Journal of Pharmaceutical Research International

33(22B): 71-82, 2021; Article no.JPRI.66964
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Evaluating the Effect of Amine-geldanamycin Hybrids on Antiviral Activity against Influenza Virus

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Authors' contributions

This work was carried out in collaboration among all authors. Author TT wrote the original concept, study design, managed the literature searches, conducted laboratory work and checked the data for validity and carried out the analyses of the study. Authors TS and CJ performed the laboratory tests. Authors WP and WSP were involved monitoring advising and guiding the progression of the study, proof reading and editing the manuscripts. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i22B31400
Editor(s):
(1) Dr. Ozlem Sultan Aslanturk, Adnan Menderes University, Turkey.
Reviewers:
(1) Qinghua Cui, Shandong University of Traditional Chinese Medicine, China.
(2) Fred Y. Aoki, University of Manitoba, Canada.
Complete Peer review History: http://www.sdiarticle4.com/review-history/66964

Original Research Article

Received 02 February 2021
Accepted 09 April 2021
Published 14 April 2021

ABSTRACT

Aims: The purpose of this study was to synthesis novel amine-geldanamycin hybrids (AGH) and evaluate their biological properties.
Study Design: Experimental study.
Place and Duration of Study: The study was carried out at the Department of Microbiology and Department of Chemistry, Faculty of Science, Silpakorn University, from December 2019 - November 2020.
Methodology: Three new amine-geldanamycin hybrids (AGH); compounds 2 to 4 were synthesised by nucleophilic substitution of geldanamycin (1). The solubility, cytotoxicity, antiviral activity and molecular docking analyses were carried out.
Results: The solubility of AGH in water was 1.918-5.571 mM, higher than that of compound 1. Compound 2 exhibited weak cytotoxicity activity against Vero and LLC-MK2 cells, with IC_{50} values

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of 229.19 and 330.58 µg/ml, respectively. All compounds inhibited influenza virus propagation in embryonated chicken eggs at the lowest amount of 1.25 µg per egg. They interacted positively with Hsp90, showing a binding free energy (ΔG) of -112.00 to -116.34 kcal/mol, which indicated lower Hsp90 affinity compared with that of geldanamycin (-133.06 kcal/mol) and 17-dimethylamino ethylamino-17-demethoxygeldanamycin (-136.55 kcal/mol), despite being bound in the similar active site. For the viral absorption, only AGH inhibited hemagglutination at a concentration of 25 µg/ml.

Conclusion: The study findings revealed, through molecular docking analysis, that the development of AGH improved the antiviral activity. The AGH inhibited not only influenza virus propagation, but also viral absorption. Therefore, AGH could be considered a new choice for antiviral agents.

Keywords: Amine-geldanamycin hybrids; antiviral activity; geldanamycin derivatives; heat shock protein 90; influenza virus; molecular docking; water solubility.

1. INTRODUCTION

Emerging infectious diseases of pandemic potential, such as influenza viruses, and Coronavirus, remain persistent threats to livelihoods and health around the world. Moreover, the existing antiviral agents mainly target the key viral enzymes that are involved in the process of replication. At the same time, many viral enzyme mutations result in drug resistance. Some antiviral agents are also of limited use because of their existing toxic side effects. These is a crucial need for developing new agents with novel antiviral mechanisms and broad antiviral activities. Particularly, influenza virus has the potential to generate a novel mutant virus through genetic reassortment. Although inactivated vaccine achieves a certain amount of protection in healthy subjects, it is less effective in elderly patients [1]. Rimantadine, amantadine [2] and neuraminidase inhibitors [3,4] have been available for prevention and therapy; however, adverse effects and the emergence of resistant viral strains have been reported [5-7].

Heat shock protein 90 (Hsp90) is a chaperone which binds to multiple proteins and facilitates the proper refolding of these proteins. It affects numerous physiological processes such as intracellular transport, protein degradation and signal transduction, it became an important target for cancer therapy [8]. Previous studies have shown that GDM blocks viral replication both *in vitro* and *in vivo* via inhibition of Hsp90 [9,10]. For example, GDM could suppress DNA replication of hepatitis B virus and its reverse transcriptase activity [11,12]. It also interferes the formation of Hsp90 and viral protein complexes which play an important role in hepatitis C virus RNA replication and in the HIV transcription process [13-16]. Inhibition of Hsp90 activity also causes inhibition of viral protein synthesis, for example, herpesvirus and some negative-stranded RNA viruses, because it facilitates the degradation of viral RNA polymerase [17-19]. It has been shown to block replication of DNA and RNA viruses in cell cultures and in animals [20,21]. Recently, GDM was isolated from *Streptomyces zerumbet* W14, and has been recognized for its potent activity against cancer cell lines [22,23]. Our previous study, GDM and its derivatives could inhibit influenza virus propagation in embryonated chicken egg [24]. However, the therapeutic utilisation of GDM has been restricted by its severe hepatotoxicity, metabolic instability and low water solubility [25]. Therefore, GDM derivatives with improved pharmacokinetic profiles have been synthesised, including 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylamino ethylamino-17-demethoxygeldanamycin (17-DMAG); however, their water solubility was limited [26]. Recently, tryptamine-geldanamycin hybrids have been synthesised. These compounds inhibited influenza virus propagation in embryonated chicken eggs. Their water solubility was increased above than that of GDM [24].

