Triterpenoid Electrophiles (Avicins) Suppress Heat Shock Protein-70 and X-Linked Inhibitor of Apoptosis Proteins in Malignant Cells by Activation of Ubiquitin Machinery: Implications for Proapoptotic Activity

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ABSTRACT

Avicins are plant-derived triterpenoid stress metabolites that have both proapoptotic and cytoprotective properties. Avicins induce apoptosis in Jurkat T leukemia cells by targeting mitochondria and release of cytochrome *c* that occurs in a p53-independent manner. However, postmitochondrial antiapoptotic barriers, such as increased expression of heat shock proteins (Hsp) and x-linked inhibitor of apoptosis proteins (XIAP), frequently exist in cancer cells and often account for resistance to chemotherapy and a poor prognosis. In this article, we show the role of avicins in the activation of stress-regulated ubiquitination and degradation of Hsp70 and XIAP. This is the first report showing the regulation of Hsp70 via the ubiquitin/proteasome pathway. We also show the induction of E3α ubiquitin ligase in avicin-treated Jurkat T leukemia cells, and its involvement in the degradation of XIAP. Avicin-mediated suppression of Hsp70 and XIAP was further confirmed in other leukemic/lymphoma cell lines and freshly isolated peripheral blood lymphocytes from Sezary syndrome patients. No change in the Hsp70 and XIAP proteins was observed in peripheral blood lymphocytes from normal donors. We propose that the ability of avicins to induce ubiquitination and regulate the degradation of Hsp70 and XIAP in leukemia cells could have important implications in the treatment of drug-resistant neoplasia and inflammatory disorders.

INTRODUCTION

Several lines of evidence indicate that cancer is a multistep process that reveals genetic changes driving a normal cell to malignancy. Cancer has been characterized by at least six essential alterations in cellular functions, including the evasion of programmed cell death (1). Thus, acquired resistance toward apoptosis is a hallmark of most types of cancer. Of the known mechanisms of acquired resistance to apoptosis, overexpression of the major stress-inducible family of heat shock proteins (Hsp; ref. 2), as well as the x-linked inhibitor of apoptosis proteins (XIAP) family (3), is prominent. Hsp70 interacts with Apaf-1 (4, 5), the apoptosis-inducing factor (6), and negatively interferes with the caspase-dependent and -independent processes of apoptosis (7). Besides Hsps, a class of proteins called the inhibitor of apoptosis proteins block cell death by inhibiting upstream and terminal caspases (8). Among the eight known mammalian inhibitor of apoptosis proteins, XIAP seems to be most potent (K_i in the low nanomolar range) and best characterized, with its ability to inhibit activated caspases-3, -7, and -9 (reviewed in refs. 8, 9). In general, elevated levels of Hsps (2) and XIAP (8, 9) are associated with drug resistance and poor prognosis. Down-regulation of Hsps (10, 11) and XIAP (12) by antisense and other interventions such as 17-AAG (an inhibitor of Hsp90; ref. 13) show the ability to overcome apoptotic resistance.

Avicins are triterpenoid electrophilic metabolite molecules isolated from an Australian desert plant, *Acacia victoriae*. These triterpene compounds induce stress resistance in human cells in a redox-dependent manner (14). Avicins' proapoptotic property seems to be independent of p53 and targets the mitochondria (15).[1] However, several proteins like Hsps and XIAP are known postmitochondrial antiapoptotic barriers. In this report, we show avicin-mediated degradation of Hsp70 and XIAP, via activation of ubiquitin/proteosomal pathway (Ub/P). Besides Jurkat T leukemic cells, other lymphoma, monocytic cell lines, and peripheral blood lymphocytes (PBL) from Sezary syndrome patients with atypical T cells (CD4+, CD26−) that are quite sensitive to apoptosis by avicin D, showed suppression of Hsp70 and XIAP proteins. We propose that the proapoptotic property of avicins may be via the coordinated regulation of several such cascades of events.

MATERIALS AND METHODS

Avicin D. Avicin D was isolated from the seedpods of an Australian desert plant, *A. victoriae* (15).

Antibodies, Plasmids, Recombinant Proteins, and Cell Lines. Human Jurkat T cell leukemia, monocytic U937 cells, cutaneous T cell lymphoma cell lines MJ (G11) and HH were

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[1] V. Haridas, et al. Avicins, a novel plant-derived metabolite lowers cellular energy metabolism via closure of VDAC in human cells, submitted for publication.

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obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 200 μmol/L glutamine. Samples of peripheral blood were obtained for *in vitro* studies from three Sezary syndrome patients with circulating atypical T cells (CD4+ CD26−). Samples were obtained during routine diagnostic assessments after informed consent was obtained in accordance with regulations and protocols sanctioned by the Human Subjects Committee of M.D. Anderson. PBL from these patient samples were isolated by using a Vacutainer CPT (Becton Dickinson, San Jose, CA). Normal PBL were prepared from buffy coat obtained from Gulf coast regional blood center (Houston, TX) using Histopaque gradient (Sigma Chemical Co., St. Louis, MO) as per the manufacturer's protocol.

Anti-Hsp70, anti-Hsp90, anti-Hsc70, anti-Hsp60, anti-heat shock factor-1 (HSF1), anti-β-actin, and anti-ubiquitin antibodies were purchased from StressGen (San Diego, CA). Anti-Ubr1, anti-calnexin, anti-grp75, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Rabbit anti-carboxyl terminus homology to Hsc/Hsp70 protein (CHIP) antibodies were purchased from Oncogene Research Products (San Diego, CA). Anti-caspase-9, anti-caspase-3, and anti-XIAP antibodies were obtained from Cell Signaling (Beverly, MA), anti-glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibodies were obtained from Ambion (Austin, TX).

Primer sequences to perform reverse transcription-PCR were obtained from StressGen. The ProBond Nickel Agarose purification kit was purchased from Qiagen (Valencia, CA).

A plasmid expressing fusion of green fluorescent protein and histidine-tagged ubiquitin (his-ub) (pDG268) for transient transfection of Jurkat T cells was a kind gift from Prof. Douglas Gray (Center for Cancer Therapeutics, Ottawa Regional Cancer Center, Ottawa, Canada). The his-ub/green fluorescent protein fusion is very efficiently processed in cells, and it is only the his-ub portion that gets conjugated to proteins.[2]

Recombinant Hsp70 protein, ubiquitin, histidine-tagged ubiquitin, and lactacystin were purchased from Sigma-Aldrich.

