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Effect of Protein Kinase Inhibitors on Activity of Mammalian Small Heat-Shock Protein (HSP25) Kinase

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ABSTRACT. The aim of this study was to investigate different protein kinase inhibitors (secondary metabolite-derived substances, synthetic compounds, and substrate-based peptides) for their potency to inhibit the mammalian small heat shock protein (HSP25) kinase (E.C. 2.7.1.37) isolated from Ehrlich ascites tumor cells. Among the secondary metabolite-derived inhibitors (staurosporine, K-252a, K-252b, KT5926, KT5720, erbstatin analog, and quercetin) and synthetic compounds (H-9, H-89, HA 1004, KN-62, ML-7, tyrphostin A25, and tyrphostin B42), KT5926, staurosporine, and K-252a inhibited HSP25 kinase most efficiently. Kinetic analysis revealed that inhibition by staurosporine (K_i = 32.4 nM) and K-252a (K_i = 13.7 nM) was competitive with ATP. Inhibition by KT5926 was competitive with the substrate peptide KKKALNRQLSVAA (K_i = 27.2 nM) and noncompetitive with respect to ATP (K_i = 38.8 nM). In comparison with other protein kinases, HSP25 kinase was relatively resistant to most of the inhibitors. KT5926 was the only tested inhibitor with certain preference for HSP25 kinase when compared with protein kinases A, C, and G. Among the tested substrate-based peptides, we identified one peptide (KKKALNRQLGVAA), which preferentially inhibited HSP25 kinase in comparison with protein kinases A and C and mitogen-activated protein kinase. This peptide inhibited HSP25 kinase competitively with the substrate peptide (K_i = 8.1 μM) and noncompetitively with ATP (K_i = 134 μM). A peptide (SRVLKEDKERWEDVK) derived from the putative autoinhibitory domain of the closely related human mitogen-activated protein kinase-activated protein kinase-2 did not inhibit HSP25 kinase activity, suggesting the existence of several species of HSP25 kinases. Furthermore, the data identified structural requirements for inhibitors of HSP25-kinase. BIOCHEM PHARMACOL 53;9:1239–1247, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. protein serine threonine kinase, inhibitors, Ehrlich ascites tumor cells, heat-shock protein, HSP25, phosphorylation

A variety of extracellular stimuli such as serum, growth factors, cytokines, GTP-binding protein-mediated agonists, tumor promoters, and cytotoxic agents (e.g. heat, arsenite, EGTA, calcium ionophores and H_2O_2) result in phosphorylation of the mammalian small heat-shock protein HSP25† [1, and references therein], which itself is thought to be involved in the organization of microfilaments [2, 3]. Phosphorylation of HSP25 is catalyzed by a new type of protein serine threonin kinase termed HSP25 kinase or mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2), largely specific for small mammalian heat-shock proteins. Protein kinases with this specific activity have been described in Chinese hamster CCL39 and HeLa cells [1], Ehrlich ascites tumor cells [4], rabbit and human skeletal muscle [5], human lung MRC-5 fibroblasts [6], and human HL-60 cells [7]. Biochemical data suggest similarity of these protein kinases, although they are not necessarily identical enzymes. The published partial sequences of MAPKAP kinase-2 derived from mouse lung [8], rabbit and human skeletal muscles [5], and a full-length cDNA derived from human HL-60 cells [7] show strong homology in the N-terminal region and the catalytic domain. However, sequence differences have been observed at the C-terminus. *In vitro*, HSP25 kinases are phosphorylated and activated by mitogen-activated protein kinase (MAPK) [1, 5, 9], although *in vivo* this reaction appears to

The E.C. number of the protein serine threonine kinases mentioned (HSP25 kinase, MAPK, MAPKAP kinase-2, CaM-PK II, PKA, PKC, PKG, MLCK, protein kinase of herpes simplex US3 gene) is 2.7.1.37. The E.C. number of the protein tyrosine kinases mentioned (EGF receptor-associated protein kinase, pp60src protein kinase) is 2.7.1.112.

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† *Abbreviations:* CaM-PK II, Ca/calmodulin-dependent protein kinase II; HSP25, 25 kDa mammalian small heat-shock protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; Hy, hydrophobic amino acid residue; MAPK, mitogen-activated protein kinase; MAPKAP kinase-2, mitogen-activated protein kinase-activated protein kinase-2; MLCK, myosin light chain kinase; DTE, dithioerythritol; EGF, epidermal growth factor; PKG, cGMP-dependent protein kinase; IC_{50}, concentration causing 50% inhibition.

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be catalyzed by a homolog of MAPK called p38 MAPK or reactivating kinase [10].