In this study, the other amine-geldanamycin hybrids were synthesised, and their antiviral activity against influenza virus was evaluated on the basis of virus propagation in embryonated chicken eggs. Their water solubility, cytotoxicity and molecular docking on Hsp90 were also determined.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out at the Department of Microbiology and Department of Chemistry,

Silpakorn University, Nakhon Pathom, Thailand from December 2019 - November 2020.

2.2 Cultivation of the Actinomycete and Product Isolation

Streptomyces zerumbet W14 was obtained as an endophyte from *Zingiber zerumbet* (L.) Smith using the surface-sterilisation technique [22]. The bacterium was grown on ISP-2 agar at 30°C for 14 days. The initial steps of antibiotic isolation and purification were as previously described [22]. The purified compound was subjected to investigation by NMR spectroscopy. The spectral data for this compound identified it as geldanamycin ($C_{29}H_{40}N_2O_9$) (**1**).

2.3 Chemical Reagents and Materials

All chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan), Sigma-Aldrich (Darmstadt, Germany) and Fluka Chemical (Buchs, Switzerland) Companies. All solvents were dried by using standard methods. The ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker Avance 300 spectrometer (Bruker, Massachusetts, USA), (300 MHz for ^{1}H, 75 MHz for ^{13}C). Mass spectra were determined with a micrOTOF (Bruker, Massachusetts, USA). Melting points were measured with a Stuart Scientific SMP 2 melting point apparatus (Cole-Parmer Ltd, Staffordshire, UK) and are uncorrected. The reaction was monitored by TLC, performed on aluminium sheets pre-coated with silica gel 60 F254 (Darmstadt, Germany). Column chromatography was performed using a Merck Kieselgel 60 column chromatograph (Darmstadt, Germany).

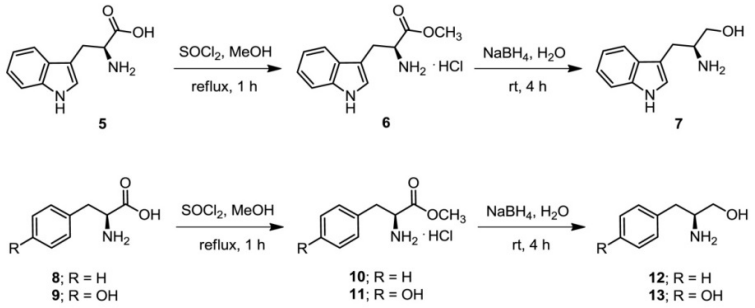
2.4 General Procedure for Esterification of Amino Acids

To a solution of amino acid; (*L*)-Tryptophan (**5**), (*L*)-Phenylalanine (**8**) and (*L*)-Tyrosine (**9**) (5 mmol) in dry methanol (10 ml), the freshly distilled thionyl chloride (5.5 mmol) was slowly added at 0 °C and refluxed for 1 h. The mixture was cooled down to room temperature and the solvent was concentrated under vacuum and recrystallized from methanol to provide amino acid methyl ester hydrochloride as white crystals (Scheme 1).

***(S)-methyl 2-amino-3-(1H-indol-3-yl) propanoate hydrochloride* (6):** (1.27 g, 99.80%); m.p. 218 °C - 219 °C; ^{1}H-NMR (D_2O, 300 MHz) δ 3.34 (dd, *J* = 4.4, 6.5 Hz, 2H, CH_2), 3.68 (s, 3H, CH_3), 4.32 (dd, *J* = 5.9, 6.6 Hz, 1H, CH), 7.07 (td, *J* = 0.9, 6.9 Hz, 1H, CH), 7.16 (m, 1H, CH), 7.18 (s, 1H, CH), 7.41 (d, *J* = 8.19 Hz, 1H, CH), 7.48 (d, *J* = 7.9 Hz, 1H, CH); ^{13}C-NMR (D_2O, 75 MHz) δ 25.6, 53.2, 53.5, 105.9, 112.0, 118.0, 119.5, 122.2, 125.3, 126.3, 136.2, 170.4.

***(S)-methyl 2-amino-3-phenylpropanoate hydrochloride* (10):** (1.08 g, 100%); m.p. 165 °C - 166 °C; ^{1}H-NMR (D_2O, 300 MHz) δ 3.23 (dd, *J* = 7.4, 14.5 Hz, 1H, CH), 3.35 (dd, *J* = 5.6, 14.5 Hz, 1H, CH), 3.84 (s, 3H, CH_3), 4.43 (dd, *J* = 5.9, 7.1 Hz, 1H, CH), 7.28-7.45 (m, 5H, ArH); ^{13}C-NMR (D_2O, 75 MHz) δ 35.5, 53.5, 54.0, 128.0, 129.2, 129.3, 133.6, 170.0.

***(S)-methyl 2-amino-3-(4-hydroxyphenyl) propanoate hydrochloride* (11):** (1.15 g, 99.8%); m.p. 195 °C - 197 °C; ^{1}H-NMR (D_2O, 300 MHz) δ 3.16 (dd, *J* = 7.5, 14.7 Hz, 1H, CH), 3.26 (dd, *J* = 5.8, 14.7 Hz, 1H, CH), 3.83 (s, 3H, CH_3), 4.25 (dd, *J* = 5.9, 7.3 Hz, 1H, CH), 6.89 (d, *J* = 8.6 Hz, 2H, CH), 7.16 (d, *J* = 8.6 Hz, 2H, CH); ^{13}C-NMR (D_2O, 75 MHz) δ 34.7, 53.5, 54.1, 115.9, 125.3, 130.8, 155.1, 170.0.