Treatment of Cells and Cytoplasmic Extract Preparation. Jurkat T cells (2 μg/mL = 1 μmol/L), U-937 (4 μg/mL), MJ (5 μg/mL), and HH cells (2.5 μg/mL) were treated for 0 to 24 hours with the indicated concentrations of avicin D. PBLs' from the patients or normal blood were treated with 5 μg/mL of avicin D for 24 hours.

At the end of the treatments, cells were harvested, washed with sterile ice-cold PBS and cytoplasmic extracts (CE) were prepared by lysing the cells in CE buffer containing 10 mmol Hepes-Cl (pH 7.5), 10 mmol KCl, 0.1 mmol EDTA, 0.1 mmol EGTA, 0.3% NP40, and a set of protease inhibitor cocktails (Sigma). After centrifugation and separating the supernatant (CE proteins), the pellet was resuspended in a buffer containing 20 mmol Hepes-Cl, 400 mmol NaCl, 1 mmol EDTA, 1 mmol EGTA, and protease inhibitor cocktail (Sigma). The nuclear protein extraction proceeded for 30 minutes on ice followed by centrifugation at 14,000 rpm for 5 minutes at 4°C. The clear supernatant containing nuclear proteins was collected, glycerol (10%) was added, and proteins stored at −80°C until use.

Western Blot Analysis. SDS-PAGE and immunoblot procedures were essentially done as described (16). Briefly, cytoplasmic and nuclear proteins were resolved on SDS-PAGE, blotted on polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and probed with various antibodies followed with anti-rabbit, anti-mouse antibody conjugated to horseradish peroxidase from Bio-Rad or horseradish peroxidase conjugated anti-goat antibody from Santa Cruz, corresponding to the primary antibody. Protein bands were detected using the enhanced chemiluminescence kit from Amersham as per the manufacturer's protocol.

Northern Blot Analysis. Total RNA from the control and avicin-treated Jurkat T cells was made using TRIzol (Invitrogen). Equal amounts of RNA were separated on formamide gels and transferred to nylon membranes (Hybond N+, Amersham, Piscataway, NJ) and UV cross-linked using UV Stratalinker (Stratagene, La Jolla, CA). Staining the membranes with 0.03% methylene blue solution in 0.3% sodium acetate (pH 5.2) monitored equal loading. The DNA probes for Hsp70 and Hsp90 were purchased from StressGen as pUC plasmids and used according to the manufacturer's protocol. The DNA fragments were radiolabeled using a Nick Translation kit from Life Technologies and [^{32}P] dCTP (Amersham). The membranes were exposed for autoradiography after hybridization using ExpressHyb (Clontech, Palo Alto, CA) solution at 58°C for 1 hour and five washes, each for 20 minutes, with 5× SSC containing 0.1% SDS at 50°C.

Reverse Transcription-PCR. Total RNA purified using TRIzol method (Invitrogen) was subjected to DNaseI (RNase-free; Sigma) treatment to remove any residual DNA, followed by heat inactivation and addition of 1 mmol EDTA. Absence of genomic DNA was confirmed by performing PCR reaction using Taq DNA polymerase. Approximately 50 to 100 ng of purified total RNA was used in a one-step reverse transcription-PCR reaction kit from Invitrogen in a Techne Genius machine. The samples were separated on 0.8% agarose-Tris-borate EDTA gels and viewed by staining with ethidium bromide.

Densitometric Analysis. Quantitation of proteins (Western) and transcripts (reverse transcription-PCR and Northern blots) was done using the NIH image software.

***In vitro* Ubiquitination.** Ubiquitination assay was done as previously described (17) with few modifications using recombinant bovine Hsp70 and NH_2-terminal (his-ub). About 0.5 μg of Hsp70 and 4 μg of his-ub were incubated in a buffer containing 50 mmol Tris-Cl (pH 7.5), 2.5 mmol $MgCl_2$, 0.05% NP40, 0.5 mmol DTT, 5 mmol ATP, 4 μmol/L MG132, and ATP-regenerating system containing 10 mmol creatine phosphate, 0.1 μg/mL of creatine kinase, and about 50 μg of CE proteins. The reaction was carried out for 1 hour at 30°C and the products were subjected to nickel agarose chromatographic purification to purify histidine-tagged proteins as per the manufacturer's protocol (Qiagen). The affinity-purified proteins were prepared for SDS-PAGE and Western analysis using anti-Hsp70 antibodies.

Transient Transfection. Jurkat T cells were transfected with a plasmid pDG268 that expresses a fusion protein of histidine-tagged human ubiquitin and enhanced green fluorescent protein. Transfection was done using Amaxa biosystems kit

[2] D. Gray, personal communication.

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and their protocol. After 24 hours of transfection, cells were harvested, resuspended at a density of 10^6 cells/mL before treatment with lactacystin or avicin D.

***In vivo* Ubiquitination Activity.** Jurkat T cells transfected with the his-ub plasmid construct were treated with lactacystin (10 μmol/L) or with avicin D (1 μmol/L) for 4 hours. Cells were harvested and CE prepared as described earlier. The his-ub-containing proteins (300 μg) were purified using nickel agarose beads as suggested by the manufacturer (Qiagen). The affinity-purified histidine-tagged proteins were separated on SDS-PAGE and analyzed on Western blots for ub-Hsp70 proteins.

20S Proteasomal Assay. Jurkat T cells were treated with 1 μmol/L of avicin D for 0 to 4 hours. Proteasomal extracts were prepared as described previously (18) in a buffer containing 50 mmol Hepes (pH 8), 5 mmol EGTA, 0.3% NP40, and 10% glycerol. The assay reaction contained 20 mmol Tris-Cl (pH 7.2), 0.1 mmol EDTA, 1 mmol β-mercaptoethanol, 5 mmol ATP, 20% glycerol, 0.02% SDS, and 0.04% NP40. About 10 μg of the proteasomal extract proteins and BocLRR-AMC (0.1 mmol), which allows measurement of the trypsin-like activity of proteasomes, was used as a substrate. The reaction was carried out at 30°C for 30 minutes and the fluorescence was read at 380 nm (excitation) and 460 nm (emission) in a Perkin-Elmer (Boston, MA) HTS 7000 Plus, Bioassay Reader.

