

## REVIEW ARTICLE

# Molecular basis for the actions of Hsp90 inhibitors and cancer therapy

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Heat-shock protein 90 (Hsp90) inhibitor downregulates c-Myc expression and upregulates the expression of tumor repressor proteins such as p53 and pRB, inhibiting the G1/S transition and causing G2/M arrest during cell cycle progression. The cycle progression is extensively controlled by the pRB/E2F signaling pathway. E2F is released from the pRB/E2F complex with the phosphorylation of pRB by cyclin–cyclin-dependent kinase (CDK) complexes. The released E2F promotes the transcription of target genes involved in cell cycle progression. The pRB/E2F signaling pathway is controlled by DNA methyltransferase-1 (Dnmt-1). The elevated expression of Dnmt-1 has been reported in carcinomas of the colon, lung and prostate. A defect of pRB expression in *Rb*–/– cancer cells is caused by the aberrant methylation of CpG in the *Rb* promoter. The Hsp90 inhibitor disrupts the Dnmt-1/Hsp90 association and upregulates pRB expression. In this review, the Hsp90 inhibitors that show promise for cancer therapy are summarized.

*The Journal of Antibiotics* (2011) 64, 635–644; doi:10.1038/ja.2011.60; published online 3 August 2011

**Keywords:** cancer therapy; Hsp90 inhibitor; signal-transduction pathway

## INTRODUCTION

### Function of heat-shock protein

Heat-shock protein (Hsp) is an ubiquitous molecular chaperone distributed from bacteria to animal cells. Hsp is involved in post-translational folding, stability and protein maturation. Hsp is an essential mediator of signal-transduction and cell cycle progression in prokaryotes and eukaryotes. Numerous Hsps including the Hsp60, Hsp70 and Hsp90 families are distributed throughout cytoplasm, mitochondria, nucleolus and cytoplasmic membrane. Human Hsp90 includes 17 genes classified as *HSP90AA*, *HSP90AB*, *HSP90B* and *TRAP*.<sup>1</sup> Major molecular chaperones related to tumorigenesis and tumor progression are classified as Hsp90. The use of Hsp90 inhibitors for cancer therapy has recently advanced. Hsp90 inhibitors lead to misfolding of client proteins of Hsp90, ubiquitination and proteasome degradation. A growing number of Hsp90 client proteins (Table 1) have been shown to be important for the development, proliferation and survival of several types of cancer. Hsp90 forms complexes together with co-chaperone molecules, such as Hip, Hop, p23, Cdc37 and immunophilins (Table 2). The Hsp90 and co-chaperone complex control the binding of client proteins, enabling them to adapt to their situation. Hsp90 family proteins should be closely associated with tumorigenesis and tumor progression. Hsp90 inhibitors disturb the cellular homeostasis and inhibit the proliferation and metastasis of cancer cells. The molecular chaperone Hsp90 has emerged as an exciting target for cancer therapy in the past decade. We describe herein the molecular mechanism responsible for the action of Hsp90

inhibitors against cancer cells and review the promise of Hsp90 inhibitors for cancer therapy.

## HSP90'S ROLES IN TUMORIGENESIS, TUMOR PROGRESSION AND TUMOR MAINTENANCE

The integral roles of Hsps in the multistep processes of tumorigenesis and tumor progression have been suggested. This can be understood by searching for the molecular mechanism of action of Hsp90 inhibitors. The Hsp90 molecular chaperone is expressed at high levels in a wide variety of human cancers, including melanoma, leukemia and cancers of the colon, prostate, lung and breast.

Hsp90 is amplified in cancer cells and is thought to induce their rapid proliferation. The increased expression of Hsp90 is crucial to tumorigenesis involving the loss of p53 function, and the higher expressions of the proto-oncogenes HER2 and c-Myc.<sup>2</sup> Hsp90 inhibitors may selectively inhibit the signal-transduction pathways amplified in cancer cells. The recent progress in understanding the complex roles of Hsp90 in tumorigenesis has been made possible in the discovery of several types of Hsp90 inhibitors exhibiting anti-tumor effect. Hsp90 inhibitor potently inhibited cellular proliferation by inducing G1 arrest in a variety of tumor cell lines. The G1 arrest by geldanamycin (GA), a benzoquinone ansamycin antibiotic, is attributed to destabilization of the Raf-1 protein, and to the resulting inhibition of mitogen-activated protein kinase (MAPK). GA triggers a rapid and marked decrease in the kinase activity of cyclin E/cyclin-dependent kinase (CDK2) complex coupled with a

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Received 26 November 2010; revised 15 March 2011; accepted 9 June 2011; published online 3 August 2011

**Table 1 Major client proteins associated with Hsp90**

<i>Hsp90</i> client protein	Character
v-Src	Avian Rous sarcoma viral oncogene protein=Src tyrosine kinase
Lyn	Src family protein tyrosine kinase
Lck	Src family protein tyrosine kinase
Yes	Avian sarcoma virus Y73 oncogene protein, non-receptor tyrosine kinase
Fps=Fes	Feline or avian sarcoma virus oncogene protein, protein tyrosine kinase
Bcr-Abl	Breakpoint cluster region-Abelson (avian Abelson leukemia virus oncogene protein)
Raf-1	Serine/threonine kinase of mouse retrovirus 3611-MSV3=MAP kinase kinase kinase
AKT/PKB	v-akt murine viral oncogene homolog, serine/threonine protein kinase=protein kinase B
ErbB2	v-erb-b2 avian erythroblastic leukemia viral oncogene protein, receptor tyrosine kinase tyrosine
Plk-1	<i>Drosophila</i> polo-like kinase 1, serine/threonine protein kinase
MET	Met proto-oncogene protein, hepatocyte growth factor receptor, protein tyrosine kinase
Wee1	Cell cycle regulator, phosphorylate cdc2 kinase, protein tyrosine kinase
Cdc2	Cell division cycle 2, G1 to S and G2 to M cell cycle regulator
Cdc4	Bind directly to cyclin E, targets cyclin E for ubiquitin-mediated degradation
Cdc6	A protein essential for a regulator at the early steps of DNA replication
Dnmt-1	DNA cytosine-5-methyltransferase
Survivin	Baculoviral inhibitors of apoptosis protein=inhibitors of caspase family proteases
Mutant p53	A mutated tumor repressor protein, loss of the function of transcription factor
c-Myc	Proto-oncogene protein of avian myelocytomatosis virus MC29 oncogene
hTERT	Human telomerase reverse transcriptase, telomerase is related to cells survival
MOK	A member of the mitogen-activated protein kinase superfamily
MAK	Male germ-cell-associated kinase, nuclear Cdc2-related kinase or MAPKK
MRK	A member of the MAPKKK family of signal-transduction molecules
DNA polymerase $\alpha$	DNA polymerase for mammalian chromosomal DNA replication
Estrogen receptor	Likely to develop tumors with high expression
Androgen receptor	Likely to develop breast cancer, prostate cancer with high expression
HIF-1 $\alpha$	Hypoxia-inducible factor-1 $\alpha$ , a family of proteins responsible for cellular oxygen deficiency
SV40 large T antigen	A potent oncogene able to transform many cell types, binds to the viral replication origin and promotes the SV40 replication
SV40 small T antigen	Promotes the efficient replication of genomic DNA, accelerating the progression of the S phase
EBV nuclear antigen	EBNA, EBNA2, EBNA5, EBNA3A and EBNA3C are essential for transformation to Burkitt's lymphoma, and so on