Specifically acting protein kinase inhibitors can help to analyze complex signal transduction systems involving protein phosphorylation. Three major classes of substances have been investigated for their potency to inhibit protein kinases: (1) secondary metabolites and their derivatives, (2) synthetic compounds, and (3) substrate-based inhibitor peptides. Secondary metabolites inhibiting protein kinases include the microbial alkaloids staurosporine (isolated from *Streptomyces* sp.) [11], K-252a (isolated from *Nocardiopsis* sp.) [12], erbstatin (isolated from *Streptomyces* sp.) [13], and plant flavonoid compounds like quercetin [14]. Several groups of specifically acting protein kinase inhibitors were obtained by chemical modification of such naturally occurring compounds (e.g. indol carbazole series compounds) or of synthetic compounds known to inhibit protein kinases (e.g. H-series compounds and tyrphostins) [14, 15]. In recent years a great number of protein kinase inhibitors showing considerable specificity for different classes of protein kinases have been described [15]. Another approach to develop specifically acting protein kinase inhibitors is the use of substrate-based inhibitor peptides. The inactive form of a number of protein kinases is maintained by the presence of an autoinhibitory region (pseudosubstrate prototope) masking the catalytic activity [16, 17]. The pseudosubstrate prototope often acts by mimicking the features of the protein substrate phosphorylation site, and the protein kinases are activated by removing the pseudosubstrate prototope from the active site. Synthetic peptides derived from pseudosubstrate prototopes of protein kinase C (PKC) [18], Ca/calmodulin-dependent protein kinase II (CaM-PK II) [19], myosin light chain kinase (MLCK) [20], and of the putative pseudosubstrate prototope of human MAPKAP kinase-2 [21] were shown to inhibit the activity of the corresponding kinases. Thus, mimicking the features of substrates is a useful strategy to regulate the activity of protein kinases.

Thus far, not much data are available concerning the effects of protein kinase inhibitors on HSP25 kinase. In this study, we report on the effects of different classes of protein kinase inhibitors on the activity of HSP25 kinase purified from Ehrlich ascites tumor cells. Furthermore, we designed synthetic peptides which can potentially mimic substrates and tested their effect on HSP25 kinase, cAMP-dependent protein kinase (PKA), PKC, and MAPK. We show that one of the peptides preferentially inhibits HSP25 kinase.

MATERIALS AND METHODS

Purification of HSP25 Kinase

Ehrlich ascites tumor cells (30 g) were grown in mice and a protein fraction (0.45S fraction) was prepared by ammonium sulfate precipitation as described [4, 22, 23]. Fifteen milliliters of the dissolved proteins were loaded on a phenyl-Sepharose CL-4B column (Pharmacia, Freiburg, Germany) and eluted with a 0.05–2.5% Triton X-100 gradient in buffer A (20 mM Tris-HCl, pH 7.4; 50 mM NaF; additives: 1 mM DTE (dithioerythritol); 1 mM EDTA; 1 mM benzamidine; 0.5 mM Na_3VO_4; 5 mM sodium pyrophosphate; 0.05% Triton X-100). HSP25 kinase eluted at 1.8–2.4% Triton X-100 and was subsequently loaded on a Biogel HT column (Bio-Rad, Munich, Germany). Elution was with a 0–250 mM potassium phosphate gradient (pH 7.4) in buffer A, and the HSP25 kinase eluted at 130–200 mM potassium phosphate. After dialysis against buffer B (20 mM HEPES–NaOH, pH 7.4; additives as in buffer A), the protein was loaded on an SP-Sepharose Fast Flow column (Pharmacia). Elution was with a 0–400 mM NaCl gradient in buffer B. HSP25 kinase eluted at 320–390 mM NaCl and, for the following reactivation, HSP25 kinase-containing fractions were concentrated to 2 mL by ultrafiltration. Reactivation of HSP25 kinase was by incubation with 0.2 μg/mL MAPK (p44mpk, Biomol, Hamburg, Germany) and 0.5 mM ATP for 30 min at 30°C. Thereafter, the mixture was diluted with buffer C (25 mM Bistris-iminodiacetic acid, pH 7.1; additives as in buffer A) and loaded on a Mono P HR5/5 column (Pharmacia). Elution was with a pI-gradient in buffer D (Polybuffer 74–iminodiacetic acid, pH 4.0; additives as in buffer A) according to the manufacturer's (Pharmacia) instruction. HSP25 kinase eluted between 5 and 10 mL after the onset of the pH gradient, and was finally loaded on a Mono Q HR5/5 column (Pharmacia). An NaCl gradient (0–400 mM) in buffer E (20 mM Tris-HCl, pH 7.4; additives as in buffer A) was used for elution. HSP25 kinase eluted at 20–80 mM NaCl. After pooling, HSP25 kinase-containing fractions were used for assays. After each step of separation, a protease/phosphatase inhibitor mix yielding final concentrations of 5 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 μg/mL apoprotinin, 0.4 μM microcystin LR (inhibitors purchased by Sigma, Deisenhofen, Germany), and 0.2 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany) was added to fractions containing HSP25 kinase. Isolated HSP25 kinase was visualized by sodium dodecylsulfate polyacrylamide gel electrophoresis [24] followed by an in-gel assay [1] using the substrate peptide P1 (cf. Table 2, purchased from BioTez, Berlin, Germany) conjugated to BSA (cf. Results section).