Statistical Analysis. Statistical significance of differences observed in the proteasomal activity in avicin-treated cells compared with the untreated cells was determined by using an unpaired Student t test. The minimum level of significance was a $P < 0.05$.

RESULTS

Effect of Avicins on the Expression of Heat Shock Proteins. To study the effect of avicins on Hsps, we first looked at the expression levels of various chaperone proteins in avicin D (1 μmol/L)–treated Jurkat cells. As shown in Fig. 1, avicin D induced a significant decrease in the protein levels of Hsp70 and Hsp90 within 1 hour of treatment that persisted up to 4 hours (Fig. 1*B*). Except for Hsp27, which showed a modest increase (1.4-fold) at 2 to 4 hours of avicin D treatment, expression of other chaperone proteins like Hsc70, the mitochondrial localized Hsp60 and grp75 and the endoplasmic reticulum resident protein calnexin did not show a significant change. In this article, we focused our attention to understand the regulation of avicin-induced decrease in Hsps, i.e., Hsp70. Therefore, we next studied their transcription. Hsps are regulated at the transcriptional level via HSF1, which under unstressed conditions resides in the cytoplasm as an inactive monomer. Under stress, HSF1 undergoes oligomerization and nuclear translocation (19), prior to the transcription of Hsp genes. We prepared nuclear and cytoplasmic proteins from avicin D-treated cells to examine changes in HSF1 protein. No apparent change in the cytoplasmic content of HSF1 protein was detected (Fig. 2*A*). But avicin treatment (4 hours) induced a modest increase (~1.5-fold) in the levels of nuclear HSF1 as determined by densitometric scanning (Fig. 2*A*). The increase in the nuclear HSF1 starting at 1 hour of avicin D treatment prompted us to examine its effect on the transcripts of Hsps. Reverse transcription-PCR was used to see the effect of avicin D on the transcripts. We observed a ~1.6-fold increase in the Hsp70α and a ~1.4-fold increase in the Hsp90β transcripts as early as 30 minutes of avicin treatment. The changes in the transcripts encoding Hsp90α, Hsp70C, Hsc70, and Hsp60 were marginal. Northern blot analysis of Hsp70 (~1.4-fold) and Hsp90 (~2-fold) transcripts also revealed an increase in both the transcripts (Fig. 2*C* and *D*). The increase in the levels of both nuclear HSF1 and Hsp transcripts (70 and 90) are possibly due to removal of the feedback inhibition of Hsp protein on HSF1 (20). These results confirmed that the avicin-induced decrease in the Hsp70/90 proteins is not at the level of transcription.

Posttranscriptional Regulation of Heat Shock Proteins. We next questioned if a posttranslational event such as proteasomal degradation could be responsible for the avicin-induced decrease in the chaperone proteins. To answer this, we studied the effect of lactacystin, an irreversible proteasomal inhibitor (21), on avicin-induced decrease in Hsp70 and Hsp90 proteins. The cells that were treated with avicins for 2 and 4 hours showed a significant decrease in Hsp70 and Hsp90 proteins (Fig. 3) compared with the untreated cells. However, pretreatment of Jurkat cells with lactacystin totally reversed the avicin-

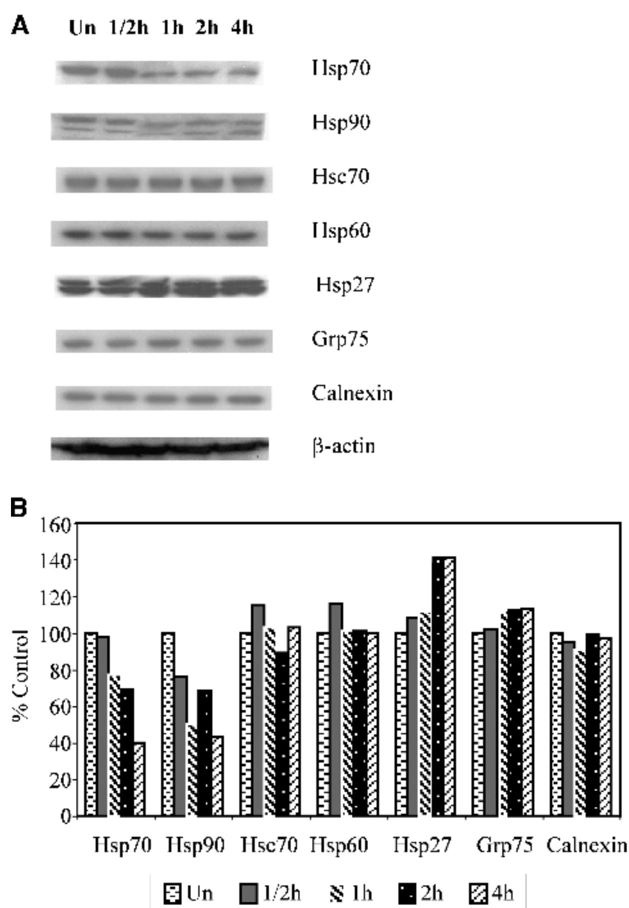

Fig. 1 Regulation of stress proteins by avicin D. *A*, Jurkat cells were treated with avicin D from 30 minutes up to 4 hours as described in Materials and Methods. Cellular proteins (25 μg) from untreated (Un) and avicin D-treated cells were probed with various antibodies (Hsp70, Hsp90, Hsc70, Hsp60, Hsp27, Grp75, calnexin, and β-actin); *B*, densitometric values obtained from scanning the autoradiographic signals of Western blots are plotted as the percentage of untreated control values (arbitrary units).

