temperature-dependent inhibition of constitutive NF-κB activity is associated with heat shock factor type-1 (HSF1) activation. HS-Sultan cells were either kept at 37 °C or subjected to heat shock for 45 min at 41, 43, and 45 °C. After the indicated periods of recovery at 37 °C, whole-cell extracts were analyzed for HSF (**a**) or NF-κB (**b**) DNA-binding activity using EMSA. Positions of HSF-HSE complex (HSF), constitutive HSE-binding activity (CHBA) and nonspecific protein-DNA interaction (NS) are indicated (**a**, top). Positions of NF-κB-DNA (NF-κB) and nonspecific protein-DNA (ns) complexes are indicated (**b**, top). The levels of HSF and NF-κB DNA-binding activity were quantitated using MDP analysis (**a, b**, bottom) and expressed as fold induction (**a**) or percentage (**b**) of control at 37 °C. The results are representative of two independent experiments with similar results.

### *Heat-induced NF-κB inhibition is rapidly followed by apoptosis in HS-Sultan cells*

The relationship between HSR activation and inhibition of constitutive NF-κB was further analyzed in HS-Sultan cells and compared with the effect on human acute leukemia K562 cells, which show low basal level of NF-κB activity.[24] HS-Sultan and K562 cells were subjected to HT at 41, 43 or 45 °C for 45 min. After a 3-h recovery period at 37 °C, whole-cell extracts were analyzed for HSF and NF-κB activity using EMSA. As expected at this time of recovery, HSF1 activation was detected only in HS-Sultan cells subjected to 45 °C HT (Figure 2a, top). Under these conditions, NF-κB activity was markedly inhibited (Figure 2a, middle), confirming the results described above. K562 cells displayed a HSR similar to HS-Sultan cells with a strong activation of HSF at 45 °C (Figure 2a, top). NF-κB DNA-binding activity was barely detectable in K562 cells at 37 °C, and no appreciable difference was detected at any of the temperatures tested (Figure 2a, middle). Next, we analyzed the levels of apoptosis in heat-shocked HS-Sultan and K562 cells using FACS analysis of annexin $V^+$ cells at 24 h after heat exposure. Concomitant with NF-κB inhibition, a tenfold increase in apoptotic cells was detected in HS-Sultan cells subjected to 45 °C HT, as compared with untreated cells (Figure 2a, bottom). The increase in apoptotic cells was also detected using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) analysis (data not shown). Conversely, K562 cells were resistant to apoptosis induction at all temperatures tested.

The effects of hyperthermic treatment are strictly dependent on the increase in temperature above physiological conditions and the duration of exposure. Consequently, we analyzed the effect of temperature versus length of treatment on HSF and NF-κB activity, and induction of apoptosis in HS-Sultan and K562 cells. Cells were subjected to 45 °C HT for 20, 30 or 45 min and, after 3-h recovery at 37 °C, whole-cell extracts were analyzed for HSF and NF-κB activity. As shown in Figure 2b, HSF was strongly induced in both cell lines independently of the time of exposure; a 20-min HT at 45 °C was sufficient to markedly inhibit constitutive NF-κB activity in HS-Sultan cells, whereas no effect was found in K562 cells (Figure 2b, top). Analysis of annexin $V^+$ cells at 6 and 24 h after HT confirmed that K562 cells are resistant to 45 °C HT, whereas in HS-Sultan cells a 20-min HT at 45 °C was sufficient to induce a ninefold increase in apoptotic cells at 24 h after treatment (Figure 2b, bottom). An increase in heat exposure time (30 and 45 min)

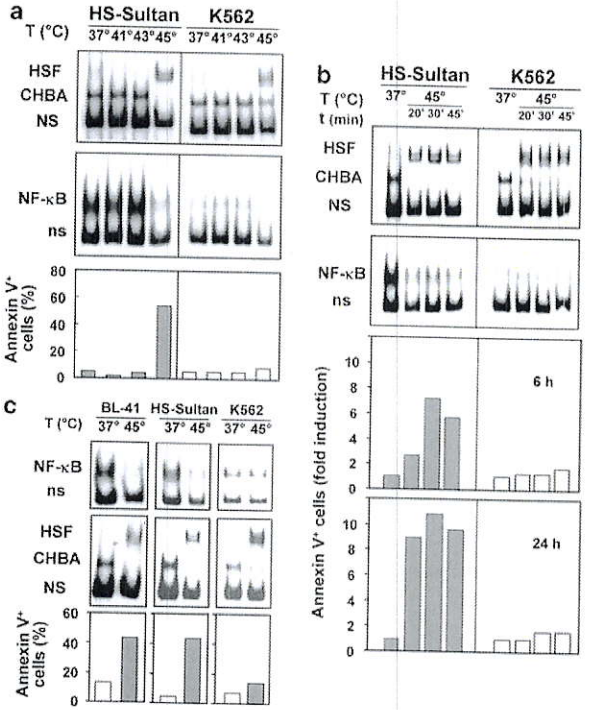


**Figure 2** Temperature-dependent NF-κB inhibition is associated with induction of apoptosis in HS-Sultan B-cell lymphoma. HS-Sultan and K562 cells were either kept at 37 °C or subjected to heat shock for 45 min at the indicated temperatures. (**a**) After 3-h recovery at 37 °C, whole-cell extracts were analyzed for heat shock factor (HSF; top) or NF-κB (middle) activity using EMSA. At 24 h after hyperthermic treatment (HT), apoptosis was determined in parallel samples using FACS analysis of annexin $V^+$ cells (bottom). (**b**) HS-Sultan and K562 cells were subjected to HT at 45 °C for the indicated periods. After 3-h recovery at 37 °C, whole-cell extracts were analyzed for HSF or NF-κB activity. In parallel samples, the number of annexin $V^+$ cells was determined at 6 and 24 h after HT, and expressed as fold induction of untreated controls. (**c**) BL-41, HS-Sultan and K562 cells that were kept at 37 °C or subjected to heat shock (45 min at 45 °C) were analyzed for NF-κB (top) or HSF (middle) activity after 3-h recovery at 37 °C. The number of annexin $V^+$ cells was determined at 24 h after HT, and expressed as a percentage of untreated controls (bottom). The results are representative of two independent experiments with similar results.

resulted in a marked enhancement of annexin $V^+$ cells already at 6 h after treatment.