Measurement of Protein Kinase Activities

The standard HSP25 kinase assay (25 μL) contained 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 1 mM DTE, 10 mM potassium phosphate, 2 μL of the purified HSP25 kinase, and 20 μM of the substrate peptide P1. The reaction was initiated by addition of 100 μM [γ-^{33}P]ATP if not otherwise indicated and incubation was for 10 min at 30°C. Aliquots of 20 μL were spotted onto phosphocellulose paper and processed as described [25] to determine [^{33}P]phosphate incorporation into P1. The activity of the catalytic subunit of PKA (Sigma) was assayed using 150 μM kemptide (Boehringer, Mannheim, Germany) as substrate, and

the activity of MAPK (p44mpk, Biomol) was assayed using 500 μM MAPK substrate peptide (Biomol). The standard assay contained 20 mM HEPES-NaOH (pH 7.4), 20 mM $MgCl_2$, 1 mM DTE, and 10 mM potassium phosphate. The reaction was initiated by addition of 100 μM [γ-^{33}P]ATP, and the samples were processed as described for the HSP25 kinase assay. The activity of PKC (Boehringer) was assayed using 75 μM of the PKC substrate peptide N-acetyl-myelin basic protein (4–14) (Boehringer). The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.25% BSA, 0.1 mg/mL phosphatidylserine (Sigma), and 2 μg/mL phorbol-12-myristate-13-acetate (Sigma). The reaction was initiated by addition of 100 μM [γ-^{33}P]ATP and the samples were processed as described for the HSP25 kinase assay. To allow direct comparison of enzymatic activities of HSP25 kinase, PKA, and PKC in the presence of inhibitor peptides, such substrate peptide concentrations were chosen which yield similar ratios to the respective K_m-values.

Protein Kinase Inhibitors

Quercetin was purchased from Sigma, with all other inhibitors from Calbiochem (Bad Soden, Germany). Stock solutions were made in dimethylsulfoxide (KN-62, K-252b, KT5926, KT5720, staurosporine: 1 mM; ML-7: 10 mM; tyrphostins A25, B42, erbstatin analog: 100 mM), in H_2O (H-9 · 2HCl, H-89 · 2HCl, HA 1004 · 2HCl: 10 mM), in ethanol (quercetin: 10 mM), or in dimethylformamide (K-252a: 1 mM). Stocks were diluted and added to the incubation mixtures assuring maximum solvent concentration of 2%, which was proven not to affect HSP25 kinase activity (not shown).

RESULTS

Purification of HSP25 Kinase

HSP25 kinase was isolated from Ehrlich ascites tumor cells as described in Materials and Methods, yielding a highly purified enzyme preparation without major contaminating proteins and a specific activity of 8.4 pmol $min^{-1}mg^{-1}$. Further data on HSP25 kinase will be presented elsewhere.* The activity of the isolated HSP25 kinase was visualized by an in-gel assay using the substrate peptide P1 (cf. Table 2) conjugated to BSA. Figure 1 shows that HSP25 kinase activity was associated with two protein bands with molecular masses of approximately 45 and 50 kDa, in accordance with earlier observations [1]. The HSP25 kinase activity could be immunoprecipitated with antibodies raised against murine MAPKAP kinase-2–derived peptides (not shown), suggesting that HSP25 kinase is closely related to or identical with MAPKAP kinase-2. The HSP25 kinase isolated from Ehrlich ascites tumor cells phosphorylated the substrate peptide P1 with a K_m-value of 2.2 μM and HSP25 with a

FIG. 1. Autoradiograph showing the in-gel assay of isolated HSP25 kinase. A 7.5–15% sodium dodecylsulfate polyacrylamide gradient gel containing the substrate peptide P1 conjugated to BSA was used for detection of HSP25 kinase activity. Bars indicate the position of molecular mass standards (from top to bottom: 200; 97.4; 69; 46; 30; 21.5; and 14.3 kDa).

K_m-value of 4.2 μM. The K_m-value for ATP was 12.8 μM (data not shown). For recombinant MAPKAP kinase-2, a similar K_m-value for HSP25 was determined, further suggesting similarity between the two protein kinases [26].

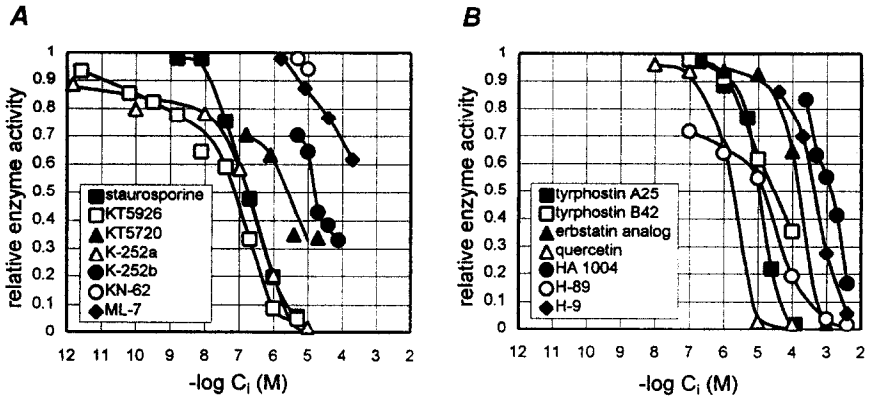
Effect of Protein Kinase Inhibitors on HSP25 Kinase Activity

The relative activity of HSP25 kinase in the presence of various concentrations of different protein kinase inhibitors is shown in Fig. 2. Among them, the indole carbazoles KT5926, K-252a, and staurosporine showed the highest inhibitory potency with IC_{50}-values (inhibitory concentration 50%) of 70, 180, and 180 nM, respectively. Two further inhibitors of this group, KT5720 and K-252b, showed a moderate effect, with IC_{50}-values of 2 and 16 μM, respectively (Fig. 2A). Similarly, the tyrosine protein kinase inhibitors quercetin, tyrphostin A25, tyrphostin B42, and erbstatin analog, as well as the H-series inhibitor H-89, showed moderate inhibitory activities, with IC_{50}-values of 2, 11, 32, 160, and 14μM, respectively (Fig. 2B). The other tested inhibitors of the H-series (H-9, HA 1004, ML-7, and KN-62) showed either a weak or no inhibitory activity with IC_{50}-values greater than 200 μM (Fig. 2A, B). The determined IC_{50}-values are listed in Fig. 2C.