Abbreviation: EBNA, Epstein-Barr virus nuclear antigen.

decline in CDK2 associated with cyclin E, accompanied by the inhibition of CDK4 kinase activity and the inhibition of pRB hyperphosphorylation.<sup>3</sup>

**Table 2 Major co-chaperone proteins associated with Hsp90**

<i>Co-chaperone molecules of Hsp90</i>	
Hip	Mammalian Hsp70-interacting protein
Hop	Mammalian Hsp70/Hsp90-organizing protein
p23	Selectively destabilizes steroid receptors
Cdc37	A regulator of Hsp90 function to bind clients
Immunophilins	Cyclophilin A and FKBP12: cyclophilin A (Cyp40) and FKBP12 are the major targets of the immunosuppressive agents, CsA and FK506, respectively. The complex of Cyp40 and FKBP51 inhibits the function of NFAT

Abbreviations: CsA, cyclosporin A; Hsp, Heat-shock protein; NFAT, nuclear factor of activated T cell.

## HSP90 INHIBITORS AND SIGNALING PATHWAYS

The rapid proliferation of cancer cells must be attributable to an unusual signaling pathway. A number of oncogene, proto-oncogene and tumor suppressor proteins are reportedly associated with Hsp90 or Hsp90-cochaperone complexes. GA was first identified as an Hsp90 inhibitor.<sup>4</sup> X-ray-diffraction studies have demonstrated Hsp90-GA complexes.<sup>5</sup> It is important to understand the biology of Hsp90 chaperone at the molecular level before proceeding to the development of Hsp90 inhibitors.

### Conformational cycle of Hsp90 chaperone

Hsp90 operates as a dimer in a conformational cycle driven by ATP binding and hydrolysis at the N terminus.<sup>6</sup> The transient N-terminal dimerization of Hsp90 has been investigated under the assumption that the C-terminal interface is stably dimerized. Using a fluorescence-based single-molecule assay, kinetic analysis of the N- and C-terminal dimerizations was performed. Surprisingly, the C-terminal dimer opens and closes with fast kinetics.<sup>7</sup> The middle domain of Hsp90 (Hsp90M) acts as a discriminator between different types of client proteins.<sup>8</sup>

GA interacts with the N-terminal ATP-binding domain of Hsp90, inhibiting its ATPase activity, and causes the release of the client protein.<sup>9</sup> Novobiocin disrupts both N- and C-terminal nucleotide binding.<sup>9</sup> Cisplatin is a selective C-terminal nucleotide competitor.<sup>9</sup> These results indicate that the conformation of the middle domain is affected by an elaborate mechanism involving the C- and N-terminus conformation of Hsp90. GA and novobiocin are important tools for the initial characterization of Hsp90 function, but do not represent clinically viable compounds.

The client proteins of Hsp90 include v-Src, Lyn, Yes, Fps, Lck, Bcr-Abl, Raf-1, ATK/protein kinase B (PKB), ErbB2/HER2/Neu, hypoxia-inducible factor-1 $\alpha$ , Plk-1, MET, Wee1, CDC2, CDC4, CDC6, CDK4, DNA methyltransferase-1 (Dnmt-1), steroid hormone receptors (estrogen and androgen receptors), survivin, telomerase human telomerase reverse transcriptase (hTERT), mitogen-activated protein kinase (MOK), male germ-cell-associated kinase (MAK), MRK, SV40 super T, large T, small T antigen, EBV nuclear antigen, mutant p53, c-Myc and DNA polymerase  $\alpha$ . The major client proteins of Hsp90 involved in the signaling pathways are illustrated in Table 1. The viral oncogenic partner proteins of pRB, including human papilloma-coded protein E7, adenovirus E1A-encoded protein and SV40 large T antigen associated with pRB, deregulate the cell cycle progression and the cellular proliferation of neoplasia.<sup>10</sup> These viral oncogenic partner proteins of pRB preferentially bind to under-phosphorylated gene products of the retinoblastoma family gene products. The association of these viral oncogenic partner proteins

of pRB causes the dissociation of the pRB/E2F complex, and the released E2F upregulates a large number of genes essential for cell cycle progression. The pRB/E2F complex releases E2F upon the phosphorylation of pRB by cyclin E/CDK2 and cyclin A/CDK2 complexes (Figure 1). Overexpression of cyclin E in tumor cells bypasses the feedback expressions of cyclin D/CDK4 and CDK6/pRB.<sup>11</sup> When the pRB binding to the partner proteins is eliminated by a pRB mutation, its partner proteins are activated and promote cell cycle progression. The major partner proteins of pRB involved in cell cycle progression are Myc and E2F. The amino-terminal domains of c-Myc and N-Myc mediate binding to the pRB carboxyl-terminal domain.<sup>12</sup> E2F contains the carboxyl-terminal domains that bind to pRB family proteins. Targeting the c-Myc expression is an important objective for cancer chemotherapy.<sup>13</sup> Hsp90 inhibitors downregulate c-Myc expression.<sup>14,15</sup> The c-Myc protein bound to TFIIIB, a RNA polymerase III-specific general transcription factor, directly activates pol III transcription, thereby stimulating ribosomal RNA production.<sup>16,17</sup> The tumor suppressors p53 and pRB repress TFIIIB expression.<sup>17</sup> The c-Myc protein itself is assumed to be necessary for c-Myc expression.<sup>18</sup> The c-Myc expression is under self-control. Hsp90 inhibitor causes the degradation of the client protein c-Myc, and consequently downregulates the synthesis of mRNA, ribosomal RNA and low-molecular-weight RNA.