Scheme 1. The synthesis of amino alcohol 7, 12 and 13

2.5 General Procedure for Reduction of Amino Acid Methyl Esters

A solution of amino acid methyl ester (6, 10 or 11) (1 mmol) in THF:H_2O (10:1) (3 ml) was added to a solution of $NaBH_4$ (4 mmol) in H_2O (5 ml) under 0 ^oC. The reaction mixture was stirred at room temperature for 4 h upon monitoring the process by TLC. The resulting mixture was acidified with 2 N HCl and extracted with ether. The aqueous phase was adjusted to base with 2 N NaOH and extracted with EtOAc. The organic phases were combined and washed with H_2O. Then dried over with anhydrous (anh.) Na_2SO_4. The organic phase was concentrated under reduced pressure to provide amino alcohol which was used in the following step without further purification.

(S)-2-amino-3-(1H-indol-3-yl)propan-1-ol **(7):** light brown oil; (0.12 g); ^{1}H-NMR (CD_3OD, 300 MHz) δ 2.71 (dd, *J* = 7.5, 14.3 Hz, 1H, CH), 2.92 (dd, *J* = 5.9, 14.3 Hz, 1H, CH), 3.11 - 3.20 (m, 1H, CH), 3.42 (dd, *J* = 7.0, 10.8 Hz, 1H, CH), 3.60 (dd, *J* = 4.8, 10.8 Hz, 1H, CH), 7.02 (td, *J* = 1.1, 7.0 Hz, 1H, CH), 7.07 (s, 1H, CH), 7.11 (td, *J* = 1.1, 7.0 Hz, 1H, CH), 7.37 (d, *J* = 8.0 Hz, 1H, CH), 7.58 (d, *J* = 7.7 Hz, 1H); ^{13}C-NMR (CD_3OD, 75 MHz) δ 30.0, 54.4, 67.0, 112.3, 112.4, 119.52, 119.8, 122.5, 124.4, 128.9, 138.2.

(S)-2-amino-3-phenylpropan-1-ol **(12):** pale yellow oil; (0.11 g); ^{1}H-NMR (CD_3OD, 300 MHz) δ 2.59 (dd, *J* = 7.7, 13.4 Hz, 1H, CH), 2.79 (dd, *J* = 6.2, 13.4 Hz, 1H, CH), 3.04 - 3.13 (m, 1H, CH), 3.41 (dd, *J* = 6.8, 10.9 Hz, 1H, CH), 3.57 (dd, *J* = 4.3, 10.9 Hz, 1H, CH), 7.18 - 7.32 (m, 5H, ArH); ^{13}C-NMR (CD_3OD, 75 MHz) δ 40.5, 55.4, 66.2, 127.5, 129.6, 130.3, 139.8.

(S)-4-(2-amino-3-hydroxypropyl)phenol **(13):** pale orange color oil; (0.14 g); ^{1}H-NMR (CD_3OD, 300 MHz) δ 2.50 (dd, *J* = 7.7, 13.6 Hz, 1H, CH), 2.70 (dd, *J* = 6.3, 13.6 Hz, 1H, CH), 2.99 - 3.04 (m, 1H, CH), 3.38 (dd, *J* = 6.9, 10.8 Hz, 1H, CH), 3.55 (dd, *J* = 4.4, 10.9 Hz, 1H, CH), 6.76 (d, *J* = 8.5 Hz, 2H, CH) , 7.05 (d, *J* = 8.5 Hz, 2H, CH); ^{13}C-NMR (CD_3OD, 75 MHz) δ 39.5, 55.6, 66.3, 116.5, 130.4, 131.3, 157.2.

2.6 Synthesis of 17-Demethoxy-geldanamycin Derivatives

A solution of amino alcohol (**7**, **12** or **13**) (0.30 mmol) in MeOH (2 ml) was added to a solution of GDM (**1**) (0.14 mmol) in CH_2Cl_2 (10 ml). The reaction was kept away from light and stirred at room temperature for 48 h (Scheme 2). The solvent was removed and diluted with EtOAc and then washed with 1 N HCl, H_2O and brine. The organic phases were combined and dried over with anh. Na_2SO_4. The organic phase was concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel eluting with 40% CH_2Cl_2 in EtOAc to afford the product as a dark purple solid.

17-((S)-2-amino-3-(1H-indol-3-yl)propan-1-ol)-17-demethoxygeldanamycin **(2):** (0.032 g, 32.1%); m.p. 144 ^oC - 146 ^oC; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.86 (d, *J* = 6.3 Hz, 3H, CH_3), 0.97 (d, *J* = 6.9 Hz, 3H, CH_3), 1.74 (m, 4H, hydrocarbon), 1.79 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 2.24 (m, 1H, CH), 2.60 (d, *J* = 13.9 Hz, 1H, CH), 2.73 (m, 1H, CH), 3.16, (d, *J* = 6.0 Hz, 2H, CH_2), 3.25 (s, 3H, CH_3), 3.33 (m, 4H, CH, CH_3), 3.42 (m, 1H, CH), 3.56 (d, *J* = 7.5 Hz, 1H, CH), 3.64 (d, *J* = 4.4 Hz, 2H, CH_2), 4.28 (d, *J* = 9.8 Hz, 1H, CH), 4.52 (m, 1H, CH), 4.93 (br, 2H, NH_2), 5.15 (s, 1H, CH), 5.85 (m, 2H, CH), 6.49 (m, 2H, CH), 6.88 (d, *J* = 11.5 Hz, 1H, CH), 7.17 (m, 4H, CH), 7.37 (d, *J* = 7.9 Hz, 1H, CH), 7.66 (d, *J* = 7.7 Hz, 1H, CH), 8.52 (br, 1H, NH), 9.08 (br, 1H, NH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 12.3, 12.5, 12.7, 22.8, 27.3, 28.6, 32.2, 34.5, 34.9, 55.4, 56.7, 57.1, 63.1, 72.6, 81.2, 81.3, 81.8, 109.0, 110.2, 111.3, 118.6, 119.7, 119.9, 122.3, 123.5, 126.6, 126.8, 127.6, 132.8, 133.7, 135.0, 135.7, 136.3, 140.8, 145.3, 156.2, 168.3, 180.2, 184.2; HRMS calculated for $C_{39}H_{50}N_4O_9$ $(M+Na)^+$ 741.3470, found 741.3473.