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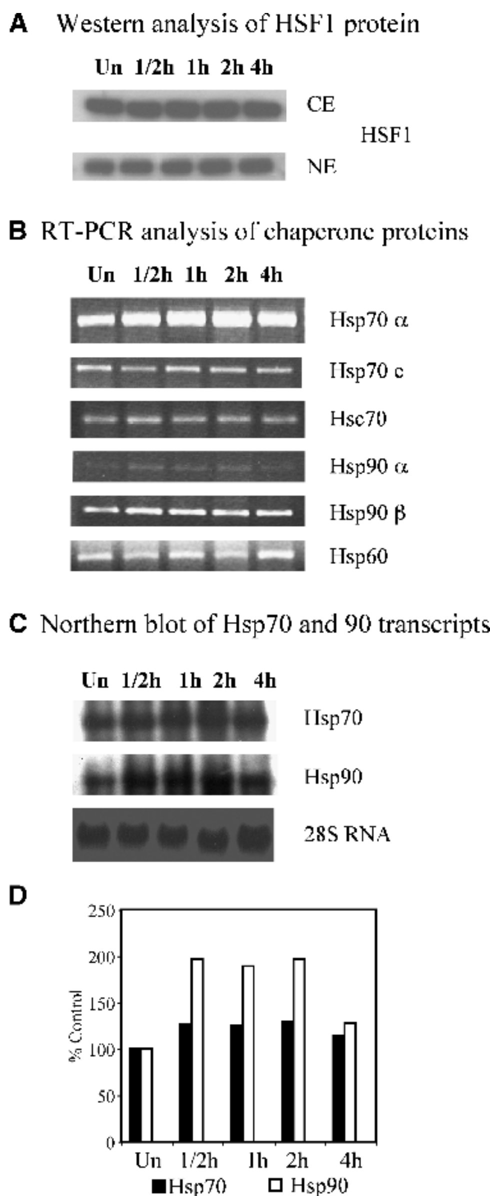

Fig. 2 Effect of avicin D on HSF1 protein and transcription of stress proteins. Translocation of the HSF1 transcription factor was examined by Western blot analysis of the cellular proteins (*A*) and nuclear proteins (*B*) prepared from Jurkat cells treated with avicin D for various time intervals. Approximately 50 μg of the proteins were resolved on SDS-10% PAGE and probed with anti-HSF1 antibodies; *B,* total RNA from avicin D-treated Jurkat cells was prepared as described in Materials and Methods and used for one-step reverse transcription-PCR assay. Twenty PCR cycles were done and the reaction products separated and viewed by ethidium bromide staining; *C,* Northern blot for Hsp70 and Hsp90 was done as described in Materials and Methods. Staining the nylon membrane with methylene blue for 18S monitored loading pattern; *D,* autoradiograph of the Northern blot was quantified by densitometric scan. The values plotted in the graph are expressed as the percent change with respect to the value of the untreated cells.

induced decrease in both Hsp70 and Hsp90 proteins, showing proteasome-based degradation of these chaperone proteins.

Avicins Induce Ubiquitination. Because most proteins destined for proteasomal degradation are marked by their ubiquitination (22), we next studied the involvement of the ubiquitin system in avicin-induced Hsp70 degradation. An *in vitro* ubiquitination assay was done using recombinant Hsp70 and his-ub with CEs of avicin D-treated cells. As shown in Fig. 4*A*, the avicin-treated extracts induced a stronger ladder of his-ub-Hsp70 compared with the extracts of the untreated cells, suggesting that avicins induce ubiquitination of Hsp70. To establish an *in vivo* relevance, Jurkat cells transfected with a plasmid expressing a fusion protein of histidine-tagged-ubiquitin (his-ub) was treated with avicin D or lactacystin. The his-tagged proteins were affinity-purified and analyzed using anti-Hsp70 antibodies. Figure 4*B* shows a significant decrease (~40%, $P <$ 0.05; Fig. 4*D*) in the levels of his-ub-Hsp70 protein band (~140 kDa) in avicin-treated cells for 2 and 4 hours, which was sensitive to lactacystin. The small amounts of his-ub-Hsp70 protein molecules synthesized *in vivo*, made it evident that the endogenous ubiquitin pool was competing with the his-ubiquitin for conjugation. Western analysis of the total CE using anti-Hsp70 antibodies showed similar change in Hsp70 protein as seen with the his-ub-Hsp70 fraction upon avicin treatment (Fig. 4*C*). Use of NEM during cellular extract preparation facilitated the visualization of additional bands of his-ub-Hsp70 (Fig. 4*E*) around the prominent 140 kDa band. These results indicate that avicins induce ubiquitination and subsequent proteolytic degradation of Hsp70.

Avicins Induce the E3α Ubiquitin Ligase that Participates in X-linked Inhibitor of Apoptosis Protein Degradation. Ubiquitination involves three steps that utilize E1 (activating enzyme), E2 (conjugating enzyme), and E3 ligases (22). Based on the importance of E3 ligases in carcinogenesis (23), we wanted to know if avicins regulate any of the known E3 ligase(s). However, there are several hundreds of known E3 ligases. We chose E3α ubiquitin ligase enzyme as it is involved in ubiquitination of several substrates via the N-end rule pathway (24). Secondly, an article by Ditzel et al. (25) reported a major connection between the ubiquitin system and apoptosis by demonstrating caspase-mediated cleavage of DIAP1 followed by its ubiquitination by E3α ligase enzyme and its subsequent degradation. Interestingly, avicins induced a dramatic increase in the E3α ligase protein with a peak at 1 hour of treatment (Fig. 5*A*). No significant change was observed in the levels of CHIP (Fig. 5*B*), another E3 enzyme, under the same conditions,

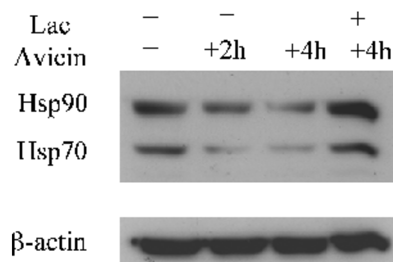

Fig. 3 Posttranscriptional regulation of Hsp70 by avicin D. Jurkat cells were treated for 2 and 4 hours with avicin D or pretreated with lactacystin (10 μmol/L, 30 minutes) followed by treatment with avicin D for 4 hours. CE proteins (50 μg) were resolved on SDS-PAGE, probed with anti-Hsp70 and anti-Hsp90 antibodies, and blots developed using standard procedures. Loading of the proteins was examined by blotting the membranes with β-actin antibodies.