Kinetic Analysis of HSP25 Kinase Inhibition by Staurosporine, K-252a, and KT5926

To elucidate the mechanisms involved in the inhibition of HSP25 kinase by staurosporine, K-252a, and KT5926, these inhibitors were tested for their ability to compete with ATP and the substrate peptide P1. In Fig. 3A, B, and C, Lineweaver-Burk plots of the inhibition of HSP25 kinase obtained by variation of the ATP concentration in the presence or absence of different concentrations of staurosporine, K-252a, and KT5962, respectively, are represented. Inhibition of HSP25 kinase by staurosporine (K_i = 32.4 nM) and K-252a (K_i = 13.7 nM) was competitive with ATP (Fig. 3A and B), suggesting the interaction of these inhibitors with the ATP binding site. Accordingly, a noncompetitive

* K. Hayess et al., manuscript in preparation.



C

	IC_{50} (nM)
KT5926	70
staurosporine	180
K-252a	180
KT5720	2,000
quercetin	2,000
tyrphostin A25	11,000
H-89	14,000
K-252b	16,000
tyrphostin B42	32,000
erbstatin analog	160,000
H-9	450,000
HA 1004	1,400,000
KN-62	>10,000
ML-7	>200,000

FIG. 2. Effects of various concentrations of protein kinase inhibitors on the activity of HSP25 kinase. (A, B) HSP25 kinase activity was measured as described in Materials and Methods using 125 μM substrate peptide P1. (C) The inhibitors used and the IC_{50}-values obtained from the curves are listed.

type of inhibition for both inhibitors with the other substrate, P1, was expected. However, determination of the type of inhibition of HSP25 kinase by staurosporine and K-252a with respect to P1 yielded different results. The intersections of the straight lines in the Lineweaver-Burk plot were not, as expected, on, but above the abscissa in the negative reciprocal substrate concentration range, probably indicating a more complex type of inhibition (not shown). The determined K_i-values for staurosporine and K-252a with P1 were 194 nM and 182 nM, respectively.

KT5926 appeared to act by a different mechanism. This substance inhibited HSP25 kinase noncompetitively with respect to ATP (K_i = 38.8 nM) and competitively with P1 (K_i = 27.2 nM) (Fig. 3C and D), suggesting binding to the peptide binding site and not, as expected, to the ATP binding site. Data obtained for KT5926 concentrations up to 40 nM fit into this model. At higher inhibitor concentrations, we observed a more complex type of inhibition (not shown). The K_i-values for ATP determined for staurosporine, K-252a, and KT5926 are listed in Table 1.

Design of Substrate and Inhibitor Peptides

The aligned phosphorylation sites of murine, hamster, and human small mammalian heat-shock protein (HSP25) and

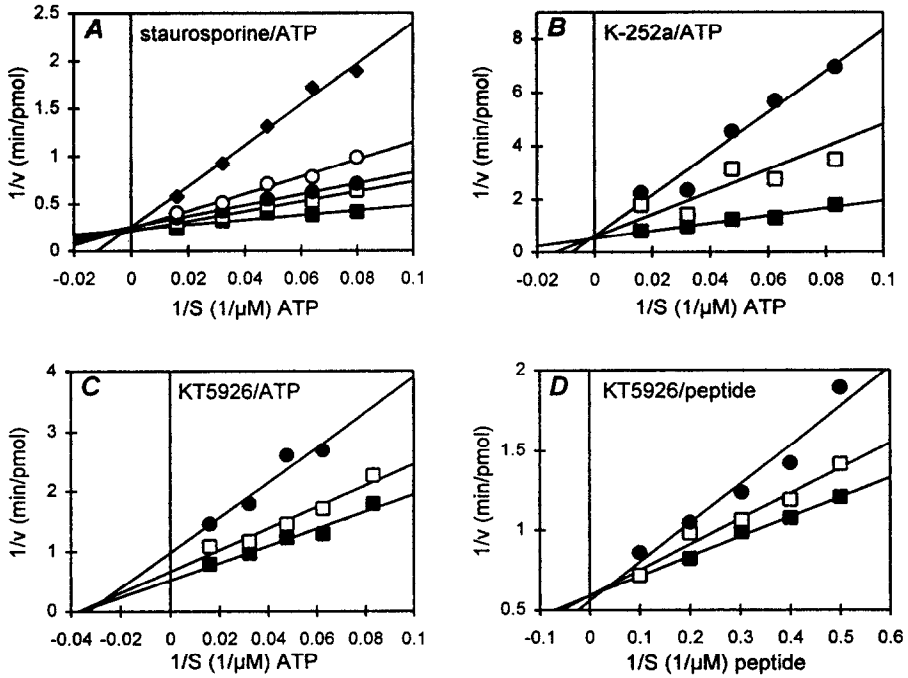
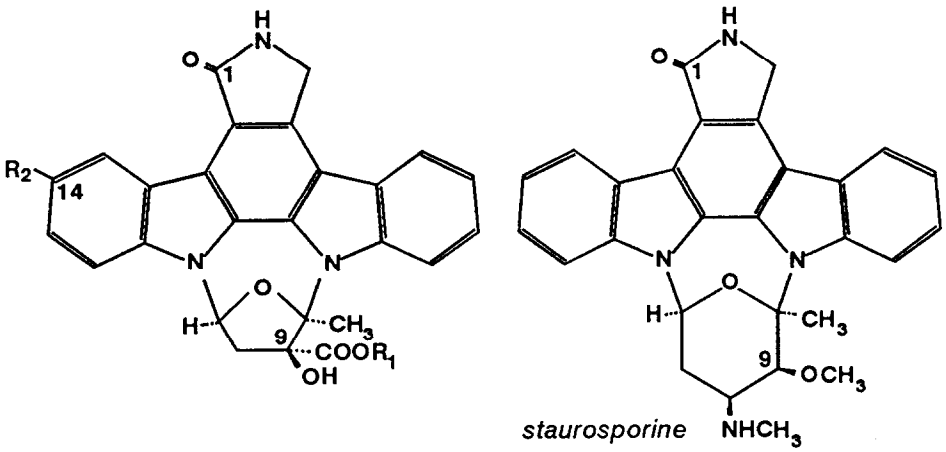