Temperature and duration of heat exposure are known to be regulated in a way that a decline of 1°C can be compensated by doubling the exposure time for temperatures above 43 °C.[30] In this context, we studied the effect of exposure of Burkitt's lymphoma cells at temperatures below 45 °C for different time intervals. HS-Sultan cells were subjected to HT at 43 °C for 90, 120 or 150 min, at 44 °C for 30, 60 or 90 min, or at 45 °C for 30 min as a positive control. Aliquots of cells were analyzed for HSF and NF-κB activity at 3 h after the end of treatment; parallel samples were collected at 24 h after treatment to determine the level of the caspase-3 substrate PARP as a marker of apoptosis. As shown in Supplementary Figure 1a, in cells exposed to 43 °C up to 150 min, HSF activity returned to basal levels after a 3-h recovery period. On the other hand, HSF activation persisted up to 3 h after the end of treatment in cells exposed to 44 °C for 60 or 90 min, and to 45 °C for 30 min. As expected, a short period at 45 °C was sufficient to cause NF-κB inhibition. The decrease of 1°C in temperature required prolonging the exposure time to 60 min to obtain complete inhibition of NF-κB activity. No significant NF-κB inhibition was detected at 43 °C up to 150 min. Under all conditions, prolonged NF-κB inhibition was associated with apoptosis induction in HS-Sultan cells, as indicated by PARP cleavage (Supplementary Figure 1b).

### *Heat stress inhibits constitutive NF-κB activity and induces apoptosis in different types of B-cell malignancies*

We then analyzed whether heat stress could induce apoptosis in other types of malignant B cells and whether this effect was associated with NF-κB inhibition. First, we determined the status of constitutive NF-κB activity in a different Burkitt's lymphoma cell line, BL-41, which was previously reported to present aberrant NF-κB regulation.[24] BL-41, HS-Sultan and K562 cells were subjected to 45 °C HT for 45 min. HSF and NF-κB activity was analyzed after a 3-h recovery at 37 °C, whereas apoptosis was determined in parallel samples at 24 h after treatment. As shown previously,[24] BL-41 cells expressed constitutively high levels of NF-κB activity similar to HS-Sultan cells (Figure 2c, top). Exposure to 45 °C HT induced HSF activation in all cell lines (Figure 2c, middle), and markedly inhibited constitutive NF-κB activity in BL-41 and HS-Sultan cells, whereas no effect was found in K562 cells (Figure 2c, top). Concomitantly, HT caused a significant increase in the number of annexin $V^+$ cells in BL-41 and HS-Sultan cells, but not in K562 cells (Figure 2c, bottom).

There is genomic evidence that aberrant NF-κB activity is a hallmark of aggressive lymphoma subtypes classified as DLBCL, and in particular in the ABC lymphomas.[11,31–33] To establish whether heat stress could induce apoptosis in DLBCL cells also and whether this effect was associated with NF-κB inhibition, we used four DLBCL cell lines with different patterns of NF-κB activation: two ABC cell lines (OCI-Ly10 and U2932) with high levels of constitutive NF-κB activity, and two GCB cell lines (OCI-Ly7 and OCI-Ly19) that did not present this type of aberration.[33] OCI-Ly10, U2932, OCI-Ly7 and OCI-Ly19 cells were subjected to 45 °C HT for 30 min. HSF and NF-κB activity was analyzed after a 3-h recovery at 37 °C, whereas apoptosis was determined in parallel samples at 7 h after treatment by determining the number of tetramethylrodamine methyl ester-negative cells, and the level of PARP cleavage. As expected, OCI-Ly10 and U2932 cells, but not OCI-Ly7 and OCI-Ly19 cells, expressed constitutively high levels of NF-κB DNA-binding activity (Figure 3a, top). Exposure to 45 °C HT induced HSF activation in all cell lines, and markedly inhibited constitutive NF-κB activity in OCI-Ly10 and U2932 cells (Figure 3a). ABC cells resulted to be much more sensitive to heat stress than GCB cells, undergoing massive apoptosis at 7 h after HT (Figures 3b and c).

Finally, we also analyzed the effect of heat stress in several cell lines of multiple myeloma (MM), a different type of aggressive B-cell malignancy characterized by constitutively high levels of NF-κB. U266, RPMI-8226, KMM1 and JJN3r MM cells were subjected to 45 °C HT for 30 min. HSF and NF-κB activity was analyzed after a 3-h recovery at 37 °C, whereas apoptosis was determined at 24 h after treatment. As expected, high levels of NF-κB activity were detected in U266, RPMI-8226 and KMM1 cells, whereas JJN3r did not present this aberration (Supplementary Figure 2a, top). Also in this case HT induced HSF activation in all cell lines and markedly inhibited NF-κB DNA-binding activity in U266, RPMI-8226 and KMM1 cells (Supplementary Figure 2a). Concomitantly, HT strongly induced