17-((S)-2-amino-3-phenylpropan-1-ol)-17-demethoxygeldanamycin **(3):** (0.016 g, 40.6%); m.p. 129 ^oC - 131 ^oC; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.84 (d, *J* = 6.4 Hz, 3H, CH_3), 0.98 (d, *J* = 6.4 Hz, 3H, CH_3), 1.75 (m, 4H, hydrocarbon), 1.78 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.21 (m, 1H, CH), 2.63 (d, *J* = 13.1 Hz, 1H, CH), 2.73 (m, 1H, CH), 2.93 (dd, J = 6.8, 13.6 Hz, 1H, CH), 3.08 (dd, *J* = 6.6, 13.6 Hz, 1H, CH), 3.26 (s, 3H, CH_3), 3.35 (s, 3H, CH_3), 3.42 (m, 1H, CH), 3.55 (dd, *J* = 1.9, 8.8 Hz, 1H, CH), 3.64 (d, *J* = 3.8 Hz, 2H, CH_2), 4.29 (d, *J* = 9.8 Hz, 1H, CH), 4.40 (m, 1H, CH), 4.95 (br, 2H, NH_2), 5.16 (s, 1H, CH), 5.86 (m, 2H, CH), 6.37 (d, *J* = 9.1 Hz, 1H, CH), 6.57 (t, *J* = 11.6 Hz, 1H, CH), 6.92 (d, *J* = 11.5 Hz, 1H, CH), 7.22 (s, 1H, CH), 7.28 (m, 5H, ArH), 9.10 (br, 1H, NH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 12.4, 12.5, 12.8, 23.0, 28.7, 32.2, 34.5, 35.0, 38.2, 56.5, 56.7, 57.1, 63.0, 72.6, 81.1, 81.3, 81.7, 109.0, 109.3, 126.5, 126.9, 128.7, 129.4, 132.8,

133.6, 134.9, 135.8, 136.7, 140.9, 144.9, 156.2, 168.3, 180.4, 184.1; HRMS calculated for $C_{37}H_{49}N_3O_9$ $(M+Na)^+$ 702.3361, found 702.3360.

***17-((S)-4-(2-amino-3-hydroxypropyl)phenol)-17-demethoxygeldanamycin* (4):** (0.028 g, 29.2%); m.p. 125 °C - 127 °C; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.89 (d, *J* = 6.2 Hz, 3H, CH_3), 1.01 (d, *J* = 6.2 Hz, 3H, CH_3), 1.78 (m, 2H, CH_2), 1.80 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.14 (m, 2H, CH_2), 2.65 (d, *J* = 13.4 Hz, 1H, CH), 2.75 (m, 1H, CH), 2.94 (d, *J* = 5.0 Hz, 2H, CH_2), 3.27 (s, 3H, CH_3), 3.36 (s, 3H, CH_3), 3.45 (m, 1H, CH), 3.59 (m, 3H, CH, CH_2), 4.30 (d, *J* = 9.8 Hz, 1H, CH), 4.38 (m, 2H, CH_2), 4.79 (br, 2H, NH_2), 5.17 (s, 1H, CH), 5.54, (br, 1H, NH), 5.86 (dd, *J* = 10.6, 10.8 Hz, 2H, CH), 6.37 (d, *J* = 8.8 Hz, 1H, CH), 6.58 (t, *J* = 11.3 Hz, 1H, CH), 6.81 (d, *J* = 8.5 Hz, 2H, CH), 6.93 (d, *J* = 11.6 Hz, 1H, CH), 7.11 (d, *J* = 8.5 Hz, 2H, CH), 7.24 (s, 1H, CH), 9.11 (br, 1H, NH); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 12.5, 12.7, 12.9, 23.1, 28.8, 32.0, 32.4, 34.5, 35.1, 37.1, 56.4, 56.8, 57.3, 62.7, 72.8, 81.3, 81.4, 82.0, 109.1, 109.3, 115.8, 126.8, 127.0, 128.0, 130.8, 133.0, 133.6, 135.1, 135.9, 141.0, 155.2, 156.4, 168.5, 180.5, 184.2; HRMS calculated for $C_{37}H_{49}N_3O_{10}$ $(M+Na)^+$ 718.3310, found 718.3309.