#### MicroRNA studies and Hsp 90 inhibitor

Humans have 800–1000 microRNAs (miRNAs), and these miRNAs are thought to regulate a significant fraction (more than 30%) of all

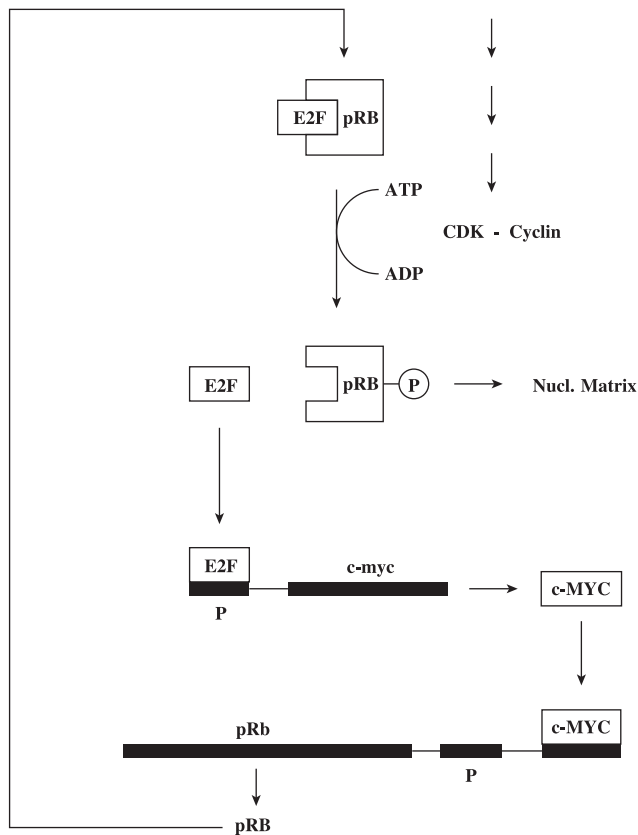
protein-coding genes.<sup>19</sup> miRNA, a form of small non-coding RNA, modulates cell cycle-dependent transcription during normal or tumoral proliferation through a complex interplay between itself and c-Myc or E2F family members.<sup>20,21</sup> E2F transcription factors are classified into eight groups, E2F-1–E2F-8.<sup>21</sup> The expression of E2F is modulated by miRNAs. The tumor suppressor miR-34a regulates the p53 pathway and downregulates c-Myc expression.<sup>22</sup> Let-7 miRNAs function as tumor suppressors by negatively regulating the expression of multiple oncogenes including *ras* and *myc*.<sup>23</sup> Argonaute proteins are associated with processing bodies and stress granules of cytoplasmic ribonucleoprotein complexes, which regulate gene expression. Argonautes are effectors of RNA interference. Hsp90 is required for the efficient targeting of hAgo2 to processing bodies and stress granules. Hsp90 inhibitor is associated with reduced miRNA and short interfering RNA-dependent gene silencing.<sup>24</sup>

Below, we describe several signaling pathways involving Hsp90.

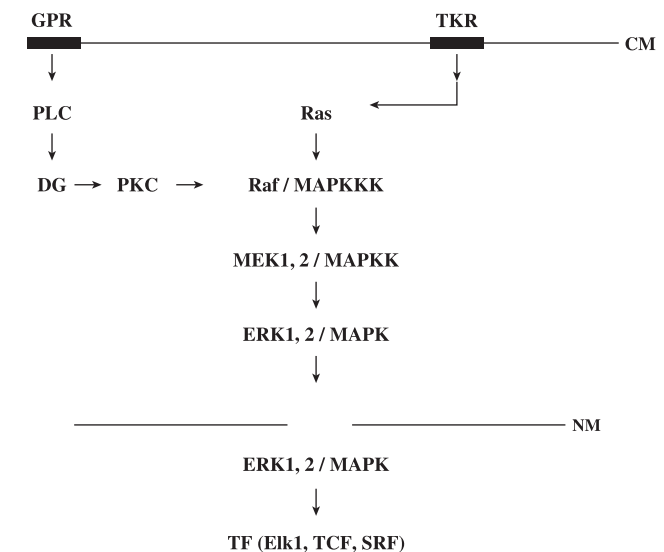
Connexin 43 (Cx43) is essential for the survival of cells. The expression of Cx43 is increased in *ras*-transformed cells, being enhanced by the Ras-Raf-MAPK pathway (Figure 2). The c-Myc–Hsp90 complex formed in Ras-transformed cells binds the AGTT-CAATCA sequence as the Cx43 promoter element. The Hsp90 inhibitor suppresses the c-Myc–Hsp90 complex binding to the Cx43 promoter, and inhibits the Cx43 promoter activity.<sup>25</sup>

The co-expression of Bcl2 and c-Myc efficiently antagonizes the effects of p53 on G1 arrest and apoptosis.<sup>26</sup>

A set of molecular chaperones Hsp90 and their co-chaperone CDC37 complex reportedly associate specifically with MOK, a member of the MAPK superfamily, and casein kinase 2 controls multiple kinases by phosphorylating the molecular chaperone CDC37. The Hsp90 inhibitors disrupt these pathways.<sup>27,28</sup>



**Figure 1** E2F-pRB signaling pathway. CDK: cyclin-dependent kinase; c-MYC: avian myelocytomatosis MC29 virus gene product; E2F: factor binding to adenovirus E2 gene promoter; P: promoter; circled P: phosphate; pRB: retinoblastoma gene product; Nucl Matrix: nuclear matrix.



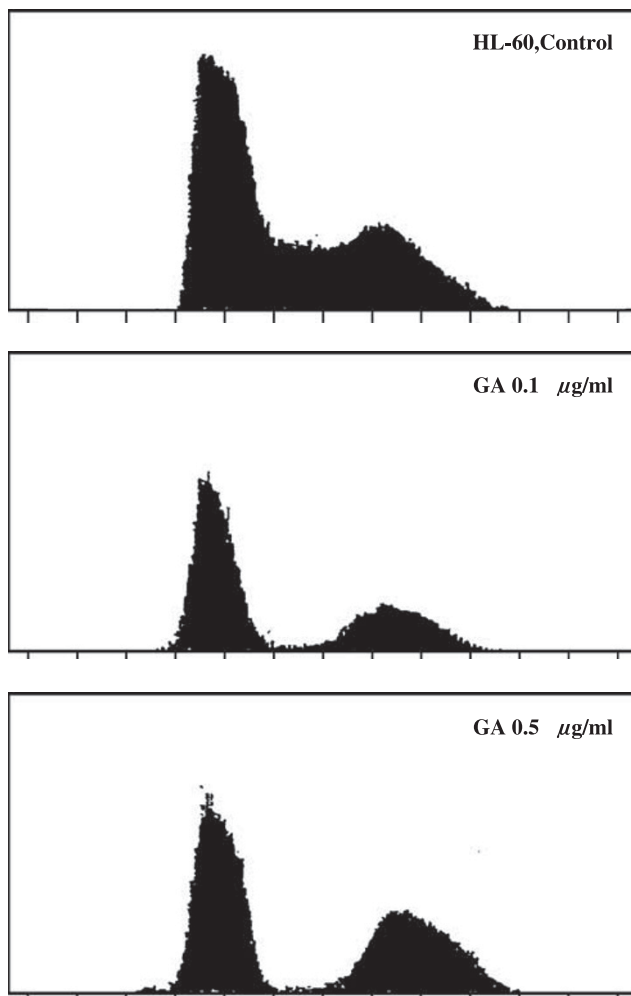
**Figure 2** Ras-Raf-MAPK signaling pathway. CM: cytoplasmic membrane; DG: diacylglycerol; Elk1: ternary complex factor; GPR: G-protein receptor; MAPK: mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPKK kinase; MEK=ERK=MAPK; NM: nuclear membrane; PLC: phospholipase C; PKC: protein kinase C; Ras: rat sarcoma gene product p21-GTP binding protein; Raf: rat fibrosarcoma gene product p74 ser/thr kinase; SRF: serum response factor; two molecules SRF and one molecule TCF or Elk1 form trimer; TCF: ternary complex factor; TF: transcription factor; TKR: tyrosine kinase receptor.