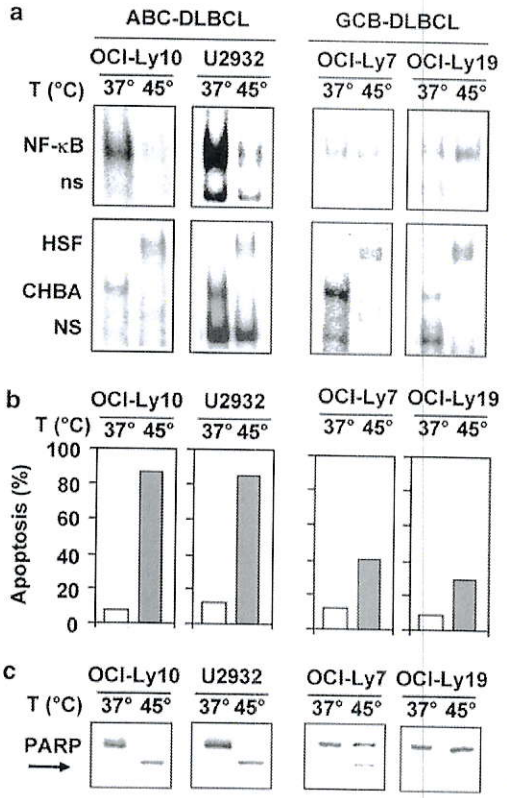


**Figure 3** Activated B-cell-like (ABC)-diffuse large-B-cell lymphomas (DLBCL) are highly sensitive to heat-induced apoptosis. ABC DLBCL OCI-Ly10 and U2932 cell lines, and germinal center B-cell-like (GCB) DLBCL OCI-Ly7 and OCI-Ly19 cells that were kept at 37 °C or subjected to heat shock (30 min at 45 °C) were analyzed for NF-κB (top) or HSF (bottom) DNA-binding activity using EMSA after 3-h recovery at 37 °C (**a**). The number of apoptotic cells was determined using flow cytometry after staining with tetramethylrodamine methyl ester (TMRM) at 7 h after hyperthermic treatment (**b**). In parallel samples, poly (ADP-ribose) polymerase (PARP) levels were determined using western blot analysis (**c**). The arrow indicates the 85 kDa PARP apoptotic fragment. The results are representative of two independent experiments with similar results.

apoptosis in U266, RPMI-8226 and KMM1 cells, whereas it had no effect in JJN3r cells (Supplementary Figure 2b). Altogether, these results indicate that aggressive B-cell malignancies presenting NF-κB-dependent disruption of survival signaling are particularly sensitive to heat-induced apoptosis.

### *Heat-induced apoptosis is independent of protein synthesis*

The results described above show that inhibition of constitutive NF-κB activity is strictly associated with HSF activation, as previously shown for mitogen-, cytokine- or virus-induced NF-κB activation.[13,21,34] Despite the large amount of literature describing a cross-talk between the HSF and NF-κB pathways, the molecular mechanism at the basis of this interaction remains elusive. Several HSPs have been indicated as possible mediators of NF-κB inhibition. In particular, HSP70, one of the main members of the HSP family, was recently shown to promote TNF-mediated apoptosis by binding to the IKKγ-subunit of the IKK signalosome and inhibiting NF-κB-dependent survival signaling.[35] To analyze whether expression of newly synthesized protein, and in particular HSP70, was necessary to trigger the pro-apoptotic signal in heat-shocked HS-Sultan cells, K562 and HS-Sultan cells were treated with cycloheximide (100 μg/ml) or vehicle for 30 min at 37 °C, and then subjected to 45 °C HT for 45 min. After 3 h at 37 °C, whole-cell extracts were analyzed for HSF and NF-κB activity. HSP70 levels and the number of annexin $V^+$ cells were determined at 24 h after HT in parallel samples. As expected, heat shock induced HSF activation and HSP70 expression in both HS-Sultan and K562 cells, whereas it completely inhibited NF-κB activity in HS-Sultan cells, resulting in massive apoptosis selectively in the Burkitt's lymphoma cell line (Supplementary Figures 3a and b). Cycloheximide treatment did not inhibit HSF activation, but prevented HSP70 synthesis and resulted in a modest apoptosis increase in both cell types. Interestingly, cycloheximide was not able to prevent the HT-induced block of NF-κB activity and the consequent apoptosis in HS-Sultan cells (Supplementary Figures 3a and b, bottom). These results indicate that heat-induced inhibition of constitutive NF-κB activity does not require *de novo* protein synthesis, and is independent of the synthesis of HSP70 or other molecular chaperones.

### *Heat-induced NF-κB inhibition leads to downregulation of anti-apoptotic protein cIAP-2*

The presence of apoptotic HS-Sultan cells early (6 h) after heat stress (Figure 2b) prompted us to analyze the possible mechanisms triggering cell death during the first few hours after heat exposure. HS-Sultan cells were subjected to 45 °C HT for 45 min, and analyzed for HSF and NF-κB activity at different times after recovery at 37 °C. Levels of the RelA/p65 NF-κB subunit, PARP, caspase-3 autocatalytic products p19/17 and different anti-apoptotic proteins were determined in parallel samples using western blot. As shown in Figure 4a, under these conditions HSF1 DNA-binding activity was sustained for at least 3 h and remained detectable up to 6 h after HT; in the same samples, NF-κB DNA-binding activity was inhibited immediately after heat treatment and for at least 8 h, whereas levels of the RelA/p65 NF-κB subunit were not altered. Interestingly, a caspase-3-dependent apoptosis was induced as soon as 2–3 h after heat stress, as indicated by the cleavage of PARP and processing of procaspase-3 (Figures 4a and c). Rapid caspase activation may be in part responsible for prolonged NF-κB inhibition, as the regulatory subunit of IKK (IKKγ or NEMO) has been shown to be proteolysed by cellular caspases, leading to NF-κB inactivation.[36]