FIG. 3. Effect of various concentrations of ATP (A, B, C) and substrate peptide P1 (D) on inhibition of HSP25 kinase activity by staurosporine (A), K-252a (B), and KT5926 (C, D). HSP25 kinase activity was measured in the absence (■) or presence of staurosporine (A: □, 25 nM; ●, 50 nM; ○, 100 nM; ♦, 200 nM), K-252a (B: □, 20 nM; ●, 80 nM), or KT5926 (C: □, 10 nM; ●, 40 nM; D: □, 10 nM; ●, 20 nM) in the presence of different concentrations of [γ-^{33}P]ATP and P1.

TABLE 1. Effect of K-252 compounds and staurosporine on HSP25 kinase, CaM-PK II, MLCK, PKC, PKA, and PKG



			K_i values for ATP/IC_{50} values (nM)					
Compound	**R1**	**R2**	**HSP25 kinase**	**CaM-PK II**	**MLCK**	**PKC**	**PKA**	**PKG**
K-252a	CH_3	H	13.7[1]	1.8[a]	20[b]	25[b]	16[b]	15[b]
KT5926	CH_3	$O(CH_2)_2CH_3$	38.8[1]	4.4[a]	18[b]	723[b]	1200[b]	158[b]
K-252b	H	H	16000*[2]	—	147[c]	20[c]	90[c]	100[c]
KT5720	$(CH_2)_5CH_3$	H	2000*[2]	—	>2000[c]	>2000[c]	56[c]	>2000[c]
staurosp.			32.4[1]/180*[2]	25*[d]	—	2.7*[e]	22*[d]	8.5*[f]

* IC_{50} value (nM).
[1] The K_i values with HSP25 kinase were calculated from the slopes of the lines shown in Fig. 3 as a function of the inhibitor concentration.
[2] IC_{50} values are taken from Fig. 2.
[a] quoted from [31]; [b] quoted from [32]; [c] quoted from [15]; [d] quoted from [29]; [e] quoted from [11]; [f] quoted from [30].

of rabbit glycogen synthase recognized by HSP25 kinases are presented in Table 2 [5, 27, 28]. Sequence comparison and studies using synthetic substrate peptides resulted in the formulation of the minimum sequence HyXRXXSXX (Hy, hydrophobic amino acid residue) required for efficient phosphorylation by HSP25 kinases [5]. Based on these data, we designed the substrate peptide P1 (KKKALNRQLSVAA) similar to the sequence around phosphorylation site serine 86 (position n) of murine HSP25 (Table 2). For efficient phosphorylation, two amino acid residues are

TABLE 2. Phosphorylation site motifs recognized by HSP25 kinases and derived peptide sequences

Mouse HSP25	S15	6	VPFSLLRSPSWEPF	19
	S86	77	FSRALNRQLSSGVS	90
Human HSP25	S15	6	VPFSLLRGPSWDPF	19
	S78, S82	73	YSRALSRQLSSGVS	86
Hamster HSP25	S15	6	VPFSLLRGPSWDPF	19
	S89	80	FHRALNRQLSSGVS	93
Rabbit glycogen synthase	S7	1	PLNRTLSVASL	11
Substrate peptide	P1		KKKALNRQLSVAA	
Phosphorylation site deficient peptides	P2		KKKALNRQLAVAA	
	P3		KKKALNRQLGVAA	
	P4		KKKALNRQLCVAA	
	P5		KKKALNRQLYVAA	
	P6		KKKALNRQLPVAA	
	P7		SRVLKEDKERWEDVK	

Upper part: Phosphorylation sites are underlined. Conserved residues of substrates of HSP25 kinases are in bold letters.

Lower part: Peptide P7 is derived from the putative autoinhibitory domain of human MAPKAP kinase-2 (amino acid residues 324–338). Residues conserved in autoinhibitory domains of PKA, PKC, and MAPKAP kinase-2 are in **bold letters.**

required C-terminal to the phosphorylation site (positions $n + 1$, $n + 2$). To avoid a potential second phosphorylation site at position $n + 1$, the residues of the murine HSP25 sequence (serine and glycine) were substituted for the corresponding residues of the rabbit glycogen synthase sequence (valine and alanine). To facilitate the HSP25 kinase assay, lysine residues not critical for the specificity of the kinase were added N-terminal.

