สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

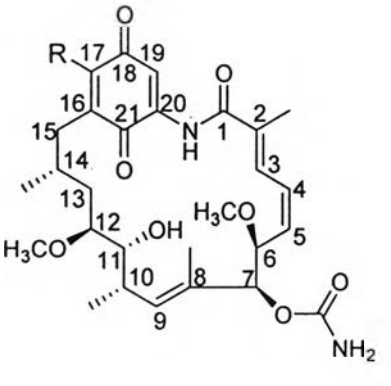
REVIEW OF LITERATURES

1. Chemistry of geldanamycins

Geldanamycin (**1**), the 17-substituted benzoquinoid ansamycin antibiotic, was previously reported as the secondary metabolite produced by the terrestrial *Streptomyces hygroscopicus* var. *geldanus* (DeBoer *et al.*, 1970; Sasaki *et al.*, 1970; Heisey and Putnam, 1986). Total synthesis of geldanamycin was subsequently achieved (Andrus *et al.*, 2002). Biosynthetic study of geldanamycin showed that the ansa chain of geldanamycin derived from four propionates, one malonate, and two 2-carbon units were still unidentified (Johnson. Haber, and Rinehart, Jr., 1974; Rinehart, Jr., and Shield, 1976).

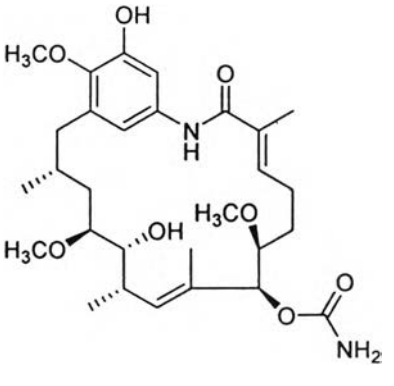
Recently, only one additional natural 17-substituted benzoquinoid ansamycin, 17-*O*-demethylgeldanamycin (**2**), was reported from the same bacterium (Barziley *et al.*, 2004). 17-*O*-Demethylgeldanamycin (**2**) was previously obtained from demethylation of geldanamycin by barium hydroxide (Rinehart and Shield, 1976).

The novel benzenoid ansamycin metabolites related to geldanamycin, reblastatin (**3**) was found as a minor component from the culture of *Streptomyces hygroscopicus* subsp. *hygroscopicus* SANK 61995. Compound **3** inhibited Rb protein phosphorylation at the cellular level and caused cell cycle arrest at the G1 phase (Takatsu *et al.*, 2000).



(**1**) R = OCH_3

(**2**) R = OH



(**3**)

2. Biological activities of geldanamycin

Geldanamycin was previously reported to possess moderately antifungal activity against several fungal plant pathogens, such as *Alternaria*, *Pythium*, *Botrytis*, and *Penicillium* and also exhibit anticancer activity (DeBoer *et al.*, 1970).

Initially, it was thought to be a nonspecific kinase inhibitor, however, geldanamycin bound to the ATP binding site of the chaperone heat shock protein 90 (Hsp90), resulting in prevention of several signaling proteins from reaching their mature forms, inhibiting their activities and affecting their stability (Stebbins *et al.*, 1997; Roe *et al.*, 1999).

Interestingly, geldanamycin prevented neurotoxic effects of anticancer drugs on cultured dorsal root ganglion neurons from chick embryo at low dose of 2 nM (Sano, 2001) and protected rat brain from focal ischemia (Lu *et al.*, 2002). The neuroprotective activity of geldanamycin was presumed that it might be mediated via suppression of Hsp90 function (Sano, 2001).

3. Neuroprotective effect of geldanamycin

Geldanamycin might protect neuron cells from apoptotic cell death by inhibiting functions of Hsp90. The result might induce receptor-interacting proteins (RIP) degradation, and inhibit nuclear factor-κB (NF-κB) activation that may protect cells against tumor necrosis factor (TNF)-induce apoptosis (Kitamura and Nomura, 2003).

4. Heat shock protein 90 (Hsp90)

Molecular chaperones maintained the appropriate folding and conformation of proteins and were crucial in regulating the balance between protein synthesis and degradation. Exposure of cells to a number of environmental stress, including heat shock, alcohols, heavy metals, and oxidative stress resulted in accumulation of a number of chaperones, commonly known as heat shock proteins (Hsps) because they were first observed in cells exposed to elevated temperature. This effect was

mediated by the transcription factor heat shock factor 1 (HSF1) and was termed the "heat shock response". Induction of Hsps protected the cells against the initial stress insult, enhanced recovery and led to the maintenance of a stress tolerant state. The Hsps also played a major molecular chaperone role under normal, stress-free conditions by regulating the correct folding, degradation, localization, and function of a relatively small but growing list of important cellular proteins (Maloney and Workman, 2002; Neckers, 2002).

A number of multigene families of Hsps existed, with individual gene products varying in cellular expression, function, and localization. They were classified according to the molecular weight, e.g., Hsp27, Hsp70, and Hsp90 (Csermely *et al.*, 1998; Maloney and Workman, 2002).