We then evaluated the effect of heat shock on the level of cIAP-2 and XIAP anti-apoptotic proteins, which are direct inhibitors of caspase-3 activity and whose expression is transcriptionally regulated by NF-κB,[37] in the same samples. As shown in Figures 4a and c, cIAP-2 levels were rapidly reduced in HS-Sultan cells with kinetics similar to caspase-3 activation and PARP cleavage. XIAP levels were also decreased, but at later times (6 h) after treatment, whereas Bcl-2 levels remained unchanged (Figure 4a). To analyze whether the decrease in cIAP-2 protein was a consequence of reduced mRNA levels after heat shock, cIAP-2, Bcl-2 and HSP70 mRNA was evaluated in HS-Sultan cells at different times after HT using reverse transcriptase-PCR. As shown in Figure 4b, the amount of the HSF-driven HSP70 mRNA increased in a time-dependent manner after treatment, as expected. No significant change in Bcl-2 mRNA level was observed; conversely, cIAP-2 mRNA level was dramatically decreased as soon as 30 min after heat shock and remained barely detectable for the following 6 h.

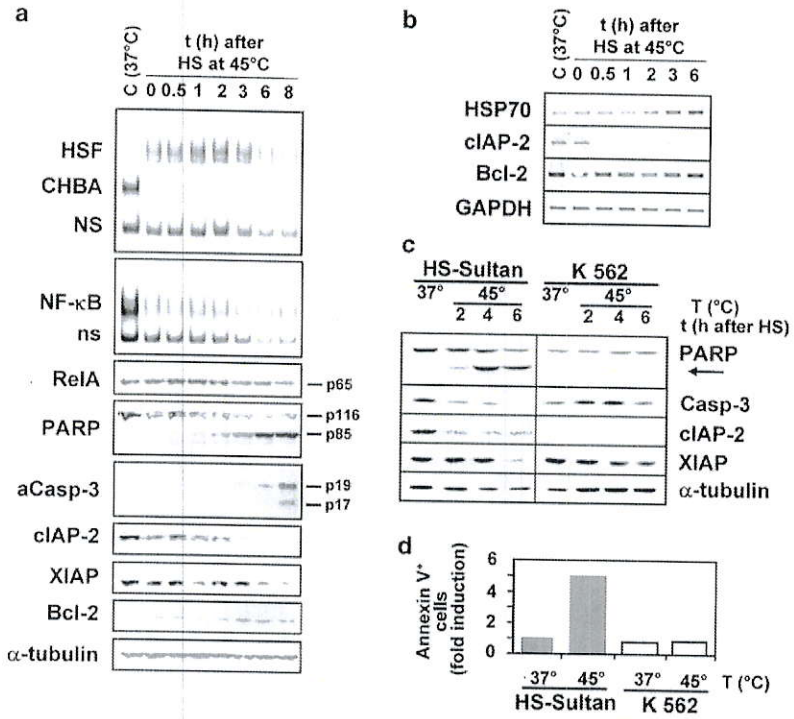


**Figure 4** Hyperthermic stress downregulates the expression of inhibitor-of-apoptosis (IAP) proteins, and induces caspase-3 and PARP processing selectively in HS-Sultan cells. (**a**) HS-Sultan cells were subjected to 45 min of hyperthermic treatment at 45 °C. After the indicated periods of recovery at 37 °C, whole-cell lysates were analyzed for HSF and NF-κB activity using EMSA, or immunoblotted with the indicated antibodies. aCasp-3 refers to the active forms of caspase 3. The levels of α-tubulin are shown as control. (**b**) In a parallel experiment, levels of HSP70, Bcl-2 and cIAP-2 mRNA were analyzed in whole-cell extracts using RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were determined and used for normalization. (**c**, **d**) HS-Sultan and K562 cells were subjected to HT as in (**a**). At 2, 4 and 6 h after heat shock (HS), whole-cell lysates were immunoblotted with the indicated antibodies (**c**). The arrow indicates the 85 kDa PARP apoptotic fragment. Casp-3 refers to the 35 kDa proform of caspase-3. In parallel samples, apoptosis was evaluated using FACS analysis of annexin $V^+$ cells after 6 h recovery at 37 °C (**d**).

To determine whether similar effects could be detected in K562 cells, HS-Sultan and K562 cells were subjected to 45 °C HT for 45 min, and after 2, 4 or 6 h recovery at 37 °C, were analyzed for caspase-3 and PARP processing, and anti-apoptotic protein expression. Western blot analysis of PARP and procaspase-3, and FACS analysis of annexin-$V^+$ cells at 24 h, confirmed that programmed cell death was induced selectively in HS-Sultan cells (Figures 4c and d). Analysis of the anti-apoptotic proteins showed that cIAP-2 basal levels were elevated in HS-Sultan cells and barely detectable in K562 cells, whereas XIAP levels seemed to be similar in both cell types (Figure 4c). In HS-Sultan cells, the levels of cIAP-2 protein were already reduced at 2 h after HT, whereas XIAP levels decreased at 6 h, confirming the results described above; instead, cIAP-2 and XIAP levels were unchanged in K562 cells. These results support the hypothesis that cIAP-2 may have an important role in resistance to caspase-3-induced apoptosis in HS-Sultan cells. Interestingly, high levels of cIAP-2 were previously shown in Epstein–Barr virus-bearing lymphoid malignancies, and cIAP-2 overexpression was correlated with resistance to caspase-3-mediated apoptosis in some types of B-lymphoid neoplasms.[38] Furthermore, compounds that selectively target cIAP-1/2 for degradation have been recently shown to induce TNFα-dependent apoptosis in cancer cells.[39] XIAP was previously identified as a key factor in apoptosis resistance of Hodgkin's lymphoma-derived B cells.[40] Although we also observed a downregulation of XIAP in our model, this occurred at later times (6 h) after the beginning of the heat-induced apoptotic process, suggesting a secondary role in triggering apoptosis.