In order to design inhibitory peptides with preference for the HSP25 kinase, the serine residue of P1 (position n) was replaced by alanine, glycine, cysteine, tyrosine, or proline residues (peptides P2–P6, respectively) (cf. Table 2). These peptides are not phosphorylated by HSP25 kinase (not shown), but may bind to the peptide binding site and thus act as inhibitors.

Effect of Peptides Derived from the Substrate Sequence and from the Putative Autoinhibitory Prototope on Activities of HSP25 Kinase, MAPK, PKA, and PKC

Peptides P2–P6 at 260 μM were used in the standard phosphorylation assay. Peptides P4, P5, and P6 (serine substituted for cysteine, tyrosine, and proline residues, respectively) had no effect on phosphorylation of P1 by the HSP25 kinase. P2 (serine substituted for alanine residue) showed a weak (10%) reduction, and P3 (serine substituted for glycine residue) a pronounced effect (40% reduction) on the phosphorylation of P1 (Fig. 4). The dependence of the phosphorylation of P1 on the concentration of P3 is shown in Fig. 5. The determined IC_{50}-value was 45 μM. To determine the specificity of P3, its concentration-dependent effect on PKA, PKC, and MAPK was also assayed. P3 also inhibited PKA (IC_{50} = 180 μM) and PKC (IC_{50} = 120 μM) although with less efficiency than HSP25-kinase, but had no effect on MAPK (Fig. 5).

The putative autoinhibitory domain of human MAPKAP

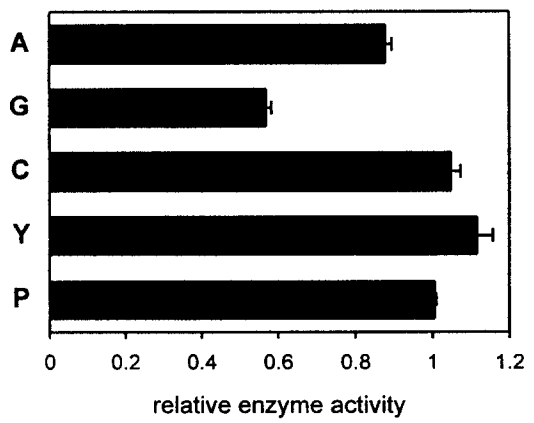


FIG. 4. Effect of peptides P2–P6 on phosphorylation of peptide P1. Phosphorylation of peptide P1 (80 μM) in the presence of peptides P2–P6 (A, G, C, Y, and P, respectively; 260 μM) is shown in comparison with phosphorylation of peptide P1 alone. Bars indicate SE.

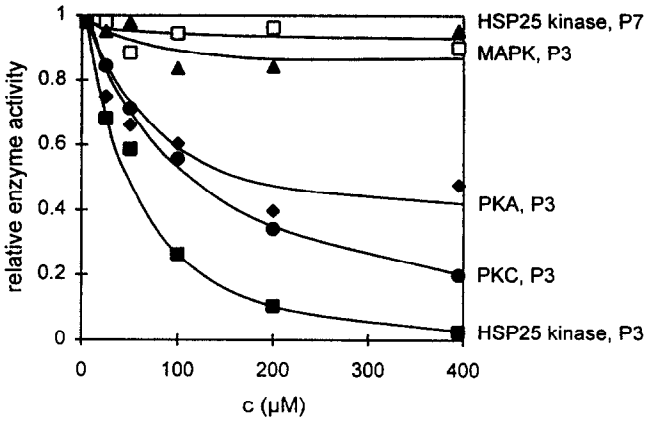


FIG. 5. Concentration-dependent effect of peptides P3 and P7 on activity of various protein kinases. The effect of peptide P3 on the activity of hsp25 kinase (■), MAPK (▲), PKA (♦), and PKC (●), and of peptide P7 on the activity of HSP25 kinase (□) is shown. Protein kinase activities were measured as described in Materials and Methods.

kinase-2 was recently described by Zu *et al.* [21]. However, the peptide derived from this domain (P7, cf. Table 2) was found to be rather unspecific, because it inhibited, in addition to human MAPKAP kinase-2, PKA and PKC as well, but not MAPK [21]. For direct comparison of the specificity of P3 and P7, the latter peptide was also included in this study. The activities of HSP25 kinase, PKA, PKC, and MAPK were measured in the presence of different concentrations of P7. We could confirm that P7 inhibits PKA and PKC, but not MAPK (not shown). Because of the similarity of isolated murine HSP25 kinase to recombinant murine MAPKAP kinase-2 (see above), we expected P7 also to inhibit HSP25 kinase. Surprisingly, P7 did not inhibit HSP25 kinase isolated from Ehrlich ascites tumor cells, as shown in Fig. 5 (see Discussion).

Kinetic Analysis of HSP25 Kinase Inhibition by P3

To determine the type of inhibition, the dependence of HSP25 kinase activity on the concentration of both P1 and ATP in the presence of different concentrations of P3 was analyzed and the data were plotted in a Lineweaver-Burk diagram. As expected, P3 inhibited HSP25 kinase competitively with P1, suggesting a common binding site for both peptides at the enzyme (Fig. 6A). The determined K_i-value was 8.1 μM for P3 with P1 as substrate. This K_i-value is below 10 μM, and P3 can thus be considered as a potent inhibitor of HSP25 kinase [16]. Because substrate-based inhibitor peptides may also act as ATP antagonists (e.g. for CaM-PK II) [16], the inhibition by P3 was also analyzed with ATP as substrate (Fig. 6B). P3 inhibited HSP25 kinase noncompetitively with ATP, suggesting that it does not interact with the ATP binding site. The determined K_i-value of P3 with ATP was 134 μM.