The 90 kDa heat shock protein, Hsp90, was a highly abundant protein in eukaryotic cell which was essential for cell viability. It constituted up to 1-2% of the cellular protein under physiological conditions, and its expression was increased several-fold in response to stress (Maloney and Workman, 2002; Neckers, 2002; Scheibel and Buchner, 1998; Toft, 1998). Up to now, members of the Hsp90 family were found in the cytosol, the endoplasmic reticulum, and chloroplast (Scheibel and Buchner, 1998).

4.1 Structure of Hsp90

The monomer of Hsp90 consists of the conserved 25 kDa N-terminal which contains the ATP-binding site and 55 kDa C-terminal domains joined together by a charged linker region, which is variable in both length and composition among species and isoforms (Buchner, 1999; Pearl and Prodromou, 2000; Maloney and Workman, 2002). The crystal structure of the yeast Hsp90 N-domain with bound nucleotide revealed structural homology between Hsp90s and the type II topoisomerase DNA gyrase B, with the conservation of residues involved in ATP binding and hydrolysis (Prodromou, *et al.* 1997; Pearl and Prodromou, 2000). Hsp90 is an ATP-dependent molecular chaperone, with dimerization of the nucleotide-binding domains being essential for ATP hydrolysis, which is in turn necessary for chaperone function. The function of Hsp90 is regulated by association with a number

of co-chaperones that combined in various ways to form a series of multimeric protein complexes. A number of co-chaperones containing a tetracopeptide repeat and binding of these proteins to Hsp90 are localized to the C-terminal MEEVD motif (Figure 1) (Pearl and Prodromou, 2000; Maloney and Workman, 2002). The middle segment in C-terminal domain contains the binding site for client protein of Hsp90 and has ATPase activity (Meyer *et al.*, 2003).

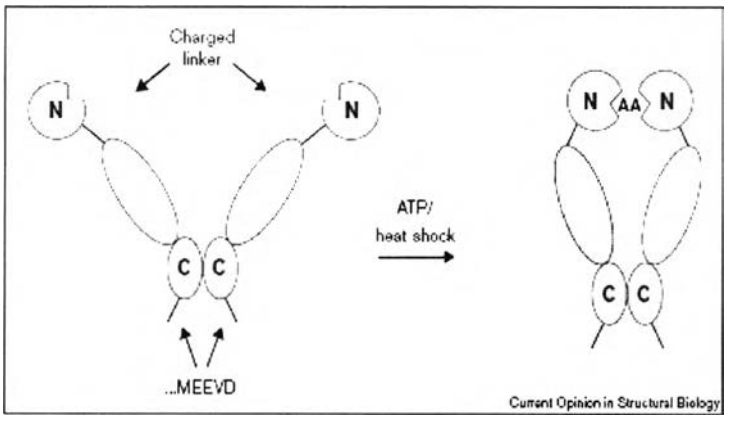


Figure 1. The conformational switch in Hsp90 (Pearl and Prodromou, 2000). The binding of ATP (A) or heat shock combines the two separated N-terminal domains in the monomer to generate the dimer structure of Hsp90.

4.2 Hsp90 function

In response to stress, expression of Hsp90 increased up to ten folds of normal cellular level. Thus, physiological levels of Hsp90 under normal conditions probably acted as a buffer to protect against the deleterious consequences of a sudden increased in the number of non-native proteins upon heat shock or other stresses (Buchner, 1999; Maloney and Workman; 2002). Hsp90 was constitutively expressed at two to ten folded higher levels in tumor cells compared to their normal counterparts, suggesting that it may play a critical regulatory role in tumor cell growth and/or survival (Neckers, Mimnaugh, and Schulte, 1999).

However, Hsp90 also has an important regulatory role under normal physiological conditions and is responsible for the conformational stability and maturation of a number of specific client proteins, which could be subdivided into

three groups: steroid hormone receptors (SHRs), serine/threonine or tyrosine kinases, and proteins with various other functions as described in Table 1 (Hostein *et al.*, 2001; Maloney and Workman; 2002; Mayer and Bukau, 1999; Scheibel and Buchner, 1998).

Table 1. Hsp90 family client proteins (Maloney and Workman, 2002).

Client protein	Function
Steriod hormone receptors	
Estrogen receptor	Ligand mediated gene transcription
Androgen receptor	Ligand mediated gene transcription
Progesterone receptor	Ligand mediated gene transcription
Serine/threonine and tyrosine kinases	
c-Src/v-Src	Signal transduction
ErbB2	Signal transduction
EGFR (Epidermal growth factor receptor, ErbB1)	Signal transduction
c-Raf-1/v-Raf-1	MAPK signaling
MEK	MAPK signaling
Akt (PkB, Protein kinase B)	PI3 kinase signaling
Proteins with various other functions	
Mutant p53	Mutant form of cell cycle checkpoint protein
Human telomerase reverse transcriptase (hTERT, catalytic subunit of telomerase)	Cell mortality and senescence
Endothelial nitric oxide synthase (eNOS)	Nitric oxide production
Calcineurin	Ca^{2+} and calmodulin dependent signaling
Tubulin	Microtubule formation
Retinoblastoma protein (Rb)	Cell cycle regulation (G1/S)
Tumor necrosis factor receptor type 1 (TNFR1)	TNF-mediated apoptosis
Immunoglobulin chains	Immune response