### *Inhibition of NF-κB by bortezomib or downregulation of endogenous p65 expression enhances heat-induced apoptosis in HS-Sultan cells*

The role of NF-κB in heat-induced apoptosis of malignant B cells was analyzed using lentiviral-mediated RNA interference to induce a knockdown of the NF-κB p65 subunit, which represents the major constituent of the NF-κB complex in HS-Sultan cells.[24] Lentiviral vectors used to knockdown p65 expression, containing shRNA for the p65 gene (LV-shp65) or a sequence in which four-point mutations were introduced to generate a control shRNA (LV-shC) and a green fluorescent protein reporter, were described previously.[24] HS-Sultan and K562 cells were mock-transduced or transduced with the LV-shp65 vector or with the mutated LV-shC vector as a control. After 72 h, approximately 90% of the cells were found to be positive for green fluorescent protein, as determined using fluorescence microscopy. At 72 h after transduction, HS-Sultan and K562 cells were either stimulated with TNF-α (50 ng/ml) or subjected to HT for 45 min at 43, 44 or 45 °C. After 24 h recovery at 37 °C, the cells were analyzed using FACScan for apoptosis detection, and p65 levels were determined in whole-cell extracts using western blot analysis. Quantitative determination of p65 protein indicated that p65 levels were only partially reduced (30–35%) in LV-shp65-transduced cells as compared with LV-shC-transduced cells. In spite of the modest reduction, the downregulation of p65 resulted in a cooperative effect in heat-treated HS-Sultan cells. As shown in Figure 5, apoptosis levels were higher in p65-interfered HS-Sultan cells than in LV-shC-transduced or mock-transduced cells at all