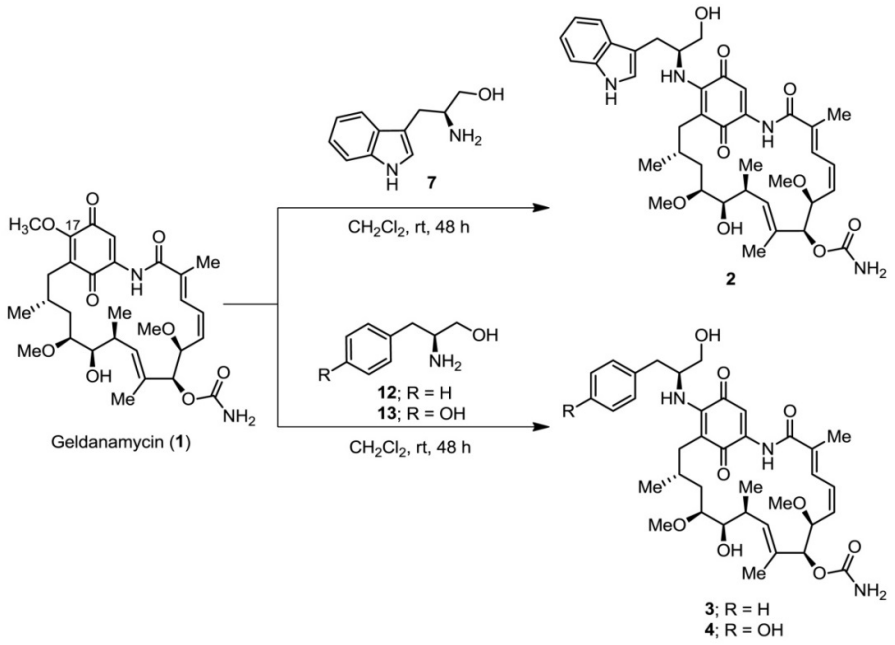
The solubility of the novel AGH in water was determined by comparison with GDM as previously described [23].

2.7 Viral Strain Propagation

Influenza viruses A/free-grazing duck/Nakhon Pathom/1/2017 (H5N2) were cultivated in embryonated eggs [27]. Viral titres were determined using the hemagglutination (HA) assay as previously described [28].

2.8 Virus Cultivation Inhibition Assay

Virus cultivation inhibition assays were carried out by 9- to 11-day-old embryonated chicken egg inoculation. One hundred microlitres of test compounds at various concentrations (12.5, 25, 50 and 100 µg/ml) was incubated with 100 µl of seed virus (2.86 x 10^8 virus particles/ml equal to 4HA units) at 37°C for 30 min, then the mixture was carried out by injection into the allantoic cavity of embryonated chicken egg and incubated at 37°C for 4 days. The allantoic fluid was harvested, and then was tested by HA assay [28]. Twenty micrograms per millilitre of heparin (Applichem, Germany) was used as a positive control.



Scheme 2. The synthesis of 17-demethoxy geldanamycin derivatives; 17-((*s*)-2-amino-3-(1*H*-indol-3-ylpropan-1-ol)-17-demethoxygeldanamycin (2), 17-((s)-2-amino-3-phenylpropan-1-ol)-17-demethoxygeldanamycin (3) and 17-((*s*)-4-(2-mino-3-hydroxypropyl)phenol)-17-demethoxygeldanamycin (4)

2.9 MTT Assay for Cell Viability

The normal cell lines (LLC-MK2: Rhesus monkey kidney cells and Vero cells: African green monkey kidney cells*)* were obtained from the Korean Cell Line Bank (Seoul, Korea). These cells were grown in DMEM medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin sulfate (100 µg/ml) at 37^{o}C in a humidified atmosphere of 5% CO_2. Cytotoxicity studies were performed in a 96-well plate. Details of the procedures have been described in a previous publication [23].

2.10 Molecular Docking Studies

The two-dimensional structures of GDM and DGH were drawn and converted to SMILES strings with ChemDraw software (http://cambridgesoft.com) and the Online SMILES Translator and Structure File Generator (https://cactus.nci.nih.gov/translate/), respectively. The energies of these compounds were optimized and converted to #D format, saved as Protein Data Bank (PDB) files using UCSF Chimera v.1.14 (University of California, CA) and further used for docking studies.

The three-dimensional structure of Hsp90 with the co-crystallized GDM (PDB ID: 1YET) was retrieved from the Research Collaborator for Structural Bioinformatics PDB and chosen for molecular docking studies. The crystal structure of 1YET is employed for docking GDM and AGH to obtain reliable predictions of ligand binding. The water molecules were removed from the crystal structure using Discovery Studio software followed by the addition of Gasteger charges to targets.

Docking simulations were undertaken with Hsp90 as the target (1YET) and GDM, 17-DMAG and AGH as the ligands using Auto Dock Vina to predict the ligand binding sites on Hsp90. The target confirmation was set as a rigid unit while the ligands were considered to be flexible and adaptable to the target. Vina explored for the lowest binding affinity conformations and provided five different conformations for the Hsp90 target. The lowest binding energy docking confirmations of each compound were selected. Auto Dock Vina was processed using an exhaustiveness of four and a grid box with dimensions for the centre of x = 35.5075, y = 47.7525, z = 54.2550 with a size of X = 45.7525, Y = 44.5035 and Z = 52.2575 for 1YET. The UCSF Chimera v.1.14 was chosen for visual inspection and preparations. The protein-ligand interactions were analyzed with the aid of Chimera and LigPlot v.4.5.3.

2.11 Statistical Analysis

Values are expressed as means ± standard deviation of three experiments. SPSS v.16.0 (SPSS Inc., Chicago, IL) software was used for data analysis. Comparisons between the two groups were analyzed using the two-tailed Dunnett *t*-tests treated compound **1** as a control group. A *p*-value <0.05 was considered to indicate statistical significance.