### CHROMOSOMAL DNA DUPLICATION AND CELL DIVISION

Eukaryotic genomes consist of multiple replicons. The human autonomous replication sequence (ARS/origin) was identified as a 36-bp determinant.<sup>29</sup> An internal 20 bp within the 36-bp consensus sequence is sufficient to act as a core origin element. Moreover, the origin activity in the tumor-transformed cells is two- to threefold higher than that of normal cells.<sup>30</sup> In chromosomal DNA replication, a plausible supermolecular assembly, the origin recognition complex, and the replisomes are thought to function in DNA replication initiation and the elongation of the DNA strands, respectively. The cell cycle starts from a G<sub>0</sub> quiescent state. The entry of G<sub>1</sub> into the S phase is accompanied by a sharp rise in DNA polymerase (Pol $\alpha$ ), a client protein of Hsp90. GA inhibits DNA replication with the partially purified Pol $\alpha$ , but the highly purified Pol $\alpha$  is refractory to GA.<sup>31</sup> This suggests that GA interacts with Hsp90 and disrupts the Hsp90/Pol $\alpha$  complex.

### Inhibition of association of pRB with nuclear matrix by Hsp90 inhibitor

GA inhibits the G<sub>1</sub>/S transition and causes G<sub>2</sub>/M arrest during cell cycle progression (Figure 3),<sup>14,15</sup> and c-Myc expression along with the



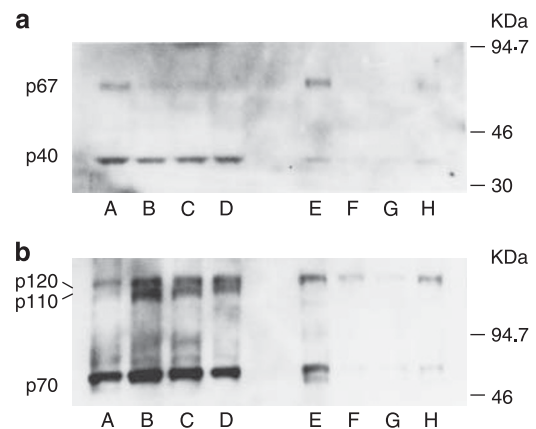
**Figure 3** Geldanamycin-induced inhibition of cell cycle progression in HL-60 cells. HL-60 cells cultured at an initial density of  $1.5 \times 10^5$  cells per ml after exposure to 0.1 or  $0.5 \mu\text{g ml}^{-1}$  GA for 20h were compared with control cells using flow cytometry. GA, geldanamycin.

upregulation of p53 and pRB expressions. The association between pRB and the nuclear matrix was inhibited by GA (Figure 4).<sup>14</sup> Lamin A, a major protein in the nuclear matrix, may be involved in the association between pRB and the nuclear matrix.<sup>14,32</sup> The G<sub>2</sub>/M arrest induced by Hsp90 inhibitor may be attributable to inhibition of the association between pRB and the nuclear matrix, presumably resulting in the inhibition of chromosome segregation and subsequent cell division.

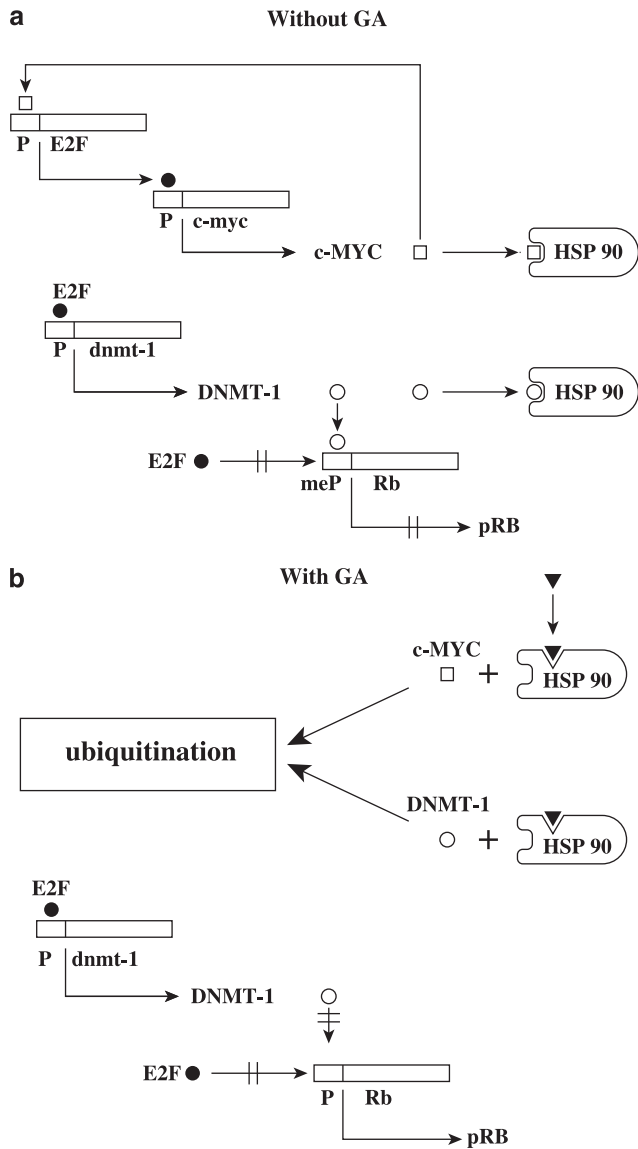
### A POSSIBLE MODEL FOR THE INHIBITION OF CELL CYCLE PROGRESSION BY HSP90 INHIBITORS

#### Dnmt-1 controls the pRB/E2F signaling pathway

We propose here a possible model of the molecular mechanism by which Hsp90 inhibitor inhibits c-Myc expression and upregulates pRB expression. Dnmt-1 expression is regulated by the pRB/E2F signaling pathway<sup>33</sup> (Figure 5). E2F binds to the promoter of Dnmt-1 and promotes the expression of Dnmt-1. Dnmt-1 catalyzes the transfer of methyl groups to cytosine in CpG dinucleotides. The methylation of CpG in the *Rb* promoter suppresses pRB expression. The elevated expression of Dnmt-1 has been shown in carcinomas of the colon, lung and prostate. The defect of pRB expression in *Rb*<sup>-/-</sup> cancer cells is caused by the aberrant methylation of CpG in the *Rb* promoter.<sup>33</sup> Histone deacetylase (HDAC) inhibitor promotes ubiquitin-dependent proteasome degradation of Dnmt-1 by Hsp90 disruption.<sup>34</sup> Dnmt inhibitors markedly decrease vessel formation and tumor cell growth.<sup>35</sup> HDAC and Dnmt-1 inhibitor should be promising for cancer therapy.