DISCUSSION

Among the tested secondary metabolite-derived and synthetic inhibitors, KT5926, staurosporine, and K-252a show

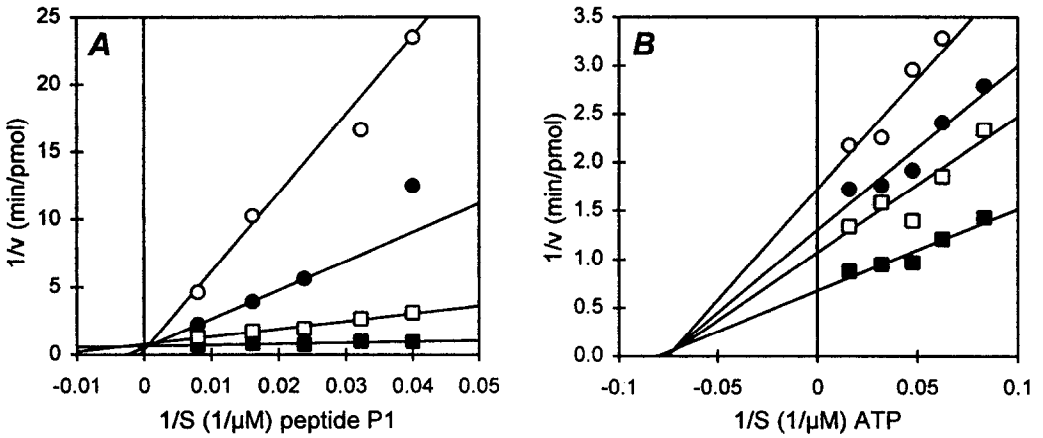


FIG. 6. Effect of various concentrations of substrate peptide P1 (A) and ATP (B) on inhibition of HSP25 kinase activity by peptide P3. HSP25 kinase activity was measured in the absence (■) or in the presence of 75 μM (□), 150 μM (●), or 300 μM (○) peptide P3 in the presence of different concentrations of peptide P1 (A) and [γ-^{33}P]ATP (B).

the highest potency to inhibit HSP25 kinase. Kinetic analysis revealed that inhibition by staurosporine and K-252a is competitive with ATP, while inhibition by KT5926 is competitive with the substrate peptide and non-competitive with respect to ATP. The determined K_i-values of these inhibitors for ATP of HSP25 kinase and the available K_i- or IC_{50}-values for ATP of other protein kinases are listed in Table 1. None of the tested inhibitors is highly specific for HSP25 kinase. Compared with other protein kinases, HSP25 kinase is relatively resistant to the PKC-specific inhibitor K-252b [15] and even to the unspecific inhibitor staurosporine [11, 29, 30]. K-252a, which is specific for CaM-PK II [31], inhibits HSP25 kinase with a similar potency as MLCK, PKC, PKA, and cGMP-dependent protein kinase (PKG). KT5926 shows certain preference for HSP25 kinase in comparison with its effect on PKC, PKA, and PKG. However, its preference is approximately 10 times higher for CaM-PK II and about two times higher for MLCK than for HSP25 kinase. KT5926 was derived from K-252a as specific inhibitor of smooth muscle myosin light chain kinase [32]. Later on, it was found that both substances are potent and preferential inhibitors of CaM-PK II (cf. Table 1) [31]. It is noteworthy that the catalytic domain of murine MAPKAP kinase-2 (which is closely related to HSP25 kinase) has the highest sequence homology to the α-subunit of rat CaM-PK II [8]. Although no calcium/calmodulin dependence of HSP25 kinase/MAPKAP kinase-2 has been observed, its relative sensitivity towards the CaM-PK II–specific inhibitors KT5926 and K-252a may reflect the evolutionary relationship of both kinases.

A common characteristic of staurosporine, K-252a, and KT5926 is the small hydrophobic moiety at position 9 (staurosporine: methoxy group; K-252a, KT5926: methoxy carbonyl group), which appears to be essential for inhibition of HSP25 kinase (Table 1). Hydrophilic (K-252b: carboxyl group) or large hydrophobic (KT5720: hexoxy group) moieties at position 9 interfere with the inhibitory effect on HSP25 kinase. A large hydrophobic moiety at position 14, as is the case for KT5926, seems to be important for the suggested interaction with the peptide binding site of HSP25 kinase (see above).

Neither the tested naphthalene sulphonamide (ML-7) nor its isoquinoline derivatives (H-89, H-9, HA 1004, KN-62) have a major inhibitory potency on HSP25 kinase. Moreover, H-7 and HA 1077 were reported to be poor inhibitors of MAPKAP kinase-2 [33]. These inhibitors are specific for MLCK (ML-7), PKC (H7), PKA (H-89, H-9, HA 1004), PKG (H-9, HA 1004), and CaM-PK II (HA1077, KN-62) [15]. Interestingly, inhibition of CaM-PK II by KN-62 is competitive with calmodulin and non-competitive with respect to ATP [15], which is in contrast to K-252a and KT5926 [31], perhaps explaining the different effects on HSP25 kinase.