3. RESULTS

The effect of GDM and AGH on influenza virus propagation was evaluated at various concentrations in embryonated chicken eggs. Virus yields were determined by the hemagglutination test. Virus propagation was obtained only in the control, while no virus was detected in compounds 1, 2, 3 and 4 treatments at the lowest amount (1.25 µg). Besides, the effect of GDM and AGH on viral adsorption to chicken erythrocytes was carried out. Interestingly, as expected compounds 2, 3 and 4 at the lowest concentration (2 µg/ml or HAI titer of 1:50) could inhibit viral binding to the cells by HAI assay, while GDM could not inhibit viral binding to chicken red blood cells. These data suggested that GDM and AGH inhibited influenza virus propagation. In addition, AGH could inhibit the viral adsorption (early step) of influenza virus infection. Heparin at a concentration of 20 µg/ml could completely inhibit both viral propagation and viral absorption (data not shown). The results show that the novel AGH can display potential application in antiviral chemoprevention and chemotherapy.

Hsp90; PBD ID: 1YET was selected for molecular docking studies. Comparative docking of 1YET with the 17-DMAG and the tested compounds was performed to support the *in vivo* antiviral activity.

The results of docking studies revealed that 17-DMAG participated in interactions through five hydrogen bonds with Ser52, Asp54, Asp93, Val136 and Phe138 to the N-terminal domain pocket of Hsp90, with a binding energy of -136.55 kcal/mol. GMD formed four hydrogen bonds with Ser52, Lys58, Asp93 and Phe138 to the N-terminal domain pocket of Hsp90, with a

binding energy of -133.06 kcal/mol. Compound 2 formed five hydrogen bonds with Asp54, Lys58, Asp93, Lys112 and Phe138 to the N-terminal part of the domain pocket, with a binding energy of -122.00 kcal/mol. Compounds 3 and 4 formed four hydrogen bonds with Asp54, Lys58, Asp93 and Lys112 to the N-terminal part of the domain pocket, with a binding energy of -116.34 and -113.65 kcal/mol, respectively (Table 1). The protein-ligand interaction plots generated using LigPlot as indicated in Fig. 1(a – d), all ligands exhibit hydrogen bonding similar to that observed by Auto Dock Vina. Also, all compounds showed highly conserved hydrophobic interactions with the same active site residues Asn51, Ala55, Asp102, Asn106, Gly135, Val136 and Thr184, except a few exceptions in case of compounds 2, 3 and 4, which are due to difference in the orientation of compounds within the active site of Hsp90. The docking presentations of crystal structures are displayed in Fig. 2(a – e), which indicated that the benzoquinone moiety of all compounds participated in the binding pocket of Hsp90. However, the amine moiety of compounds 2, 3 and 4 molecules did not form any hydrogen bonds with Hsp90, these caused the binding orientation of the amine moiety was laid outside the binding pocket site.

Table 1. Molecular docking results of 17-DMAG, geldanamycin (1) and amine-geldanamycin hybrids (2 - 4) to Hsp90 (1YET)

Compounds	Binding energy (Kcal/mol)	Docking site
17-DMAG	-136.55	in active pocket site
1	-133.06	in active pocket site
2	-112.00	in active pocket site
3	-116.34	in active pocket site
4	-113.65	in active pocket site

17-DMAG: 17-dimethylamino-ethylamino-17-demethoxygeldanamycin

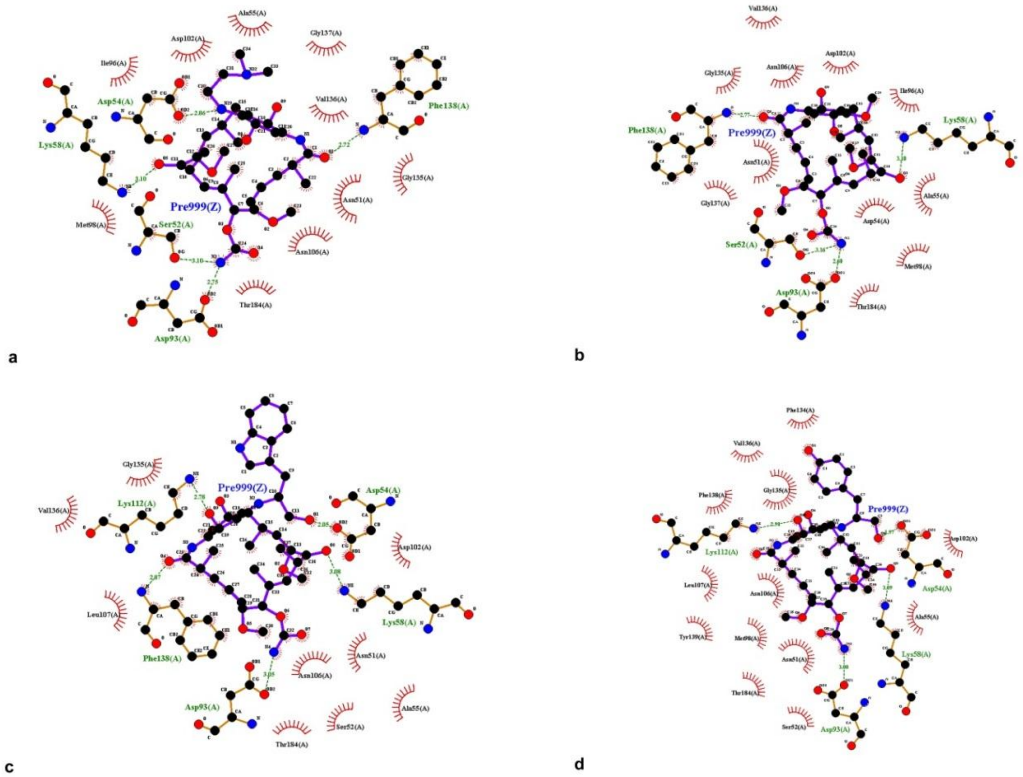


Fig. 1. LigPlot showing hydrogen bonding interactions (with green dashed lines) and hydrophobic contacts (red arcs with radiating lines) for the ligand : 17-DMAG (a), compound 1 (b), compound 2 (c) and compound 4 (d) molecules in active pocket of Hsp90.