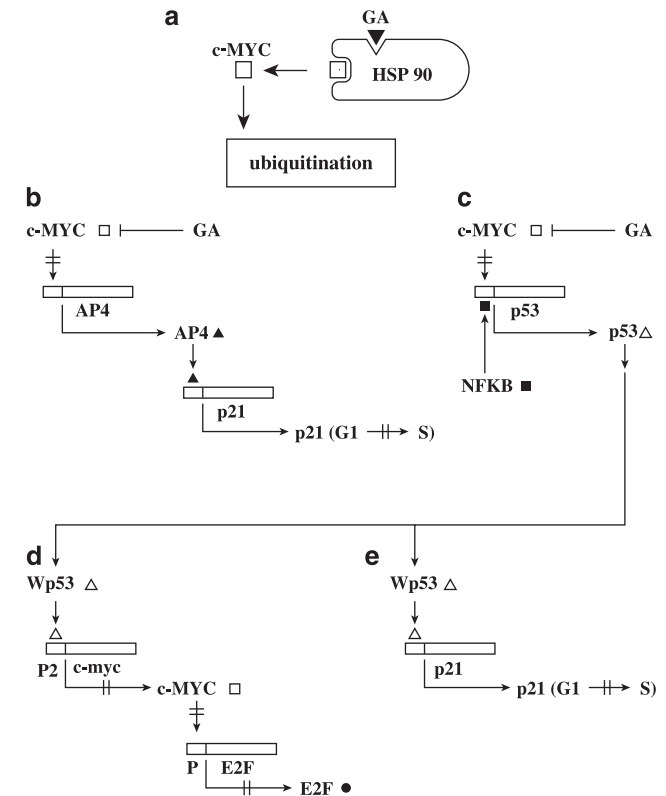
**Figure 4** Effect of geldanamycin on the expressions of c-MYC, p40, pRB and p70, and their associations with the nuclear matrix in HL-60 cells. (a) Effect of geldanamycin on the expressions of c-MYC and p40 and their associations with the nuclear matrix in HL-60 cells. Proteins were transferred from a polyacrylamide gel to a nitrocellulose sheet and analyzed using western blotting. c-MYC and a 40-kDa protein crossreactive with anti-human c-MYC monoclonal antibody are indicated by p67 and p40, respectively. Lanes A to D indicate nuclear extracts, and lanes B to H indicate nuclear matrices from HL-60 cells. Lanes A and E: control; lanes B and F:  $1 \mu\text{g ml}^{-1}$  GA; lanes C and G:  $0.2 \mu\text{g ml}^{-1}$  GA; lanes D and H:  $0.04 \mu\text{g ml}^{-1}$  GA. The MWs of the marker proteins are expressed in kDa. GA: geldanamycin. (b) Effect of geldanamycin on the expressions of pRB and p70 and their associations with the nuclear matrix in HL-60 cells. Proteins were transferred from a polyacrylamide gel to a nitrocellulose sheet and analyzed using western blotting. pRB and a 70-kDa protein crossreactive with anti-pRB monoclonal antibody are indicated by p110 (under-phosphorylated pRB) and p120 (hyper-phosphorylated pRB), respectively. Lanes A to D indicate nuclear extracts, and lanes B to H indicate nuclear matrices from HL-60 cells. Lanes A and E: control; lanes B and F:  $1 \mu\text{g ml}^{-1}$  GA; lanes C and G:  $0.2 \mu\text{g ml}^{-1}$  GA; lanes D and H:  $0.04 \mu\text{g ml}^{-1}$  GA. The MWs of the marker proteins are expressed in kDa. GA: geldanamycin.



**Figure 5** A model for the regulation of c-MYC and pRB expression in the E2F/pRB signaling pathway and the effect of geldanamycin on this pathway. (a) A model for the regulation of c-MYC and pRB expression. (b) A model for the regulation of c-MYC and pRB expression in the presence of GA. DNMT-1, dnmt-1: DNA methyltransferase-1; GA: geldanamycin; meP: methylated promoter.

**Dnmt-1 controls the c-Myc expression and the upregulation of p53**

The molecular mechanism for the downregulation of c-Myc expression and the upregulation of p53 by GA in L5178Y cells<sup>15</sup> can be explained in four steps (Figure 6). The Hsp90 inhibitor inactivates Hsp90 and destabilizes its client c-Myc protein (Figure 6a). The p53 promoter region from -96 to -41 contains nuclear factor-κB and c-Myc binding sites.<sup>36</sup> In addition, nuclear factor-κB binding to the p53 promoter causes the upregulation of p53 expression (Figure 6c). Wild-type p53 suppresses c-Myc expression (Figure 6d). Wild-type p53 binds to p21 (CDK inhibitor) promoter, triggering the expression of p21 and inhibiting G1/S transition during cell cycle progression. c-Myc directly regulates the expression of AP4 via CACGTG motifs in the first intron of the AP4 gene, and AP4 directly represses p21 expression by occupying four CAGCTG motifs in the p21 promoter



**Figure 6** A model for the regulation of c-MYC and p53 expression and the effect of geldanamycin (GA) on c-MYC and p53 expression. (a) A model for the degradation of c-MYC in the presence of GA. (b) A model for the expression of p21 in the presence of GA. (c) A model for the expression of p53 in the presence of GA. (d) A model for the inhibition of E2F expression by wp53. (e) A model for the expression of p21 by wp53. AP4: activator protein 4; NF-κB: NF-κB; p21: p21cdk inhibitor; wp53: wild-type p53.

(Figure 6b).<sup>37</sup> These findings indicate that c-Myc protein downregulates p21 expression (Figure 6e).

**HSP90 INHIBITORS ARE PROMISING FOR CANCER THERAPY**

Our interest lies in how to exploit the therapeutic potentials of Hsp90 inhibitors. Numerous Hsp90 inhibitors have been reported since GA was first identified as an Hsp90 inhibitor in 1994. The molecular mechanism responsible for the signaling pathway involved in Hsp90 action has been identified by exploring the mechanism of Hsp90 action.

17-AAG (17-allylamino-17-demethoxy GA) is currently being examined in Phase 3 clinical trials. 17-AAG is less toxic than GA and depletes some proteins involved in phosphoinositide 3-kinase (PI3K)/Akt signaling, such as ErbB2, epidermal growth factor receptor (EGFR) and phosphorylated AKT.<sup>38</sup> 17-AAG decreases a group of Myc-regulated mRNAs and increases the mRNA expression of Hsp90β, Hsp72, Hsp70, Hsp47 and Hsp27.<sup>39</sup> The major molecular chaperones Hsp90, Hsp70 and Hsp60 interact with newly synthesized polypeptides, and maintain the proteins in unfolded states that are suitable for translocation across the membrane. The nuclear translocation of heparanase is abolished by GA and 17-AAG in HL-60 cells, indicating that Hsp90 is involved in the nuclear translocation of heparanase.<sup>40</sup>

17-Dimethylaminoethylamino-17-demethoxy GA has been shown to be active against multiple myeloma in a synergistic manner when

administered in combination with the Akt inhibitor perifosine.<sup>41</sup> Dimethylaminoethylamino-17-demethoxy GA also acts in synergy with the effect of radiation therapy against non-small-cell lung carcinoma by interfering with base excision and ATM-mediated DNA repair.<sup>42</sup> DMGA-N-oxide, a novel cell-impermeable Hsp90 inhibitor, was reported to show a dramatic impact on the metastatic phenotype of the lung colonization of melanoma cells and be related to the hepatotoxic property of benzoquinone ansamycins.<sup>43</sup>

The O-carbamoylmethyloxime derivative of radicicol, KF25706, inhibits v-Src- and k-Ras-activated signaling.<sup>44,45</sup>

Macbecin<sup>46</sup> and its non-quinone compound that target Hsp90 have been reported,<sup>47</sup> indicating that the quinone moiety of macbecin is not a prerequisite for Hsp90 inhibition. The non-quinone derivative of macbecin has significantly improved the binding affinity to Hsp90 *in vitro*.