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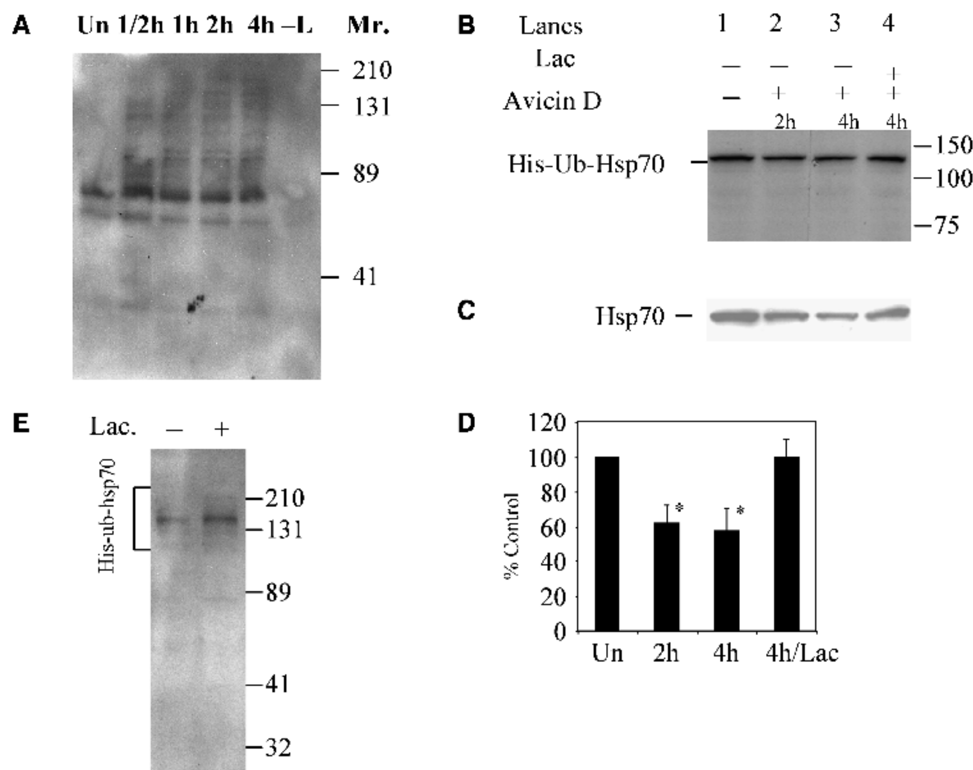

Fig. 4 Avicin D induces ubiquitination. *A,* an *in vitro* ubiquitination assay using recombinant Hsp70, his-tagged ubiquitin, and CE proteins from avicin D treated cells was done, followed by affinity purification of the his-tagged proteins to probe with anti-Hsp70 antibodies. Lane -L, control reaction where no CE proteins were used. M_r is shown on the right; *B, in vivo* ubiquitination of Hsp70 was monitored by transfection of Jurkat cells with his-ub expressing plasmid that were treated with avicin D (1 µmol/L) for 2 hours (*lane 2*) and 4 hours (*lane 3*) as indicated, or pretreated with lactacystin (10 µmol/L) 30 minutes prior to incubation with avicin D for 4 hours (*lane 4*). His-tagged proteins were affinity-purified and probed with anti-Hsp70 antibodies. M_r is shown on the right; *C,* total CE proteins (25 µg) from the same experiment were resolved on SDS-PAGE and probed with anti-Hsp70 antibodies; *D,* the his-ub-Hsp70 protein band was quantitated by densitometry and expressed as percent change of untreated cells. * $P < 0.05$ (Student's *t* test); *E,* visualization of his-ub-Hsp70 protein ladder from *in vivo* ubiquitination of Hsp70: Jurkat cells transfected with his-ub plasmid were treated with lactacystin (10 µmol/L, 4 hours). During cell lysis, 0.2 mmol NEM was added to the CE buffer to stabilize the his-ub-Hsp70 bands. His-tagged proteins were affinity-purified from CE proteins as described earlier and probed with anti-Hsp70 antibodies. M_r is shown on the right.

thereby indicating the specificity of E3α ligase enzyme induction by avicins.

Based on Ditzel's findings (25) and our discovery that avicins induce E3α ubiquitin ligase enzyme (Fig. 5*A*), we turned our attention to XIAP. Avicin-treated Jurkat cells showed a significant decrease in XIAP protein starting at 1 hour posttreatment (Fig. 5*C*). Lactacystin blocked the avicin-induced XIAP decrease, confirming a proteasome-based degradation of XIAP as shown in Fig. 5*E*. To explore if E3α ligase regulates XIAP protein for which caspase activity is necessary, we employed zVAD-fmk to block the caspases and monitor its effect on avicin D-mediated XIAP degradation. Avicin-induced XIAP degradation was partially blocked (~22%) by zVAD-fmk (Fig. 5*F*, *lane 4* compared with *lane 3*), suggesting that besides the degron pathway (involving E3α ligase activity), other pathways (autoubiquitination) are involved in the degradation of XIAP. The presence of several other proteins that could be targets of E3α ubiquitin ligase cannot be ruled out.

Caspase Activation Occurs After the Down-regulation of Heat Shock Protein-70 and X-linked Inhibitor of Apoptosis Proteins. Hsp70 has been shown to associate with Apaf-1 and prevent activation of the initiator procaspase-9 (4, 5). These reports therefore prompted us to investigate the status of caspase-9 in avicin D-treated Jurkat cells. An increased cleavage of caspase-9 was observed at 2 hours of avicin treatment (Fig. 6*A*). However, the ability of XIAP to block the activity of even the proteolytically active caspases by binding to them (8, 9) prompted us to investigate if the cleaved caspase-9 acts on its downstream target, i.e., caspase-3. As shown in Fig. 6*B*, a fractional cleavage of caspase-3 was observed at 2 hours of avicin D treatment that increased at 4 hours. Interestingly, the activation of caspase-9 (at 2 hours) seems to closely follow the degradation of Hsp70 (Fig. 1*A*) and XIAP (Fig. 5*C*), which occurs at 1 hour of avicin treatment in Jurkat cells. The kinetics of these two events suggests that a decrease in the two antiapoptotic proteins, Hsp70 and XIAP, is necessary for the activation of caspases, thereby leading to apoptosis.