4.3 The Hsp90 as a novel drug target

Recently, Hsp90 served as the target for a potent new antitumor drug. Screening of natural compounds for substances inhibiting the proliferation of tumor cells identified geldanamycin as a promising candidate. Geldanamycin inhibited growth of a large number of cancer cell lines when was administered in sub-micromolar range. Initally, geldanamycin was supposed to be an inhibitor for cell cycle kinases, since they were found to be inactive in the presence of geldanamycin. Later on, it turned out that the effects on the kinase activity were indirect and the only pharmacologically specific drug target was Hsp90 (Buchner, 1999; Scheibel and Buchner, 1998; Pearl and Prodromou, 2000). Incubation of cell lines with geldanamycin led to the rapid dissociation of Raf-1-Hsp90 complex, concomitant with a markedly decrease half-life of the Raf-1 protein. Apparently, geldanamycin induced the disruption of the Raf-1-Hsp90 complex, resulting in the loss of Raf-1 protein from the cell, although Raf-1 synthesis was actually increased. Prevention of Raf-1-Hsp90 complex formation by geldanamycin interfered with trafficking of newly synthesized Raf-1 from the cytosol to the plasma membrane, indicating the essential association with Hsp90 for both Raf-1 protein stability and its proper localization in the cell. As for the kinase, geldanamycin abolished the effects of Hsp90 on the conformation of p53 mutants (Scheibel and Buchner, 1998). Geldanamycin treatment led to an arrest of the receptor cycle at a stage in which the substrate protein was complexed with Hsp90, Hsp70, and p60, resulting in ubiquitination and degradation by the proteasome of the client protein (Buchner, 1999; Neckers, 2002; Neckers, Mimnaugh, and Schulte, 1999; Scheibel and Buchner, 1998). The antitumor activity of geldanamycin and its specific binding to Hsp90 identified the Hsp90 chaperone family as a novel target for anticancer drug development (Neckers, Mimnaugh, and Schulte, 1999).

4.4 Natural product origins of Hsp90 inhibitors

So far, only two natural products were found as Hsp90 inhibitors, geldanamycin and radicicol, similarly to ATP, altered the affinity of the N-terminal chaperone site of Hsp90 for non-native proteins, while the C-terminal site was not influenced by the drug. The elucidation of the structure of the N-terminal domain in

the presence of geldanamycin or radicicol revealed that geldanamycin and radicicol bind to the ATP-binding site of Hsp90 (Scheibel and Buchner, 1998; Roe *et al.*, 1999).

Radicicol (**4**), an antitumor antibiotic, was first isolated from the fungus *Monosporium bonorden* which was able to reverse the tumor phenotype of *src* and *ras* transformed cell lines and disrupted mitogenic signaling via Ras- and Raf-1 dependent signal transduction pathway. Although radicicol was apparently able to inhibit tyrosine kinase activities *in vivo*, it had no inhibitory activity against the serine/threonine kinases of mitogen-activate pathway. Similar manner to that of geldanamycin, radicicol was also found to be Hsp90 inhibitor (Roe *et al.*, 1999; Westwell, 2003). The crystal structure of radicicol-Hsp90 complex showed that radicicol was orientated in the opposite sense to geldanamycin, so that the aromatic ring was directed toward the bottom of the pocket and the macrocycle containing the conjugated bond system and epoxide were toward the top of the pocket (Figures 2, 5c).

Comparing to the Hsp90-bound structures of geldanamycin and Mg^{2+}-ADP, radicicol produced the direct hydrogen bond to Asp 79 via the 3-hydroxyl of the aromatic ring which also participated with the ester carbonyl of the macrocycle in a bidentate interaction with the bound water (Figures 2, 4). Radicicol retained the water that bound to N6 of adenine but replaced the N7 binding water with the 5-hydroxyl of its aromatic ring. The N3- O2′ bridging water was displaced by radicicol, but unlike the geldanamycin complex, the conformation of Asn 92 was not disturbed. The Mg^{2+}-water network bound by the phosphates of the ADP was also disrupted by binding of radicicol, which occupied the space with its macrocycle and epoxide group, but without any direct replacement of water sites by functional groups. Lys 44 was involved in an interaction with epoxide oxygen of radicicol (Roe, *et al.*, 1999).