The carbazol-4-one benzamide derivatives, SNX-2112 and SNX-5422, exhibit potent antitumor activities against HER kinase-dependent cancers. These compounds cause HER2 degradation and the regression of HER2-dependent xenografts, potently inhibiting tumor cell growth, angiogenesis and osteoclastogenesis in multiple myelomas and other hematological tumors by abrogating signaling via Akt and ERK.<sup>48,49</sup>

Two 17-AAG formulations, tanespimycin and IPI-504, are Hsp90 inhibitors. IPI-504 has been found to display antitumor activity against pancreatic cancer xenografts and is a promising compound against gastrointestinal stromal tumors of platelet-derived growth factor receptor- $\alpha$  mutants.<sup>50</sup>

Genistein, an isoflavone, inhibits EGFR tyrosine kinase,<sup>51</sup> and also inhibits the Hsp90 deacetylase (HDAC6)–Hsp90 co-chaperone function, which downregulates the expression of androgen receptor in androgen-dependent prostate cancer cell lines.<sup>52</sup>

Quercetin, a flavonoid, inhibits cancer cells that over-express ErbB2 (Her-2/neu) trans-membrane tyrosine kinase and downregulates the downstream PI3K–Akt signaling pathway.<sup>53</sup>

Novobiocin binds to the C-terminal domain of Hsp90 and disrupts the Bcr-Abl/Hsp90 complex.<sup>54,55</sup> The novobiocin derivatives, 4TCNA and 7TCNA, destabilize Raf-1, HER2 and CDK4 in human cancer cells.<sup>56</sup>

The Hsp90 inhibitor STA-9090 inhibits Kit-dependent and -independent malignant mast cell tumors.<sup>57</sup>

The pyrazole-based compounds, CCT018159 and 3,4-diarylpyrazole resorcinol, and their amide derivatives, VER-49009 and VER-50589, have been reported to be Hsp90 inhibitors. Consistent with Hsp90 inhibition, these analogs cause the induction of Hsp72 and Hsp27 alongside the depletion of Hsp90 client proteins, including c-Raf and b-Raf. The cellular potency of these analogs is independent of NAD(P)H quinone oxidoreductase 1/DT-diaphorase and P-glycoprotein expression.<sup>58</sup> Hydroquinone ansamycins converted with quinone oxidoreductase 1 lead to a greater Hsp90 inhibitory activity and reduced toxicity.

The isoxazole derivatives NVP-AUY922,<sup>59</sup> PU-H71,<sup>60</sup> CUDC-305<sup>61</sup> and BIIB021 (CNF2024)<sup>62</sup> are Hsp90 inhibitors that have been tested clinically. 17-AAG and other benzoquinone ansamycins are inactive in P-glycoprotein/MRP-1-expressing tumors, but BIIB021 is active independently of NQO1/DT-diaphorase and P-glycoprotein expression. BIIB021, which can be orally administered, decreases Hodgkin's lymphoma cell viability at low nanomolar concentrations in synergy with doxorubicin and gemcitabine.<sup>63</sup> The use of an inhibitor of PI3K/Akt/mTOR (mammalian target of rapamycin: ser/thr kinase signaling pathway), such as NVP-BEZ235, and targeting Hsp90 chaperone improves the efficacy of rapamycin against hepatocellular carcinoma.<sup>64,65</sup>

Hsp90 inhibitors target Raf-1. The disruption of the Raf-1/Hsp90 complex by GA results in the loss of Raf-1–Ras associations.<sup>66</sup> PU-H17 downregulates components of the Ras/Raf/MAPK pathway and G2/M phase progression and induces the degradation of activated Akt and Bcl-Abl inducing apoptosis. PU-H17 shows potent antitumor effects against triple-negative breast cancers (defined by the absence of estrogen, progesterone and HER2 receptors).<sup>60</sup> Hsp90 inhibitors are thought to downregulate Ras expression and also be active against triple-negative breast cancers. PI3K and MAPK kinase inhibitors are active against k-Ras G12D, PIK3CA and H1047R murine lung cancers.<sup>67</sup> The combined use of NVP-BEZ235 and ARR-142886, an MAPK kinase inhibitor, showed marked synergy in shrinking k-Ras-mutant murine lung cancers.<sup>67</sup> Hsp90 has an important role in 3-phosphoinositide-dependent protein kinase-1–Akt signaling pathway.<sup>68</sup> 3-Phosphoinositide-dependent protein kinase-1 binds Hsp90, and Hsp90 inhibitor causes proteasome-dependent degradation of 3-phosphoinositide-dependent protein kinase-1.

#### Antibodies against tumor-specific cell surface antigens

Antibodies against tumor-specific cell surface antigens could be a favorable means of targeting cancer cells. Transtuzumab, a monoclonal antibody directed against p95-HER2, has been proven to be effective in the treatment of Her2-overexpressing breast cancer.<sup>69</sup> IPI-504 is a highly soluble small-molecule inhibitor of Hsp90. Combination of IPI-504 with transtuzumab significantly enhances tumor growth delay.<sup>70</sup> Combination of IPI-504 with lapatinib also enhances tumor growth delay.<sup>70</sup>

The provirus integration site for Moloney murine leukemia virus tumor-specific cell surface antigens (PIM-1) is a proto-oncogene that encodes ser/thr kinase with multiple cellular functions. Overexpression of PIM-1 has a critical role in the progression of prostatic and hematopoietic malignancies.