The natural flavonol quercetin is a rather unspecific protein kinase inhibitor. It inhibits PKA (IC_{50} = 1 μM) [34], PKC (IC_{50} = 15 μM; K_i = 2.6 μM) [14, 35], the protein kinase of the epidermal growth factor (EGF) receptor (IC_{50} = 15 μM) [14], the pp60^{src} protein kinase (K_i = 6–11 μM) [36], and the protein kinase of herpes simplex US3 gene (K_i = 10 μM) [37], all competitively with respect to ATP. As shown here, inhibition of HSP25 kinase by quercetin is in the same range (IC_{50} = 2 μM, cf. Fig. 2). Erbstatin analog is a more selective inhibitor of protein tyrosine kinases. Accordingly, it is a poor inhibitor of HSP25 kinase, with an IC_{50}-value of 160 μM (Fig. 2), which is approximately 150 times less efficient than inhibition of EGF receptor-associated tyrosine kinase (IC_{50} = 0.9 μM) [13] and about 15 times less efficient than inhibition of PKC (K_i = 11 μM) [38]. Tyrphostins, low-molecular-mass organic compounds resembling tyrosine and erbstatin moieties, were specifically designed to inhibit tyrosine protein kinases. They are considered to be 100- to 10000-fold less potent against serine/threonine protein kinases [39]. Tyrphostin A25 inhibits the EGF receptor-associated protein kinase with an IC_{50}-value of 15 μM [40]. Surprisingly, inhibition of the serine/threonine-specific HSP25 kinase by this drug is in the same range (IC_{50} = 11 μM) (Fig. 2). Thus, tyrphostin A25

probably has less specificity for protein tyrosine kinases than generally assumed. This may be due to the ability of tyrphostins to compete with both ATP and peptide substrates [14, 39]. The second tyrphostin tested, B42, appears to discriminate between the two types of protein kinases. It inhibits the EGF receptor-associated protein kinase with an IC_{50}-value of 0.1 μM [41] and the HSP25 kinase with an IC_{50}-value of 32 μM (Fig. 2).

We designed a substrate peptide (P1, KKKALNRQLSVAA) similar to the sequence around phosphorylation site serine 86 of the murine HSP25. In order to create inhibitory peptides with preference for HSP25 kinase, the phosphorylatable serine of P1 was replaced by alanine, glycine, cysteine, tyrosine, or proline residues (peptides P2–P6, respectively). Peptide P3 (KKKALNRQLGVAA) inhibits HSP25 kinase competitively with P1 (K_i = 8.1 μM) and noncompetitively with ATP (K_i = 134 μM), suggesting a common binding site of P1 and P3 at the enzyme. Because the K_i-value of P3 with respect to P1 is smaller than 10 μM, P3 can be considered as a potent inhibitor of HSP25 kinase [16]. P3 also inhibits PKA and PKC with less efficiency but has no effect on MAPK activity. It is likely that the similarity of the phosphorylation site motifs recognized by PKA (XRRXSX), PKC (XRXXSXRX) [42], and HSP25 kinase (HyXRXXSXX) [5] is the basis for inhibition of PKA and PKC by P3.

The peptide P7 derived from the putative autoinhibitory domain of the human MAPKAP kinase-2 was reported to be rather unspecific, since it inhibits, in addition to human MAPKAP kinase-2, PKA and PKC as well [21]. The reason for this may be the presence of conserved amino acid residues which have been identified in the pseudosubstrate prototopes of several protein kinases, including PKA and PKC, and which are important for recognition by the catalytic domain (cf. Table 2) [20, 21, 43]. Surprisingly, P7 does not inhibit HSP25 kinase isolated from Ehrlich ascites tumor cells. This is in contrast to results published previously [21], but can be explained by the existence of several species of HSP25 kinases which may show a different sensitivity towards inhibitor peptides. The observed sequence divergence at the C-terminus described for MAPKAP kinase-2 supports this view [5, 7, 8]. Furthermore, the autoinhibitory domain of the murine MAPKAP kinase-2 was not found to function as a pseudosubstrate [17].

Both the efficacy and preference of the inhibitor peptide P3 presented in this study are probably based on its similarity with natural phosphorylation site motifs. Characteristic features of these motifs include the arginine residue (position n − 3) and the leucine residue (position n − 5). The valine residue (position n + 1) may also be of functional importance, because hydrophobic residues (valine, isoleucine, and leucine) have been observed at this position in pseudosubstrate prototopes of several protein kinases [16], including the human MAPKAP kinase-2 (Table 2) [21]. Comparison of pseudosubstrate prototopes of several protein kinases reveals that alanine is the most common residue occupying the phosphate acceptor site at position n, although glycine, threonine, and serine residues can also occur [16]. In this light, it is noteworthy that the alanine peptide (P2) is less effective than the glycine peptide (P3) in inhibiting HSP25 kinase.

In summary, we have shown that secondary metabolite-based protein kinase inhibitors as well as a substrate peptide-based inhibitor peptide are able to inhibit HSP25 kinase. However, so as to interfere selectively with the mitogen- and stress-related phosphorylation of small heat-shock proteins, efforts should be undertaken to develop inhibitors that are more specific for HSP25 kinase. As shown here, KT5926 and peptide P3 appear to be suitable initial products, because they are effective inhibitors of HSP25 kinase and show certain preference for this enzyme in comparison with PKC, PKA, and, in the case of KT5926, with PKG.

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