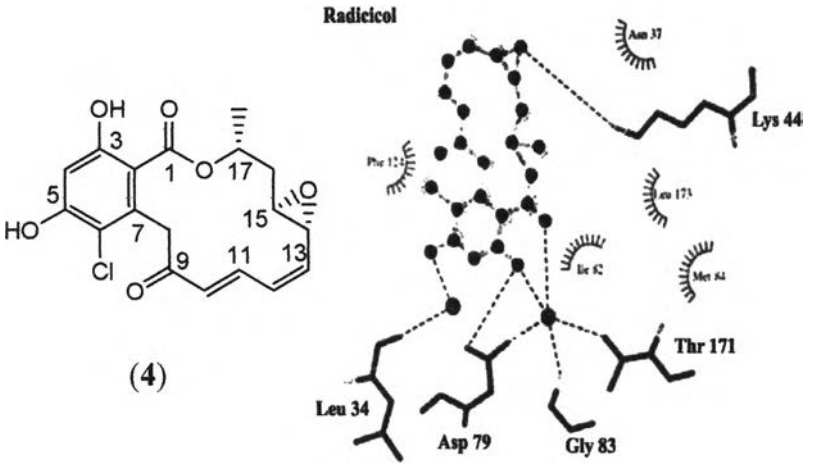
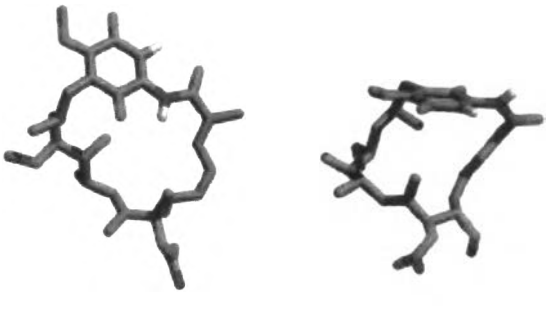


Figure 2. Interaction between radicicol and yeast Hsp90 (Roe *et al.*, 1999).

Geldanamycin consists of the ansa ring closed by an embedded benzoquinone with a pendant carbamate group approximately half-way around the ansa ring. (Scheibel and Buchner, 1998). Geldanamycin bound to intact dimeric yeast Hsp90 with stoichiometry of approximately one antibiotic molecule per Hsp90 monomer. Geldanamycin was oriented with the macrocyclic ansa ring and pendant carbamate group directed toward the bottom of the binding pocket and the benzoquinone ring directly toward the top of the pocket as it opens to the surface of the domain. In contrast to the extended structure adopted by the unliganded geldanamycin, the bound antibiotic was almost folded over, so that the planes of the benzoquinone and the macrocycle were closed to parallel as shown in Figure 3 (Roe *et al.*, 1999; Jez *et al.*, 2003).



free bound

Figure 3. Conformations of geldanamycin in the free (left) and the Hsp90-bound (right) forms (Jez *et al.*, 2003).

The Hsp90 N-terminal domain in the presence of geldanamaycin or ATP indicated that geldanamycin mimics ADP/ATP, using the interactions offered by the nucleotide binding site of Hsp90. Almost all of the hydrophobic interactions between geldanamycin and Hsp90 had precise equivalents in the interactions between Hsp90 and ADP/ATP (Roe *et al.*, 1999).

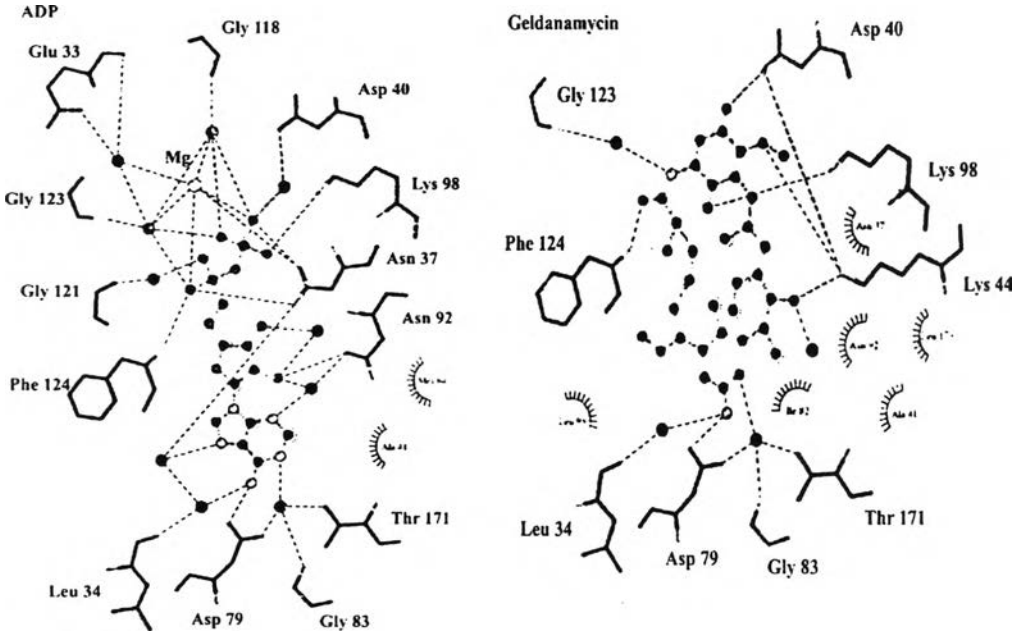


Figure 4. Interaction between ADP (left), geldanamycin (right) and yeast Hsp90 (Roe *et al.*, 1999).