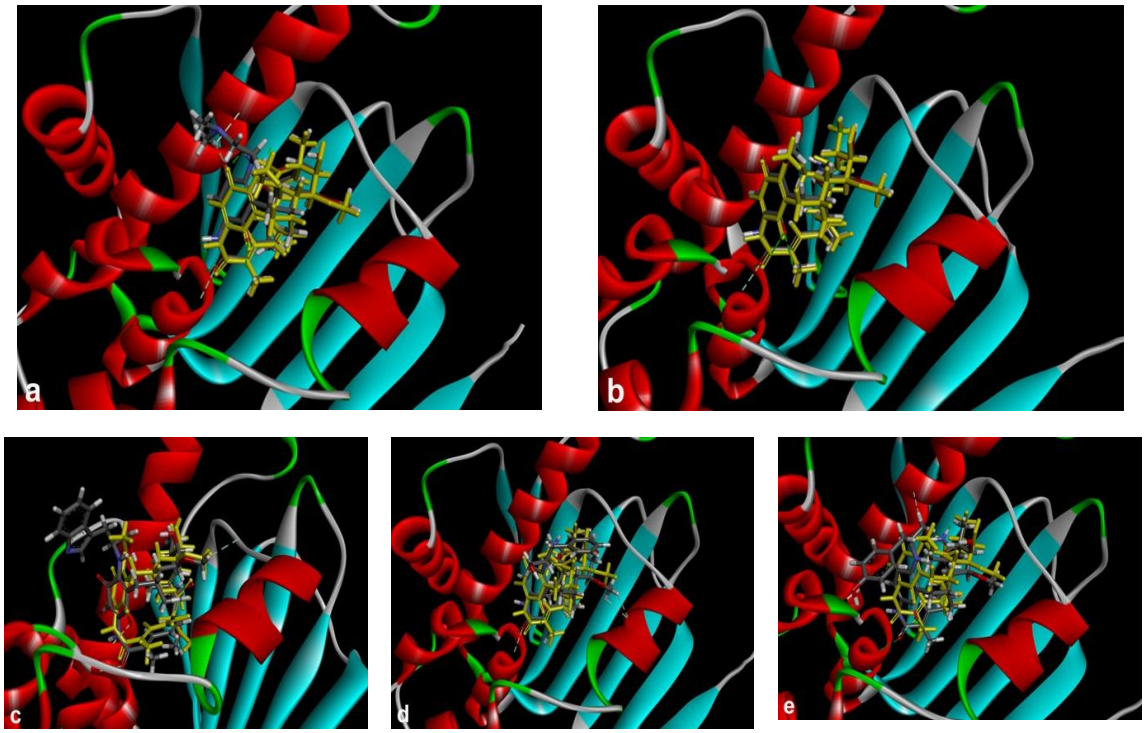


Fig. 2. Crystal structure superimposed on Hsp90 (1YET) docked 17-DMAG (a), compound 1 (b), compound 2 (c), compound 3 (d) and compound 4 (e). The yellow-brown ligand is represented geldanamycin molecule

The water solubility of GDM (**1**) was found to be 0.152 mM (Table 2). In contrast, the solubility of AGH (**2, 3** and **4**) in water was 5.571 mM, 1.963 mM and 1.918 mM, respectively, approximately 36.65, 12.91 and 12.62 times, respectively, higher than that of GDM. These data suggest that the conjugation of an amine moiety to GDM at the C17 position greatly enhanced their water solubility.

GDM and AGH were evaluated for cytotoxicity activity against two normal cell lines (Vero and LLC-MK2 cells) using the MTT assay. Compounds 2 exhibited weak cytotoxicity activity toward Vero and LLC-MK2 cells with IC_{50} values of 229.19 and 330.58 µg/ml, respectively (Table 3). Compounds 3 and 4 exhibited cytotoxicity activity against Vero cells with IC_{50} values of 79.02 and 94.21 µg/ml, respectively. Compound 4 exhibited weak cytotoxicity activity toward LLC-MK2 cells with IC_{50} values of 339.98 µg/ml. However, The results show that the AGH possesses low toxicity to normal cells compared with GDM.