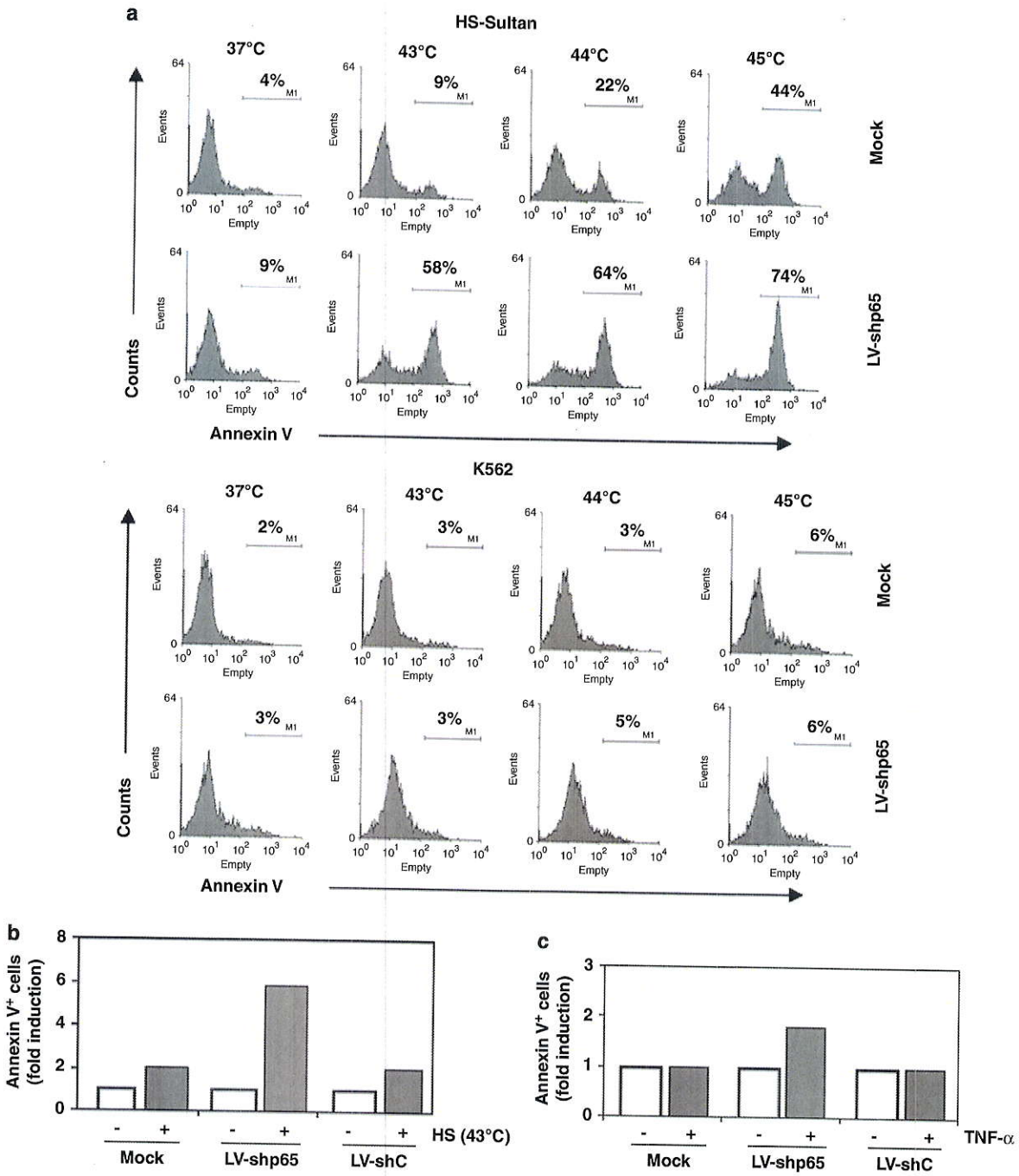


**Figure 5** Inhibition of p65 expression sensitizes HS-Sultan cells to heat-induced apoptosis. HS-Sultan and K562 cells were mock-transduced (Mock) or transduced with the LV-shp65 vector (LV-shp65) or the mutated LV-shC vector (LV-shC). At 72 h after infection, transduced and mock-transduced cells were either kept at 37 °C or subjected to heat shock at the indicated temperatures for 45 min. (**a**) Histograms of FACS analysis of annexin V staining 24 h after hyperthermic treatment in HS-Sultan (top) and K562 (bottom) mock-transduced and LV-shp65-transduced cells are shown. Percentages of apoptotic cells in each sample are indicated. (**b**, **c**) At 72 h after infection, transduced and mock-transduced HS-Sultan cells were either kept at 37 °C (−) or subjected to 43 °C heat shock (HS) for 45 min (+) (**b**). Alternatively, transduced and mock-transduced cells were stimulated with tumor necrosis factor-α (TNF-α; 50 ng/ml) (**c**). Apoptosis was evaluated using FACS analysis of annexin $V^+$ cells at 24 h after stimulation. The number of annexin $V^+$ cells is expressed as fold induction of untreated control. The results are representative of three independent experiments with similar results.

temperatures tested. A particularly relevant synergistic effect was detected in cells subjected to a 43 °C HT, which did not induce apoptosis in LV-shC-transduced or mock-transduced cells (Figure 5a, top, and 5b). No significant difference between p65-interfered and mock-transduced cells was instead detected in K562 cells under all conditions (Figure 5a, bottom). In addition, p65-interfered HS-Sultan cells were found to be more sensitive to TNF-α-induced apoptosis, which was increased by

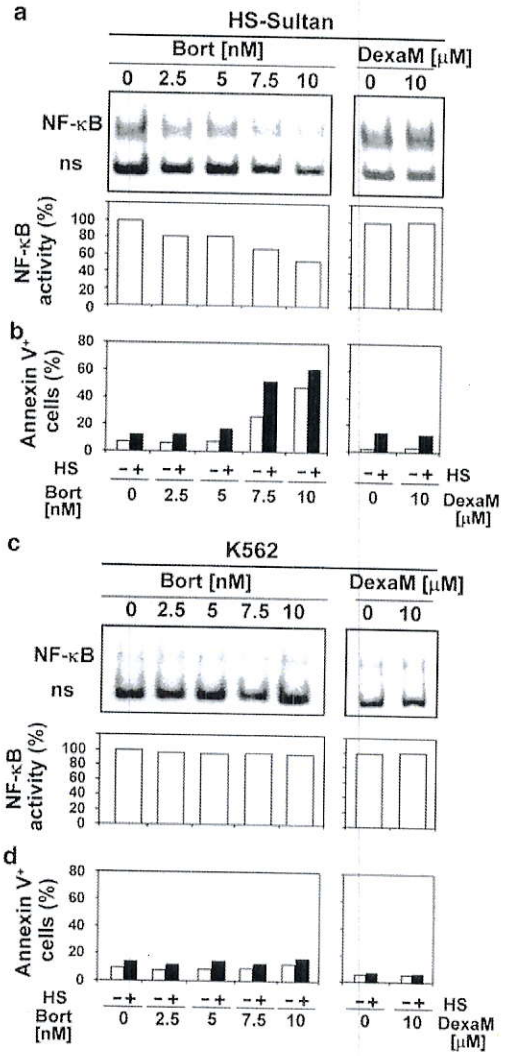


**Figure 6** The proteasome inhibitor bortezomib enhances heat-induced apoptosis in HS-Sultan cells. HS-Sultan (**a**, **b**) and K562 (**c**, **d**) cells were treated with the indicated doses of bortezomib, dexamethasone (DexaM) or control diluent. After 16 h, whole-cell extracts were analyzed for NF-κB activity using EMSA (**a**, **c**, top). The levels of NF-κB DNA-binding activity were quantitated using MDP analysis (**a**, **c**, bottom). In a parallel experiment, HS-Sultan (**b**) and K562 (**d**) cells treated with bortezomib or dexamethasone for 16 h were subjected to heat shock (HS) at 43 °C for 45 min (+) or kept at 37 °C (−). After 24 h, the number of annexin $V^+$ cells was determined in heat-shocked (empty bars) or not heat-shocked (filled bars) cells using FACS analysis. The results are representative of two independent experiments with similar results.

twofold as compared with mock-transduced or LV-shC-transduced cells (Figure 5c). This result is in line with the well-known role of NF-κB in counteracting the apoptotic stimulus triggered by TNF-α [41].

To confirm that NF-κB inhibition could enhance the effect of mild heat treatment in Burkitt's lymphoma cells, HS-Sultan and K562 cells were treated with different concentrations of the proteasome inhibitor bortezomib (Velcade, Millenium Pharmaceuticals), which is known to block NF-κB activity by preventing IκBα degradation,[42] or with the conventional drug dexamethasone. After 16 h, whole-cell extracts were analyzed for NF-κB DNA-binding activity. As shown in Figure 6a, at this time bortezomib was found to inhibit NF-κB activity in HS-Sultan cells dose dependently, whereas dexamethasone had no effect, confirming previous observations.[24] In a parallel experiment, HS-Sultan and K562 cells treated for 16 h with different concentrations of bortezomib or with dexamethasone were subjected to 43 °C HT for 45 min. Analysis of annexin $V^+$ cells at 24 h after HT indicated that, in the absence of heat treatment, bortezomib induced apoptosis in HS-Sultan cells starting at the concentration of 7.5 and 10 nM concomitantly with NF-κB inhibition (Figure 6b). As expected, a 43 °C HT caused a modest increase in the number of apoptotic cells (Figure 6b). The number of apoptotic cells was increased in HS-Sultan cells pre-treated with 7.5 nM bortezomib, whereas treatment with dexamethasone did not enhance the effect of hyperthermic stress (Figure 6b). It should be noted that pretreatment with bortezomib was unable to further enhance apoptosis under conditions of maximal response to heat (45 min at 45 °C; data not shown). Finally, neither bortezomib nor dexamethasone had any effect on NF-κB DNA-binding activity or apoptosis in K562 cells (Figures 6c and d).