A common superimposition of the Hsp90-bound structures of geldanamycin and Mg^{2+}-ADP (Figures 4, 5a, 5b) revealed a remarkably nucleotide mimicry of geldanamycin, both in shape complementary and in the interactions they made both with the protein and with the tightly bound water molecules implicit in the structure. Mg^{2+}-ATP/ADP bound to the N-domain of yeast Hsp90 in an unusual compacted conformation, which brought the sugar and α-phosphate group into close proximity to the five-membered ring of the adenine. The major specific interaction between the adenine base and the protein involved a direct hydrogen bond from the exocyclic N6 of adenine to the carboxylate side chain of Asp 79. Simultaneously, Asp 79 provided one of three protein ligands for a tightly bound water molecule which donated a hydrogen bond to N1 imino nitrogen of the adenine. These interactions were critical to specific binding of adenine nucleotides, and mutation of

Asp 79 to asparagines was sufficient to abolish Hsp90 function *in vivo* (Roe *et al.*, 1999).

In the geldanamycin complex (Figures 4, 5b), equivalent interactions were provided by the carbamate group pendant on the macrocycle to Asp 79, directly. The remaining hydrogen bonding interactions from carbamate pendant made by geldanamycin were with bound water molecules to Leu 34, Gly 83, and Thr 171. Those providing hydrogen bonds to N6 and N7 of adenine were retained in the geldanamycin complex, Asp79, but the water bridging N3 of the base and O2′ of the sugar was displaced, and the side chain of Asn 92 which provided the protein anchor for that water rotates around 90° and packs against a methyl group of the macrocycle. The two hydrophobic faces of bound adenine experienced very different environments, with one being substantially buried against a hydrophobic surface provided by the side chains of Met 84, Leu 93, Phe 124, Thr 171, and Leu 173. The other face was exposed and coated by a network of bound water molecules which connect to the waters and Mg^{2+} bound by the phosphates of the ADP toward the open mouth of the pocket. Binding of geldanamycin displaced much of this extended water network, occupying the volume with part of the macrocycle and the benzoquinone ring, quinone, and methoxy oxygens of which replace two of the water positions associated with the β-phosphate of a bound ADP. The groups forming the hydrophobic face of the adenine binding site made extensive hydrophobic interactions with the macrocycle of geldanamycin. The phosphate groups of bound ADP made only two direct contacts with proteins; the peptide nitrogen of Gly 123 at the N-terminus of the α-helix from 123 to 130 donated a hydrogen bond to the α-phosphate group, while the β-phosphate was involved in an ion pair/hydrogen bond with the side chain of Lys 98. Both of these interactions were produced by geldanamycin, with Gly 123 hydrogen bonding to the macrocycle peptide oxygen and Lys 98 to a quinone oxygen. Lys 44 was involved in an interaction with the macrocycle hydroxyl of geldanamycin but made no interactions with a bound nucleotide (Roe *et al.*, 1999).

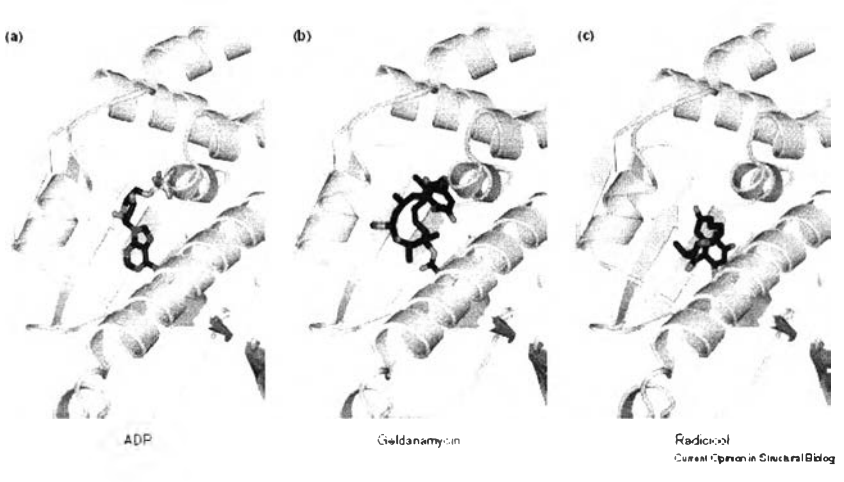


Figure 5. Drug binding to the nucleotide-binding site of Hsp90 N terminus. Crystal structures of the N-terminal domain of yeast Hsp90 with (a) bound ADP and the antitumor HSP90 inhibitors (b) geldanamycin (c) radicicol (Pearl and Prodromou, 2000).

5. Structure-activity relationship of geldanamycin

Geldanamycin had been subjected to an extensive synthetic program in an attempt to improve its potency in reducing the activity of the ErbB2 oncogenic tyrosine kinase *in vitro* an *in vivo* (Schnur *et al.*, 1995 (a); Schnur *et al.*, 1995 (b)). At that time of those studies, it was not recognized that the effects of geldanamycin on cellular ErbB2 activity was mediated by binding to Hsp90. Later, became possible to rationalize the effects in terms of the structure of the yeast Hsp90-geldanamycin complex. However, as the potency of these derivatives was primarily assessed in terms of ErbB2 levels in cells or whole animals, factors such as stability and bioavailability will clearly play a part (Roe *et al.*, 1999).