Effect of Avicin D on the Proteasomal Activity. The ubiquitin/proteasome machinery has been proposed to play a key role in the regulation of apoptosis. Specific inhibitors of proteasomes have been shown to induce apoptosis by accumulation of proapoptotic molecules and other less characterized mechanisms (26). Therefore, we wanted to investigate the effect of avicin D on the proteasome function in Jurkat leukemia cells. A

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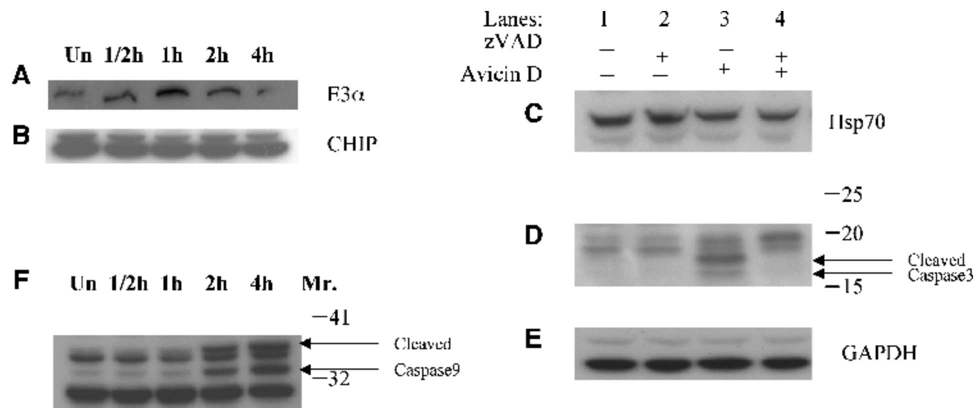

Fig. 5 Avicins induce the E3α ubiquitin ligase enzyme that participates in XIAP degradation. Western analysis of CE proteins (50 μg) from avicin D-treated Jurkat T cells probed with anti-E3α ubiquitin ligase (*A*), anti-CHIP (*B*), anti-XIAP (*C*) and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies (*D*); *E,* Jurkat cells were either treated with lactacystin (10 μmol/L, *lane 2*), avicin alone (4 hours, *lane 3*), or pretreated with lactacystin followed by avicin D (1 μmol/L, 4 hours; *lane 4*). CE proteins (50 μg) were resolved in SDS-PAGE and probed for XIAP protein. Jurkat cells were treated with zVAD-FMK (50 μmol/L, *lane 2*) or avicin D (1 μmol/L, 4 hours; *lane 3*) or pretreated with zVAD-FMK 30 minutes prior to avicin D treatment (*lane 4*). CE proteins (50 μg) were probed for XIAP (*F*), β-actin (*G*), and caspase-3 (*H*) protein. The cleaved products of caspase-3 are marked with an arrow and relevant molecular weight markers are shown on the right.

time-dependent decrease in the 20S proteasomal activity was observed upon avicin D treatment with the maximum and significant decrease of 33% and 41% at 2 and 4 hours, respectively (Fig. 7*A*). The decrease in the proteasomal activity from 2 hours matches with the protein conjugates observed in avicin D-treated cell extracts at around the same time (Fig. 7*B*). Recently, Sun et al. (27) showed that caspase activation inhibits the proteasome function during apoptosis, a process that leads to accumulation of proapoptotic factors. Interestingly, the 30% to 40% decrease in the proteasome activity during 2 to 4 hours of avicin treatment is in agreement with our observation of caspase-9 (Fig. 6*A*) and caspase-3 activation (Fig. 6*B*). It is, however, important to mention that the known antiapoptotic proteins such as Hsp70, Hsp90 (Fig. 1), and XIAP (Fig. 5*C*) are degraded to a great extent within 2 hours of avicin treatment when the proteasome activity shows only a marginal decrease.

Effect of Avicin D on Other Leukemic/Lymphoma Cell Lines and Fresh PBL from Sezary Syndrome Patients. To rule out the possibility that the effects of avicins described here could be cell type–specific, we next evaluated some leukemic/lymphoma cells treated with avicin D. Although the effects of avicin D on modulation of Hsp70 and XIAP vary at 4 hours in the different cell lines tested (Jurkat, U937, MJ-1, and HH), a significant decrease in Hsp70 and XIAP seemed to be consistent at 24 hours of avicin D posttreatment in all the cells (Fig. 8*A-C*). This observation suggests that the ability of avicins to regulate Hsp70 and XIAP is not restricted to a cell type. This result prompted us to examine the effect of avicins in fresh cells from patients. When PBL from Sezary syndrome patients were treated with avicin D for 24 hours, we observed a decrease in both Hsp70 (25-35%) and XIAP (30-40%) proteins (Fig. 8*D*, *E*, and *F*). Interestingly, avicin D treatment also causes apoptosis in these cutaneous T cell lymphoma cells (data not shown). PBL from normal blood samples treated with avicin D showed no significant change in both the Hsp70 and XIAP proteins (Fig. 8*G*) and seem to be resistant to apoptosis.[3] Thus, avicins' ability to regulate the two antiapoptotic proteins in various cells may contribute to its proapoptotic function.

DISCUSSION

Stress is a fundamental aspect of cellular life. Thus, the ability to cope with various environmental or internal stressors is essential for the maintenance and survival of organisms (28). One of the early characteristics of resistance or tolerance to stress is activation of the Hsp, which can be traced in evolution to the earliest prokaryotes, including archea (29). Because Hsps promote cell survival in multicellular organisms, elimination of damaged or mutated cells may become compromised when Hsps are continuously activated. During neoplastic transformation, cells activate a stress response to protect themselves against elimination (30). As a consequence, cancer cells are eventually selected for their antiapoptotic phenotype. Activation of Hsps in various cancers is common and is responsible, in part, for the antiapoptotic phenotype of cancer cells and contributes to resistance to anticancer drugs (2, 31, 32).