These observations suggest the interesting possibility that, in some types of cancers, co-treatment of sub-optimal doses of NF-κB inhibitors with mild heat stress may induce cell death under conditions in which the single treatment is not effective.

## Conclusion

The HSR represents the major defense mechanism that cells use against the deleterious effects of environmental and physiological stress, which provoke cell damage because of protein misfolding, degradation and insoluble aggregation.[16] However, the role of HSR in cell fate is bimodal. Whenever the damage can be overridden with HSP chaperoning activity, the cell will survive; conversely, the stress response will trigger apoptosis. HSPs have an extremely complex role in the regulation of apoptosis. In particular, HSP70, HSP27 and HSP90 were shown to inhibit apoptosis by direct physical interaction with apoptotic molecules[20,43] and their expression was linked to cancer apoptosis-resistance induced by chemotherapy and radiation therapy.[44] In fact, it was recently shown that inhibition or silencing of HSF1 leads to enhanced sensitivity of some types of solid tumors to hyperthermia or chemotherapy.[45–47] On the other hand, hyperthermia is one of the modalities used in the clinical setting to treat various forms of malignancies, and has been proven to be especially effective in combination with radiotherapy and chemotherapy in different types of cancer.[48,49] In addition, several potent HSF1 inducers provoke apoptosis in some types of cancer cells.[24,27] Recently, the clinical application of heat as a coadjuvant in cancer treatment is gaining new interest because of the substantial technical improvements achieved;[30] however, the molecular mechanisms at the basis of the differential sensitivity of different types of neoplasias to hyperthermic treatment are still mainly unknown, constituting a major obstacle in the use of this type of therapy.

Starting from our early discovery of a cross-talk between HSF1 and NF-κB,[13] there is a large amount of evidence that a variety of chemical HSR inducers, which include cyclopentenone prostanoids, sodium arsenite, the serine protease inhibitors N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and 3,4-dichloroisocoumarin, as well as heat shock itself, prevent NF-κB activation triggered by cytokines, mitogens or viruses.[16,21,22,34]

In this study, using as a model HS-Sultan cells, a B-cell lymphoma characterized by high levels of constitutive NF-κB activity,[8,24,50] we show for the first time that heat stress is also

able to inhibit constitutive NF-κB DNA-binding activity in B-cell malignancies. Heat-induced NF-κB inhibition leads to rapid downregulation of the anti-apoptotic protein cIAP-2, followed by activation of caspase-3 and cleavage of the caspase-3 substrate PARP, causing massive apoptosis under conditions that do not affect viability in cells not presenting NF-κB aberrations. NF-κB inhibition by the proteasome inhibitor bortezomib and by shRNA interference results in increased sensitivity of lymphoma cells to hyperthermic stress. We also show that, in addition to HS-Sultan cells, heat stress is able to inhibit constitutive NF-κB activity and induce massive apoptosis in other types of aggressive B-cell malignancies presenting aberrations in the regulation of the nuclear factor. These include multiple myeloma and the ABC subtype of DLBCL, the most common type of non-Hodgkin's lymphoma, which represents an important clinical challenge as patients with ABC DLBCL respond poorly to conventional chemotherapy and present a distinctly inferior prognosis.[11,31,32]

Altogether, these findings indicate that aggressive B-cell malignancies presenting constitutive NF-κB activity are sensitive to heat-induced apoptosis, and suggest the interesting possibility that aberrant NF-κB regulation may be a marker of heat stress sensitivity in cancer cells.

## Acknowledgements

This work was supported by the Italian Ministry of University and Scientific Research (MIUR), the Italian Ministry of Public Health (ISS projects Lotta ai Tumori), the EU EICOSANOX project and Regione Piemonte (A361 Project). We thank Dr Cristina Ferreri for technical help.