As expected from its role in reproducing the functions of the exocyclic amino and imino nitrogens of adenine, all modifications of the carbamate group resulted in substantial loss of potency. The significant loss of activity resulting from the reduction of the 2-3-double bond was less easily understood but might result from the loss of the rigidifying effect of this bond in stabilizing the folded-over conformation of bound geldanamycin. The only improvements that were obtained over unmodified geldanamycin resulted from modification of the ansa ring hydroxyl and the methoxy of the benzoquinone ring, both of which participated in a hydrogen bond network

involving Asp 40 and Lys 44. In the ADP complex, Asp 40 provided a hydrogen bond to one of the ligand waters of the Mg^{2+} cation, and its carboxyl headgroup was directed away from Lys 44, which made no contacts to the bound nucleotide and was relatively disordered. In the geldanamycin complex, the side chain of Asp 40 rotated toward Lys 44 and formed a hydrogen bond to its ε-amino group, stabilizing its conformation. The ε-amino group of Lys 44 was simultaneously hydrogen bonded to the ansa ring hydroxyl of geldanamycin. Oxidation of this hydroxyl to a ketone would provide a better hydrogen bond acceptor for this interaction and would explain the increase in potency observed with ketone derivatives and the substantial loss of potency when amines were introduced at this position. As well as interacting with Lys 44, the carboxyl of Asp 40 was in hydrogen-bonding contact with both the benzoquinone methoxy oxygen and the adjacent quinine oxygen. Replacement of the methoxy oxygen, a poor hydrogen bond acceptor, with an amino group would provide a much better hydrogen-bonding interaction for Asp 40. Consistent with this, introduction of a variety of alkylamino groups at this position give improved potency (Roe *et al.*, 1999). Among the successful analogues, 17-allyl-17-demethoxy-geldanamycin (17-AAG) was currently in Clinical Trial phase II which found bound to Hsp90 and shared important biological activities with geldanamycin (Cheng *et al.*, 2005; Schulte, and Neckers, 1998)

Many series of modified geldanamycin had been synthesized during passed years by the means of Hsp90 inhibitor such as 17-amides, carbamates, or aryl type substituted geldanamycin (Le Brazidec *et al.*, 2004). Geldanamycin was also modified by linked with a series of carbohydrate for enzyme-specific activation to increase tumor selectivity (Cheng *et al.*, 2005). A series of novel hormone-geldanamycin hybrid such as estradiol (Kuduk et al., 1999) or testosterone (Kuduk et al., 2000) were synthesized and evaluated for their ability to induce the selective degradation of the estrogen receptor or androgen receptor. Furthermore, geldanamcyin was also modified by conjugated with herceptin, the monoclonal antibody, to enhance their anticancer activity and reduced hepatotoxicity and cardiotoxicity caused by geldanamcyin and herceptin itself, respectively (Mandler *et al.*, 2002; Mandler *et al.*, 2004). All data mentioned above were shown in Figure 6.