PIM-1-specific monoclonal antibody suppresses human and mouse tumor growth by decreasing PIM-1 levels, reducing Akt phosphorylation and activating apoptosis.<sup>71</sup>

#### Heparanase is involved in tumor metastasis

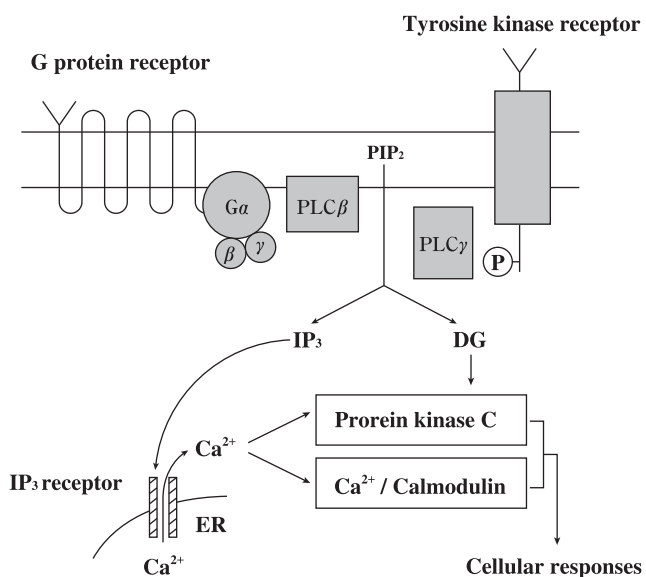
Heparanase displays pleiotropic effects on cancer progression. Heparanase cleaves carbohydrate chains of heparan sulfate proteoglycans (HS chains), which are an important component of the extracellular matrix. Hsp90 is involved in nuclear translocation and angiogenesis through the degradation of HS chains in the extracellular matrix on the cell surface. Heparanase is involved in angiogenesis through the direct induction of Cox-2 (cyclo-oxygenase-2, prostaglandin endoperoxide synthase or prostaglandin H synthase). Cyclo-oxygenase-2 induces vascular endothelial growth factor and basic fibroblast growth factor and is involved in neovascularization.<sup>72</sup> Heparanase is involved in tumor metastasis by inducing endothelial cell migration by the activation of the Akt/PKB signaling pathway in a manner that is independent of its endoglycosidase activity. The combination of Hsp90 inhibitor and heparanase inhibitors, such as suramin,<sup>73</sup> could be a promising treatment for blocking cancer metastasis.

#### HSP90 INHIBITORS IN VARIOUS STAGES OF CLINICAL TRIALS

A large multi-faced Phase 1 program was designed to evaluate the pharmacokinetics, safety, efficacy and targeted biological activity of a once-daily oral dose. The maximum-tolerated dose identified in Phase 1 trials is typically used to determine the dose level for Phase 2 and 3 trials. Several Hsp90 inhibitors are currently being evaluated. The chemical structures of major Hsp90 inhibitors under clinical trials are shown<sup>74</sup> (Figure 7).



**G-protein-coupled receptors.** G-protein-coupled receptors have trans-membrane domains that penetrate the plasma membrane seven times. The receptors include rhodopsin (a light sensor), hormone receptors, and nerve signaling and smell signaling receptors. The characteristic structure of a G-protein-coupled receptor is a trans-membrane domain consisting of a hydrophobic 25-amino-acid repeat. G protein is activated by GTP binding. The G-protein-GTP complex moves to the G-protein-GDP complex catalyzed by G protein itself in the presence of GTPase-activating protein. The inactive G-protein-GDP complex progresses to the G-protein-GTP complex by guanine nucleotide exchange factor. The GTP binding proteins are classified into two groups consisting of those with three heterogeneous subunits ( $\alpha$ ,  $\beta$  and  $\delta$ ) and low-molecular-weight GTP binding proteins, such as Ras, Rho, Rac and Cdc42. G protein consists of three heterogeneous subunits. The G protein dissociates into  $G_\alpha$  and  $G_{\beta\delta}$  complex through GTP binding to the  $G_\alpha$  subunit. In mammalian species, 16 kinds of  $G_\alpha$ , 5 kinds of  $G_\beta$  and 11 kinds of  $G_\delta$  genes are known. The mammalian  $G_\alpha$  proteins have multiple roles in signaling pathways involving, for example, the regulation (activation or repression) of adenylyclase, Rho guanine nucleotide exchange factor and the activation of cGMP phosphodiesterase and phospholipase  $C_\beta$ . The activation of adenylyclase generates the second messenger cAMP, which activates protein kinase A (ser/thr kinase). The phospholipase  $C_\beta$  generates the second messengers phosphatidylinositol ( $IP_3$ ), inositol 1,4,5-trisphosphate and diacylglycerol. Diacylglycerol activates PKC (ser/thr kinase) with  $Ca^{2+}$  released from the endoplasmic reticulum by  $IP_3$  binding to  $IP_3$  receptor on the endoplasmic reticulum. Calmodulin displays pleiotropic effects in the presence of  $Ca^{2+}$  (Figure 8). When CaM is occupied by two  $Ca^{2+}$  molecules, the CaM- $Ca^{2+}$  complex binds to myosin light-chain kinase (MLCK). The CaM- $Ca^{2+}$ -MLCK complex phosphorylates myosin light chain (MLC). The phosphorylated MLC binds to actin molecule. The interaction of the phosphorylated MLC-actin complex with  $Ca^{2+}$ -troponin induces muscle contraction. The CaM activates adenylyclase, phosphorylase kinase, membrane  $Ca^{2+}$ ,  $Mg^{2+}$ -ATPase, NDA kinase and CaM-dependent protein kinases, among others, in the presence of  $Ca^{2+}$ . Thus, G protein affects multiple signaling pathways.



**Figure 8** G-protein tyrosine kinase signaling pathway. DG: diacylglycerol; ER: endoplasmic reticulum;  $IP_3$ : inositol 1,4,5-trisphosphate;  $PIP_2$ : phosphatidylinositol 4,5-bisphosphate.

**Low-molecular-weight GTP binding protein.** The GTP binding proteins p21 (p20~p30 monomer) include Ras, Rho, Rab, Arf, Sar and Ran families. Ras was first identified as an oncogene. Ras is activated by several plasma membrane receptor signals. The activated Ras family proteins activate Raf kinase, which triggers the MAP (mitogen-activated protein=microtubule-associated protein) kinase cascade. The downstream factors of Ras are sequentially Raf (MAPKKK), MAPK kinase 1, 2 (MAPKK) and ERK1, 2 (MAPK) (Figure 2). These kinases finally activate transcription factors such as Elk1, ternary complex factor and serum response factor. Ras-MAP signaling regulates gene expression, cell proliferation, differentiation and apoptosis. Rho family proteins are mainly involved in the regulation of actin filaments and the cytoskeleton arrangement, as well as cell division. Rab, Arf and Sar family proteins are involved in the regulation of protein translocation in the endoplasmic reticulum. Ran family proteins are involved in the regulation of RNA and protein transport into the nucleus. The MAPK group pathways include ERK/MAPK, Janus kinase-signal transducers and activators of transcription, p38 MAPK and BMK1/ERK5 signaling pathways. The tyrosine kinase signaling pathways activate the Ras-MAP and Janus kinase-signal transducers and activators of transcription signaling pathways, among others. These factors are involved in the intracellular signaling induced by the stimulation of cytokines, hormones and growth factors.

**Tyrosine kinase.** Tyrosine kinase includes groups of both trans-membrane receptor tyrosine kinase and membrane-associated tyrosine kinase.