Table 2. Water solubility of geldanamycin (1) and its derivatives (2, 3 and 4)

Compounds	MW	Solubility in water (mg/ml)[a]	Solubility in water (mM)[a]	Relative solubility
1	560	0.085 ± 0.004	0.152 ± 0.002[b]	1.00
2	718	4.000 ± 0.000	5.571 ± 0.000[c]	36.65
3	679	1.333 ± 0.577	1.963 ± 0.850[d]	12.91
4	695	1.333 ± 0.577	1.918 ± 0.830[d]	12.62

[a]; The results presented represent the average of three separate experiments (mean ± SD).
[b, c, d]; Significant differences ($P < 0.05$)

Table 3. Cytotoxicity activity (IC_{50}) of geldanamycin (1) and its derivatives (2 and 3)

Compounds	IC_{50}[a] (µg/ml)	
	Vero[c]	LLC-MK2
1	54.25	45.61
2	229.19	330.58[e]
3	79.02[d]	97.30[e]
4	94.21[d]	339.98[e]

[a]: *IC_{50} values represent the concentration causing 50% growth inhibition. They were determined by linear regression analysis of the average of three separate experiments.*
[b]: *Therapeutic index is defined as the ratio of the median toxic dose on normal cells (Vero) to the median effective dose on cancer cells.*
[c]: *Vero; African green monkey kidney cell line, LLC-MK2; Rhesus monkey kidney cell line,*
[d, e]:*Significant differences from the compound **1** ($p < 0.05$)*

4. DISCUSSION

The development of AGH was carried out through nucleophilic substitution reaction at the C17-position of GDM. The introduction of an amine group at the C17-position of GDM did not interfere with the binding of GDM derivatives to Hsp90, but greatly deceased their toxicity and increased their water solubility. As indicated by the crystal structure of the GDM-Hsp90 complex [29], the substitution in the C17 methoxyl of GDM is revealed to the external cavity of the Hsp90 protein, while the 19-substituted GDM is crucial for the interaction with the Hsp90 protein, which is not a possibility in terms of Hsp90 inhibitors [30]. AGH inhibited not only viral propagation but also viral absorption, which suggests that AGH could protect against viral infection at both steps. It has been reported that curcumin interrupts virus-cell attachment, which leads to inhibition of influenza virus propagation. Curcumin had a direct effect on viral particle infectivity that was reflected by the inhibition of hemagglutination [31]. Due to the similar structures between curcumin and amine moiety of AGH molecules contained the phenyl group which affected by the inhibition of hemagglutination. To this end, AGH could inhibit influenza virus infection at both steps. The toxicity and water solubility of GDM has been a marked hindrance to its development for chemotherapy use. These have incentivised scientists to torn their attention to developing less toxic GDM derivatives. For example, 17-amino-17-demethoxygeldanamycins has been reported to be novel agents with potential antiviral activity [30]. However, drug related toxicity of these compounds was unfavorable, as they caused hepatotoxicity [32,33]. In our study, the synthesised AGH exhibited less cytotoxicity than GDM in normal cell lines and showed a greater increase in water solubility. These properties are due to the structural relationships with amine moieties in the AGH molecules. For the synthesised derivatives, our results are in good agreement with those of previous reports [34,35], that the amino or amine moieties increases water solubility. This study will help the researcher to uncover structural modifications of compounds to improve their biological activities.

By the molecular docking studies, these AGH bound to the N-terminal ATP pocket of Hsp90. The binding mode of these compounds using the crystal structure has been predicted to understand the protein-ligand contacts and their interaction strength docking of these compounds along with 17-DMAG, the co-crystallised control compound in the active site of Hsp90 (1YET). These AGH exhibited similar pose as that of co-crystallised ligands 17-DMAG and GDM in the binding region. The interaction plots generated using LigPlot indicated that the AGH exhibit hydrogen-bonding with most similar to that observed in 17-DMAG, and also they showed highly conserved hydrophobic interactions with the same active site of amino acid residues. Similar to the findings of previous studies, the molecular docking in the present study has shown that the pocket of Hsp90 is of associated with polar, hydrophobic and charged amino acids, including Asn51, Asp54, Ala55, Lys58, Asp93, Ile96, Met98, Asn106, Leu107, Lys112, Gly135, Phe138 and Thr184. Although, the bottom of the pocket becomes increasingly hydrophobic. However, it retains polar residues at its deepest portion as Ser52, Asp93 and Thr184 [29,36]. This result supported that AGH specifically target and deactivate Hsp90 to inhibit virus replication similar to GDM. Therefore, the inhibition of virus replication through the cellular regulation by Hsp90 should be a new approach for the emergence of a new virus, such as influenza virus, coronavirus (SARS and COVID-

19) and resistant viral strain. Altogether, these data indicate that since AGH have a significant inhibitory effect against influenza virus propagation in embryonated chicken egg, there is justification for development of these compounds as a new agent for viral infection. In this study, the synthesis of AGH and their antiviral activity have been developed and initiated. The characterizations of AGH on ADMET, pharmacokinetic, *in vivo* toxicity studies and broad-spectrum antiviral activity against various viruses should be investigated in further studies.

5. CONCLUSION

In summary, novel AGH with antiviral activity, low cytotoxicity and enhanced waster solubility were presented in this work, in comparison with GDM. All AGH compounds showed antiviral activity in terms of viral propagation; moreover, they inhibited not only viral propagation but also viral absorption and had higher water solubility and lower toxicity than GDM. These results show these compounds can inhibit the function of Hsp90 and hence, virus propagation. This suggests a new antiviral approach. Therefore, Hsp90 could be an excellent antiviral target, and AGH could be useful for alternative antiviral agents in the future. This study will help researchers to uncover novel AGH derivative compounds as potential alternative agents for treatments of influenza virus infection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was approved by the Committee of the Scientific Study of Humane Technique in Laboratory Animal Experiments, and Human Ethics, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

ACKNOWLEDGEMENTS

The authors are grateful to Ms Chanakan Winyakul in the Department of Chemistry, Faculty of Science, Silpakorn University, Thailand, for measuring NMR and MS data, respectively. This work was supported by the Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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