In this study, we wanted to understand how avicins regulate the postmitochondrial events in apoptosis, and discovered the ability of avicins to down-regulate antiapoptotic proteins Hsp70 and Hsp90, as well as the XIAP family. Several other chaperone proteins, including Hsp60, Hsc70, grp75, and calnexin were not affected. Based on the ability of avicins to regulate expression of transcription factors in a redox-dependent manner, we expected that down-regulation would be secondary to transcription of HSF. We were surprised to find that the avicins did not affect the transcription of HSF1, a redox-regulated event (19). To explain the down-regulation of Hsp70, we then turned to the possibility that the Ub/P pathway may be involved. First we showed that the proteasome inhibitor, lactacystin, reversed the down-regulation of Hsp70. Next, we

[3] C. Zhang, X. Ni, A. Gaikwad, J. Gutterman and M. Duvic et al., unpublished data.

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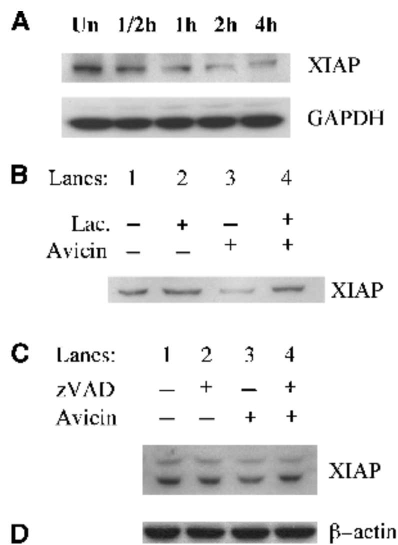

Fig. 6 Caspase activation occurs after the down-regulation of Hsp70 and XIAP proteins. Western analysis of CE proteins (50 μg) from avicin D-treated Jurkat T cells for 0 to 4 hours and probed with caspase-9 (*A*) and caspase-3 antibodies (*B*). An arrow marks the cleaved products of both caspase-9 and caspase-3. M_r markers are shown on the right. A protein band cross-reacting with caspase-3 antibody is shown to examine the loading pattern.

showed that Hsp70 is polyubiquitinated prior to down-regulation of the protein. This is the first report, to our knowledge, demonstrating regulation of a chaperone protein via enhanced ubiquitination.

The ability of avicins to activate the Ub/P pathway led us to evaluate the effect on the other important postmitochondrial antiapoptotic proteins like XIAP. The results suggest that avicins enhance both auto-ubiquitination, as well as degradation of XIAP by the ring finger E3α/degron pathway. Recently, a report of a small molecule antagonist of XIAP that overcomes resistance to apoptosis was revealed (33). These observations, therefore, suggest that regulation of XIAP selectively, or together with Hsps, may offer a new approach to cancer therapy.

Twenty years ago, Varshavsky's group (34) showed that ubiquitin is a stress protein with functional properties of a chaperone. His group showed that ubiquitin and Hsps often work in concert and, not infrequently, in a reciprocal manner (35). The fact that Ub/P is a stress-activated system explains its critical role in the regulation of apoptosis. Thus, various stresses are known to activate the Ub/P response, either to repair cellular damage or to remove defective cells. Cellular stress, as well as the maintenance of cancer cells with increased expression of Hsps, confers a high metabolic cost to the organism. Even though the avicins decrease cellular ATP production,[1] sufficient energy seems available to activate the Ub/P pathway and to execute the apoptotic process. Because proteolysis is an exergonic process (36), apoptosis of cancer cells and recycling of high-energy amino acids ultimately conserves energy and removes a principal source of reactive oxygen species.

Avicins were isolated from the pods and germinating seeds of a desert tree. As plants are stationary, they must respond quickly to environmental perturbations, in part by preserving or removing proteins necessary for hormonal action. For example, hormones such as ethylene and abscisic acid, prevent the degradation of positive regulators necessary for their action (37). Avicins' ability to activate the Nrf2 transcription factor, which increases expression of a battery of genes responsible for antioxidant defenses and cellular detoxification, is an important example of this principle. Thus, the cell is protected against external threats. On the other hand, plant hormones such as auxin, gibberlin, and jasmonates activate the Ub/P system to remove negative factors (37). The removal of negative regulators of apoptosis described in this article adds to the important examples of this principle. Thus, avicins' effect on stress is 2-fold, cytoprotection by preventing access of the Ub/P system to certain transcriptional factors, and proapoptotic by enhancing removal of antiapoptotic proteins. The ability of the avicin molecule to regulate stress responses by rapid modulation of the Ub/P pathway is consistent with its anti-inflammatory (38) and antioxidant properties (14). In addition, the similarity between avicins' biological effects and certain properties of calorie restriction is consistent with up-regulation of protein

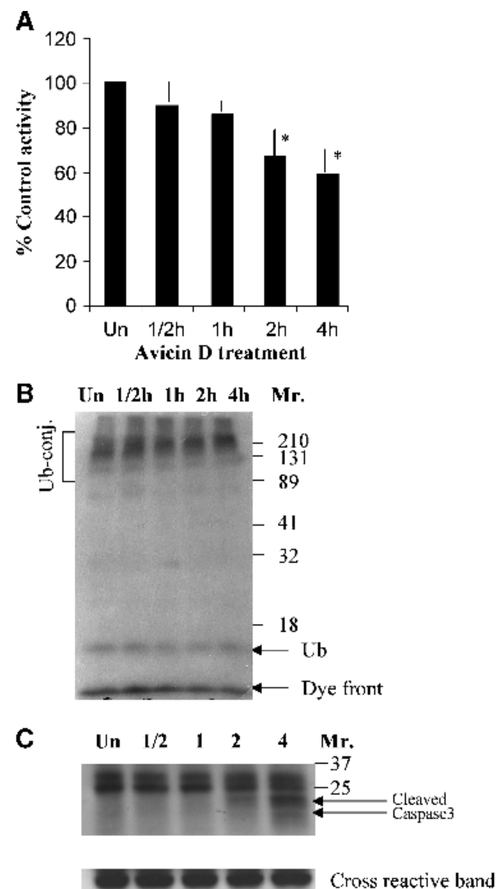

Fig. 7 Effect of avicin D on proteasomal activity. *A,* Jurkat cells treated with avicin D were used to determine proteasomal activity as described in Materials and Methods. The fluorescence measurement values shown are obtained from three independent experiments and represented as the percentage of control with respect to untreated cells. *t* Test significance shows * $P < 0.05$; *B,* approximately 50 μg of CE proteins from Jurkat cells treated with avicin D were separated on SDS-12.5% PAGE and probed with anti-ubiquitin antibodies to detect ubiquitin-protein conjugates. Position of M_r is shown on the right. Free ubiquitin and the dye-front are appropriately marked.