**Trans-membrane receptor tyrosine kinases:** Trans-membrane receptor tyrosine kinases include the EGFR family, the fibroblast growth factor receptor family, the PDGFR family and the insulin receptor family. The fibroblast growth factor receptor family includes EGFR, ErbB-2, ErbB-3 and DER. The insulin receptor family includes insulin receptor, insulin-like growth factor receptor, Ros, Sea, Trk, Met, DILR, Sev and Ltk. The PDGFR family includes PDGFR $\alpha$ , PDGFR $\beta$ , colony-stimulating factor 1 receptor, Kit, Ret and Flt. The fibroblast growth factor receptor family includes fibroblast growth factor receptor, Flg, Bek, K-Sam and Cek-1. When the trans-membrane is activated by extracellular signals, GTPase-activating protein, phospholipase  $C_\beta$ ,  $IP_3$  kinase and Raf-1 bind together and exhibit their respective functions (Figure 8).

**Non-trans-membrane receptor-type tyrosine kinases:** Non-receptor-type tyrosine kinases include the Src family and the Abl family of tyrosine kinases. The Src family tyrosine kinases are those associated with membranes, such as Src, Yes, Fgr, Lyn, Lck, Hck, Tkl, Blk, Dsrc 64 and Dsrc 24. The Abl family tyrosine kinases are localized in the cytoplasm or nucleus, such as Abl, Arg, Dash, Nabl, Fes/Fps and Fer. The Src family tyrosine kinases have Src homology domains SH1, SH2 and SH3. The SH1 domain is a tyrosine kinase domain itself located at the C-terminal region. The SH2 domain is involved in the protein-protein interaction between phosphorylated tyrosine peptides. The SH2 and SH3 domains are closely related to proliferation, differentiation and tumorigenesis. The SH2 and SH3 domains have important roles in the signal-transduction pathway. The SH2 domain is involved in the interaction between the ErbB receptor tyrosine kinase and PI3K.  $IP_3$  interacts with the pleckstrin homology domain of Akt/PKB and activates Akt/PKB.

**Hsp90 inhibitor decreases Hsp90-pp60v-Src complex:** Hsp90 specifically forms a complex with pp60v-Src, and the pp60v-Src/Hsp90 complex is unable to function as a protein tyrosine kinase. When pp60v-Src is subsequently found inserted into plasma membrane, it is



active as a kinase is no longer associated with Hsp90.<sup>80</sup> Benzoquinoid ansamycins suppress the phosphorylation of Src in v-Src-transformed cells.<sup>81</sup> Wild-type v-Src expression regulates c-Src kinase activity through a direct interaction between v-Src and c-Src, inducing the hyper-phosphorylation of c-Src Tyr<sup>416</sup>.<sup>82</sup> A C-terminal peptide containing c-Src-Tyr<sup>527</sup> is deleted in v-Src.<sup>83</sup> When Fyn-Tyr<sup>531</sup> corresponding to c-Src-Tyr<sup>527</sup> is deleted, NIH3T3 cells are transformed. This result indicates that the phosphorylation of Fyn-Tyr<sup>531</sup> and c-Src-Tyr<sup>527</sup> is indispensable for the cells to have a normal status.<sup>83</sup> The substitution of phenylalanine for Try<sup>416</sup> in c-Src suppresses the biological activity of the mutant proteins as assayed using colony formation using soft agar and the induction of morphological alterations. These results suggest that the phosphorylation of Tyr<sup>416</sup> has a positive regulatory effect on the activity of the protein tyrosine kinase pp60c-Src.<sup>84</sup> The phosphorylation of Tyr<sup>416</sup> and Tyr<sup>527</sup> of c-Src in NIH3T3 cells has been reported to regulate cell proliferation and cell-to-cell communication.<sup>85</sup> These results indicate that the phosphorylations of Tyr<sup>416</sup> and Tyr<sup>527</sup> exert positive and negative effects on c-Src tyrosine kinase activity, respectively.

**Ser/thr kinases.** Protein kinase A, PKC, PKB/Akt and protein kinase G are activated by cAMP, diacylglycerol and Ca<sup>2+</sup> in the IP<sub>3</sub> signaling pathway, PI (3, 4) P<sub>2</sub> and cGMP, respectively. Na diuretic peptide ligands such as A-type natriuretic peptide, B-type natriuretic peptide and C-type natriuretic peptide stimulate the GC receptor. The GC activated by nitrogen oxide generated by nitrogen oxide synthetase is activated by Ca<sup>2+</sup> bound to CaM in the nerve signaling pathway. CaM-dependent protein kinase II is activated by Ca<sup>2+</sup> bound to CaM. MAPK and Cdc2 phosphorylate serine, threonine and tyrosine residues (Figure 8).

**Ion-channel receptors.** The ion-channel receptors are classified into two groups. The cation-channel receptors for acetylcholine, glutamate, serotonin and ATP selectively transport Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>, and the anion-channel receptors for glycine and GABA selectively transport Cl<sup>-</sup>. The nerve signal-transduction substances, other than glycine, act on G-protein-coupled receptors as well as ion-channel receptors. The nicotin-dependent acetylcholine receptor is a trans-membrane receptor with four domains consisting of five subunits, and the 20 domains work as one ion channel. Over 10 subunits of glutamate receptor channels are known. N-methyl-D-aspartate,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid and kinin acid receptors are formed using combinations of these subunits. The glutamate receptors have important roles in the nerve signal-transduction pathways, for example, the plasticity of synapse transduction in the hippocampus, cerebellum, memory and highly organized nerve functions of the brain. NP in the nuclear membrane consisting of the inner and outer membrane has a diameter of 100 nm. Protein with an MW of 20~40 kDa is permeable through the NP. The NP is enclosed by a caged structure of large protein complexes, and NP channel transports RNA and proteins.

**NP channel.** Proteins containing a nuclear localization signal are imported into the nucleus. The export mechanism of RNA, such as mRNA and low-molecular-weight RNA, is not clear yet. The cap structure of mRNA is known to be important for its export, and the Rev of HIV and the Rex of HTLV-I are essential for export.

**Nuclear transcription factors.** Transcription factors are imported from the cytoplasm and localized in the nucleus. Ets, Fos, Jun, Maf, Myb, Myc, Rel and Ski are important transcription factors involved in transcription activation or regulation. Nuclear factor- $\kappa$ B consists

of the hetero-subunits p50 and p65 (Rel).  $\kappa$ B associates with nuclear factor- $\kappa$ B negatively, regulating the nuclear translocation of nuclear factor- $\kappa$ B. The hetero-dimer Fos/Jun (p38/p34) complex is a constituent of activator protein-1, which is regulated by PKC. *Ets* (avian retrovirus E26 transformation specific), *fos* (FBJ mouse osteosarcoma), *jun* (avian sarcoma virus 17), *maf* (musculo aponeurotic fibrosarcoma), *myb* (avian myeloblastosis virus), *myc* (avian myelocytomatosis MC29 virus), *rel* (avian retrovirus) and *ski* (avian leukemia virus/Sloan-Kettering Institute) are oncogenes.

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