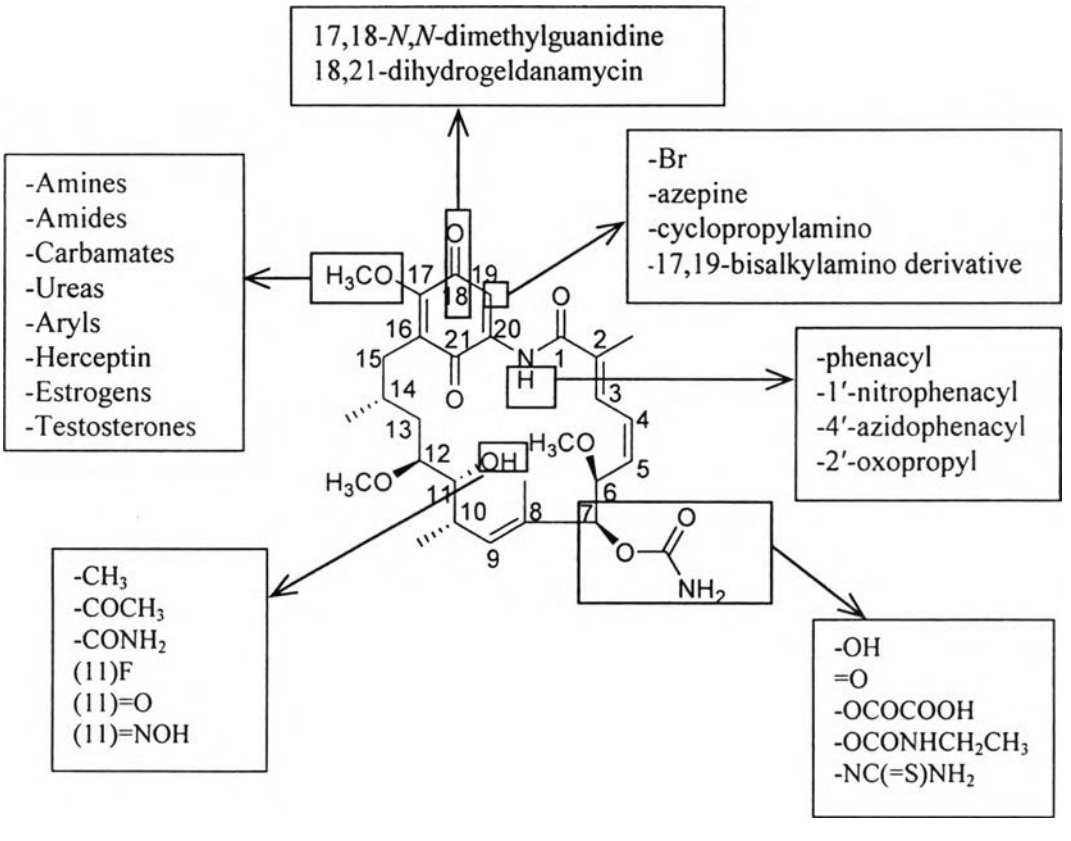


Figure 6. Chemical modifications of geldanamycin for antitumor activity.

6. P19 Embryonal carcinoma cells

P19 embryonal carcinoma cell line was the pluripotent stem cells of murine teratocarcinoma. It was established directly from a primary tumor of C3H/He mice, produced by grafting an embryo at 7 days of gestation to the testes of an adult male mouse. The resulting tumor was excised, dissociated and plated for growth in tissue culture. The karyotype of P19 was normal male 40;XY. This line proliferated and differentiated in cell culture. However, these cells differentiated poorly under normal condition, extensive differentiation can be induced when suitable chemical agents, such as retinoic acid, were included in the differentiating cultures (Bain *et al.*, 1994; MacPherson and McBurney, 1995; McBurney and Roger, 1982).

Undifferentiated P19 cells were polygonal in shape (Figure 7) and expressed a number of embryonic cell surface antigens. Many of these antigens were also expressed on neuroepithelial germinal cells which expressed in Table 2 (Jones-Villeneuve *et al.*, 1982; MacPherson and McBurney, 1995).

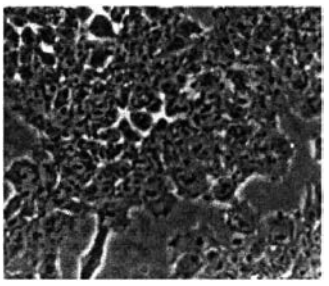


Figure 7. Undifferentiated P19 cells (MacPherson and McBurney, 1995).

7. Differentiation of P19 cells into P19 neuron-like cells

Treatment of P19 cells with retinoic acid (RA) in the range of 10^{-8} – 10^{-6} M induced their differentiation into a limited variety of cell types which appeared to be similar to those derived from the neuroectoderm (Jones-Villeneuve *et al.*, 1982; Staines *et al.*, 1994). Cultures at low cell density were more efficiently induced to differentiation than cultures at high cell density. The monolayers of P19 cells when treated with RA did undergo differentiation, the resulting cell population was dominated by fibroblast-like cells and contained few neurons. Differentiation of P19 cells into P19 NLCs was greatly enhanced by allowing cells to aggregate into solid spheres of cells (MacPherson and McBurney, 1995).

The differentiation of P19 cells into P19 neuron-like cells (P19 NLCs) was dependent on the concentration of RA in the culture medium. At the concentrations > 5×10^{-8} M, all of the aggregates contained cells with neuron-like processes by 72 h after plating. Between 10^{-8} and 5×10^{-8} M, many aggregates, which did not contain neuron-like cells, contained fibroblast-like cells. Below 10^{-8} M, the cultures resembled untreated controls and contained only embryonal carcinoma cells and small amounts of extraembryonic endoderm-like cells (Jones-Villeneuve *et al.*, 1982). The more effective ranges of RA in induction of neuronal differentiation from P19 cells are 10^{-7} to 10^{-6} M (MacPherson and McBurney, 1995).

P19 NLCs which appeared as early as 4 to 5 days after RA treatment, had round cell bodies 6-10 μm in diameter with at least one long branching process as shown in Figure 8. Within the first few days following RA treatment, P19 cells lose

the embryonic cell surface antigen SSEA-1 and the neurons began expressing the HNK-1 antigen, a glycoprotein found on embryonic neurons. The intermediate filament, vimentin, presented in undifferentiated P19 cells was rapidly replaced by the neurofilament proteins including both NF68 and NF160 as shown in Table 2 (MacPherson and McBurney, 1995).

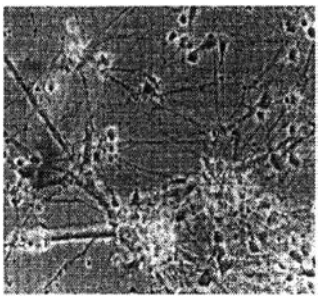


Figure 8. P19 neuron-like cells (MacPherson and McBurney, 1995).

Table 2. Lineage specific markers in undifferentiated and differentiated P19 cells (MacPherson and McBurney, 1995).