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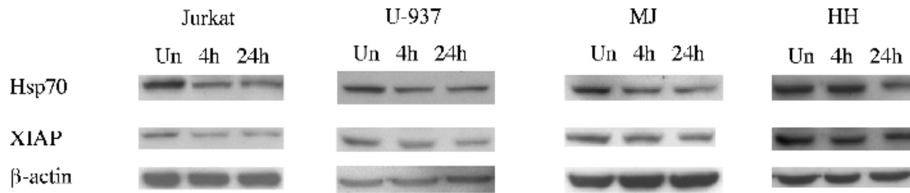

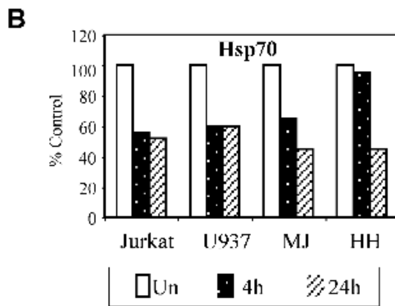

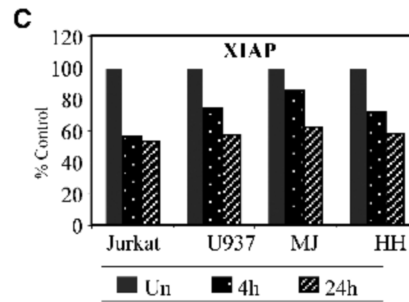

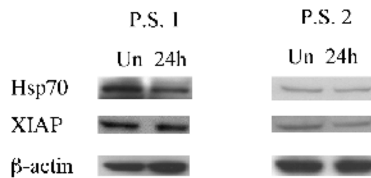

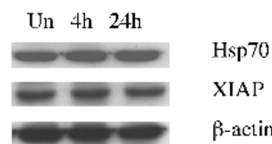

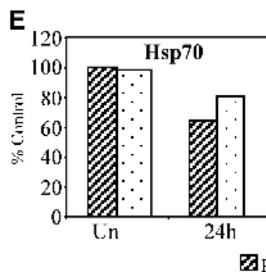

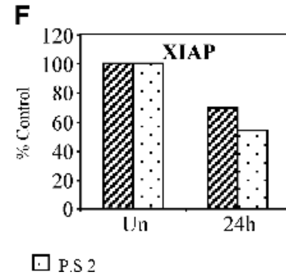

Fig. 8 Effect of avicin D on Hsp70 and XIAP proteins. *A,* various cell lines (Jurkat, U-937, MJ, and HH) were treated with avicin D for 4 and 24 hours as described in Materials and Methods. CE proteins were resolved on SDS-10% PAGE and probed with anti-Hsp70, anti-XIAP, and anti-β-actin antibodies. The autoradiographic signals were quantified by densitometry and the values represented as percentage control values of untreated cells (graphs *B* and *C* for Hsp70 and XIAP proteins, respectively); *D,* PBL cells from two Sezary syndrome patients (*P.S. 1* and *P.S. 2*) were treated with avicin D as described in Materials and Methods and CE proteins probed with anti-Hsp70, anti-XIAP, and anti-β-actin antibodies. The intensity of the signal was quantified and represented as mentioned earlier (*E* and *F* for Hsp70 and XIAP proteins, respectively); *G,* normal PBL cells were treated with avicin D and CE proteins probed with anti-Hsp70, anti-XIAP, and anti-β-actin antibodies.

turnover and enhanced Ub/P activity (39). Interestingly, a similar sequence of events described in this paper for regulation of Hsp70 has been shown in cell division, where the ubiquitin protein ligase (APC/C complex) degrades a chaperone (securin) to activate a cysteine protease (separase) during mitosis (40).

Recently, drugs that inhibit the proteasome have shown promising results as anticancer agents (41, 42). Although avicins share some properties of proteasome inhibitors such as PS341, significant differences exist. The proteasome inhibitors such as PS341 generally completely suppress 20S activity (43). The avicins only partially suppress 20S activity. Both compounds suppress nuclear factor κB, but the avicins do so by redox regulation (38). Both PS341 (42) and avicins (44) inhibit the PI3K/Akt pathway. However, the proteasome inhibitors potently activate stress responses and up-regulate expression of Hsp70 and Hsp90 (42), in contrast to the avicins. Thus, we propose that the avicins offer a unique anticancer profile and could be synergistic with proteasome inhibitors. Recently, a synthetic triterpene has been shown to be synergistic with Velcade, a proteasome inhibitor (45).

Many questions remain to be answered. For example, does the activation of ubiquitination occur in tumor cells other than a T cell lineage? Interestingly, our preliminary data suggest that avicins can activate ubiquitination in *Schizosaccharomyces pombe* cells and a yeast mutant for ubiquitin ligase is resistant to cytotoxicity.[4] What are the upstream signaling events responsible for Ub activation? What is the nature of chemical interaction of avicins with the SH groups in E1, E2, and E3? Considering the probable interaction of the avicin side chain with thiol groups, it will be important to understand how avicins chemically regulate these enzymes.

[4] J. Gutterman, et al. The tumor inhibitory triterpenoid, avicin G, inhibits cytokinesis and causes up-regulation of protein ubiquitination in the fission yeast, *Schizosaccharomyces pombe*. (submitted for publication).

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In conclusion, these results, combined with our previously published data, have important implications for biological and potential clinical application. We propose that the avicins regulate a very highly coordinated programmed response to stress, in which transcription factors are regulated by redox-modification to maintain homeostatic balance and other proteins are removed to enhance destruction of damaged cells. The overall effect is to shift energy requirements from immediate needs to that associated with repair or maintenance of somatic health. Thus, a rapid and selective regulation of stress by the avicins acts as a molecular switch to control cell death and life, inflammation, and other aspects of metabolism. Therefore, in addition to regulation of neoplastic growth, avicins' down-regulation of Hsps, an important component of innate immune system, almost certainly contributes to their anti-inflammatory effects and regulation of the innate immunity (14, 46). It is likely that the covalent adduction of avicins to protein substrates acts as a molecular tag to regulate this balance in human cells.

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