## References

1 Li Q, Verma I. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002; **2**: 725–734.
2 Santoro MG, Rossi A, Amici C. NF-kappaB and virus infection: who controls whom. *EMBO J* 2003; **22**: 2552–2560.
3 Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002; **3**: 221–227.
4 Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004; **25**: 280–288.
5 Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature* 2006; **441**: 431–436.
6 Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 1999; **18**: 6938–6947.
7 Baldwin Jr AS. The transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; **107**: 3–6.
8 Ni H, Ergin M, Huang Q, Qin JZ, Amin HM, Martinez RL *et al.* Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. *Br J Haematol* 2001; **115**: 279–286.
9 Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W *et al.* Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997; **100**: 2961–2969.
10 Pham LV, Tamayo A, Yoshimura LC, Lo P, Terry N, Reid PS *et al.* A CD40 signalosome anchored in lipid rafts leads to constitutive activation of NF-kappaB and autonomous cell growth in B cell lymphomas. *Immunity* 2002; **16**: 37–50.
11 Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* 2001; **194**: 1861–1874.
12 Orlowski RZ, Eswara JR, Lafond-Walker A, Grever MR, Orlowski M, Dang CV. Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. *Cancer Res* 1998; **58**: 4342–4348.
13 Rossi A, Elia G, Santoro MG. Inhibition of nuclear factor kappa B by prostaglandin A1: an effect associated with heat shock transcription factor activation. *Proc Natl Acad Sci USA* 1997; **94**: 746–750.
14 Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* 1998; **12**: 3788–3796.
15 Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J Natl Cancer Inst* 2000; **92**: 1564–1572.
16 Morimoto RI, Santoro MG. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat Biotechnol* 1998; **16**: 833–838.
17 Kline MP, Morimoto RI. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Mol Cell Biol* 1997; **17**: 2107–2115.
18 Pirkkala L, Nykanen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J* 2001; **15**: 1118–1131.
19 Anckar J, Sistonen L. Heat shock factor 1 as a coordinator of stress and developmental pathways. *Adv Exp Med Biol* 2007; **594**: 78–88.
20 Takayama S, Reed JC, Homma S. Heat-shock proteins as regulators of apoptosis. *Oncogene* 2003; **22**: 9041–9047.
21 Rossi A, Elia G, Santoro MG. Activation of the heat shock factor 1 by serine protease inhibitors. An effect associated with nuclear factor-kappaB inhibition. *J Biol Chem* 1998; **273**: 16446–16452.
22 Amici C, Belardo G, Rossi A, Santoro MG. Activation of I kappa B kinase by herpes simplex virus type 1. A novel target for anti-herpetic therapy. *J Biol Chem* 2001; **276**: 28759–28766.
23 Piva R, Ruggeri B, Williams M, Costa G, Tamagno I, Ferrero D *et al.* CEP-18770: a novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. *Blood* 2008; **111**: 2765–2775.
24 Piva R, Gianferretti P, Ciucci A, Taulli R, Belardo G, Santoro MG. 15-deoxy-D12,14-prostaglandin J2 induces apoptosis in human malignant B-cells: an effect associated with inhibition of NF-κB activity and down-regulation of anti-apoptotic proteins. *Blood* 2005; **105**: 1750–1758.
25 Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M *et al.* Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 2000; **403**: 103–108.
26 Mosser DD, Theodorakis NG, Morimoto RI. Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. *Mol Cell Biol* 1988; **8**: 4736–4744.
27 Ciucci A, Gianferretti P, Piva R, Guyot T, Snape TJ, Roberts SM *et al.* Induction of apoptosis in estrogen receptor-negative breast cancer cells by natural and synthetic cyclopentenones: role of the IkappaB kinase/nuclear factor-kappaB pathway. *Mol Pharmacol* 2006; **70**: 1812–1821.
28 Kanda K, Hu HM, Zhang L, Grandchamps J, Boxer LM. NF-kappaB activity is required for the deregulation of c-myc expression by the immunoglobulin heavy chain enhancer. *J Biol Chem* 2000; **275**: 32338–32346.
29 Wu C. Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol* 1995; **11**: 441–469.
30 Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T *et al.* The cellular and molecular basis of hyperthermia. *Crit Rev Oncol Hematol* 2002; **43**: 33–56.
31 Dave SS, Fu K, Wright GW, Lam LT, Kluin P, Boerma EJ *et al.* Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* 2006; **354**: 2431–2442.
32 Hummel M, Bentink S, Berger H, Klapper W, Wessendorf S, Barth TF *et al.* A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med* 2006; **354**: 2419–2430.
33 Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G *et al.* A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 2006; **441**: 106–110.
34 Santoro MG. Heat shock factors and the control of the stress response. *Biochem Pharmacol* 2000; **59**: 55–63.
35 Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H *et al.* Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and

impairing NF-kappa B survival signaling. *Genes Dev* 2004; **18**: 1466–1481.
36 Frelin C, Imbert V, Bottero V, Gonthier N, Samraj AK, Schulze-Osthoff K *et al.* Inhibition of the NF-kappaB survival pathway via caspase-dependent cleavage of the IKK complex scaffold protein and NF-kappaB essential modulator NEMO. *Cell Death Differ* 2008; **1**: 152–160.
37 Vaux DL, Silke J. IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* 2005; **6**: 287–297.
38 Dürkop H, Hirsh B, Hahn C, Stein H. cIAP2 is highly expressed in Hodgkin-Reed-Sternberg cells and inhibits apoptosis by interfering with constitutively active caspase-3. *J Mol Med* 2006; **84**: 132–141.
39 Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P *et al.* IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007; **131**: 669–681.
40 Kashkar H, Haefs C, Shin H, Hamilton-Dutoit SJ, Salvesen GS, Kronke M *et al.* XIAP-mediated caspase inhibition in Hodgkin's lymphoma–derived B cells. *J Exp Med* 2003; **198**: 341–347.
41 Ghosh S. Regulation of inducible gene expression by the transcription factor NF-kappaB. *Immunol Res* 1999; **19**: 183–189.
42 Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J *et al.* The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 2001; **61**: 3071–3076.
43 Beere HM. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J Clin Invest* 2005; **115**: 2633–2639.
44 Mosser DD, Morimoto RI. Molecular chaperones and the stress of oncogenesis. *Oncogene* 2004; **23**: 2907–2918.
45 Westerheide SD, Kawahara TL, Orton K, Morimoto RI. Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. *J Biol Chem* 2006; **281**: 9616–9622.
46 Zaarur N, Gabai V, Porco JA, Calderwood S, Sherman MY. Targeting heat shock response to sensitize cancer cells to proteasome and Hsp90 inhibitors. *Cancer Res* 2006; **66**: 1783–1791.
47 Rossi A, Ciafrè S, Balsamo M, Pierimarchi P, Santoro MG. Targeting the heat shock factor 1 by RNA interference: a potent tool to enhance hyperthermochemotherapy efficacy in cervical cancer. *Cancer Res* 2006; **66**: 7678–7685.
48 Wust P, Hildebrandt B, Sreenivasa G, Rau B, Gellermann J, Riess H *et al.* Hyperthermia in combined treatment of cancer. *Lancet Oncol* 2002; **3**: 487–497.
49 Takahashi I, Emi Y, Hasuda S, Kakeji Y, Maehara Y, Sugimachi K. Clinical application of hyperthermia combined with anticancer drugs for the treatment of solid tumors. *Surgery* 2002; **131**: S48–S54.
50 Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T *et al.* Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood* 2002; **99**: 4079–4086.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)