Antigen	Antigenic determinant	Undifferentiated P19 cells	RA-treated P19 cells
SSEA1	Cell surface antigen present on most embryonal carcinoma cells	+	-
Vimentin	Intermediate filament of mesoderm and neuroepithelia	+	-
HNK-1	Carbohydrate antigen of N-CAM	-	+
NF68	Intermediate filament of neurons	-	+
NF160	Intermediate filament of neurons	-	+
GFAP	Glial fibrillary acidic protein intermediate filament of astrocytes	-	+
Tau	Neuron-specific microtubule-associated protein	-	+
NeuN	Neuron-specific nuclear antigen	-	+
N-c-src	Neuron-specific c-src	-	+
Synapto-physin	Presynaptic membrane protein	-	+

Differentiation of P19 cells into P19 NLCs required exposure of the aggregated cells to RA (Coleman and Taylor, 1996; Jones-Villeneuve *et al.*, 1982). RA appeared to be an important regulator of growth and differentiation in mammalian cells (Bain and Gottlieb, 1994). The RA signal was transduced by ligand-inducible transcription factors belonging to the super family of nuclear receptors (Costa and McBurney, 1996; Nakshatri *et al.*, 1996). There are two major families of receptors – the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The three types of RARs, RARα, β, and γ, could bind and respond to all- *trans* and 9-*cis* RAs, whereas the RXRs, RXRα, β, and γ, could bind and respond to 9-*cis* RA but not to all- *trans* RA. Both the RARs and RXRs bound cooperatively as heterodimers to retinoic acid response elements (RAREs) of target gene and could activate their expression in the presence of cognate ligands (Nakshatri *et al.*, 1996).

8. Specific markers in neuronal cells

The P19 NLCs expressed a variety of neuronal markers such as 68,000 and 160,000 mol. wt. neurofilament proteins, tetanus toxin binding sites, synaptophysin and HNK-1 surface binding sites (Staines *et al.*, 1994). The P19 NLCs were irreversibly postmitotic and exhibited many characteristics of mature CNS neurons containing various neurotransmitters such as γ-aminobutyric acid (GABA) (MacPherson and McBurney, 1995) and acethylcholine (Jones-Villeneuve *et al.*, 1982; Staines *et al.*, 1994). Differentiated P19 cells were shown to express a number of cholinergic neuronal markers such as choline acetyltransferase (ChAT) and acetyl cholinesterase (AChE) (Jones-Villeneuve *et al.*, 1983) and GABAergic neuronal marker, glutamic acid decarboxylase (GAD) (Parnas and Linial, 1995).

Both GABAergic and cholinergic phenotypes coexisted at day 8 and could be clearly followed in 10-day-old cultures which monitored the major neurotransmitter phenotype of P19 NLC by immunofluorescence detection. When cells were plated at low density (5×10^4 cells/cm^2), antibodies for the cholinergic markers ChAT and AChE stained more than 85% of the neurons in sister cultures. In contrast, only 10% of the cells were positive for GAD, an essential enzyme in GABA biosynthesis (Parnas and Linial, 1995).

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The cholinergic phenotype of P19 NLCs was dramatically, down-regulated as culture density increased (Table 3). When cells were plated at low density (5×10^4 cells/cm^2), ChAT immunoreactivity was very intense and at equal levels in all labeled cells. Surprisingly, at 2×10^5 cells/cm^2, ChAT immunoreactivity dropped to background levels. No such effect of culture density was seen for GAD-positive neurons (Parnas and Linial, 1995).

Table 3. Cholinergic and GABAergic phenotypes in different culture densities (Parnas and Linial, 1995).

Neuron markers enzymes	**Cells/ cm^2**		
	0.5×10^5	1.0×10^5	2.0×10^5
ChAT	91.02 ± 5.85 (n = 903)	Not detected	Not detected
AChE	88.07 ± 12.76 (n = 624)	Not detected	Not detected
GAD	11.01 ± 2.96 (n = 1110)	8.40 ± 3.37 (n = 1121)	9.29 ± 2.20 (n = 628)

n = number of neuron cells.

Values given are % of cells ± S.D.

Values for GAD staining are within one S.D. under all conditions.

9. P19 NLCs as a model in drug screening

P19 NLCs was used as an *in vitro* model for reveal neurodegeneration elicited by reactive oxygen intermediated from hydrogen peroxide. The result showed that oxidative stress induced degeneration of neurons presumably resulting form increased intracellular Ca^{2+} concentration and subsequent activation of calpain (Ishihara *et al.*, 2000).

The differentiated P19 neuroglial cultures have been used for cytotoxicity evaluation of cysteinylcatechols which formed by nucleophilic addition of cysteine to oxidized catechols. Cysteinylcatechols were identified as markers of catechol

oxidation in brain tissue. The result showed that 5-*S*-cysteinyl-3,4-dihydro-phenylacetate (cysdopac) was specifically cytotoxic to differentiated P19 NLC cultures. (Montine *et al.*, 1997).

According to the data mentioned above, P19 NLCs can be used as a promising *in vitro* model for study of biological effects of drugs or natural products on neuronal cells.

Herein, we reported the neuritogenic effect and neuroprotective activity of geldanamycin and its derivatives by using P19 NLCs as an in vitro model in our study. The method used in our study and results were shown in Chapters III and IV, respectively.